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Regulation of Cyclooxygenase-2 pathway by HER2 receptor

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Emerging lines of evidence suggest that in addition to growth factors, the process of colorectal tumorigenesis may also be driven by the upregulation of the inducible form of cyclooxygenase-2 (COX-2), an enzyme responsible for the conversion of arachidonic acid to PGEs. The present study was undertaken to investigate the expression and activation of the HER family members, and to explore the regulation of COX-2 expression by the HER2 pathway in human colorectal cancer cells. Here, we report that human colorectal cancer cell lines express abundant levels of HER2 and HER3 receptors, and are growth-stimulated by recombinant neu-differentiation factor-beta 1 (NDF). NDF-treatment of colorectal cancer cells was accompanied by increased tyrosine phosphorylation and heterodimerization of HER3 with HER2. In addition, we demonstrated that HER2 and HER3 receptors in colorectal cancer cells are constitutively phosphorylated on tyrosine residues and form heterodimeric complexes in the absence of exogenous NDF. Inhibition of HER2/HER3 signaling by an anti-HER3 mAb against the ligand binding site resulted in a decrease in the levels of constitutively activated HER2/HER3 heterodimers, and the unexpected reduction of COX-2 expression. Activation of the HER2/HER3 pathway by NDF induced the activation of COX-2 promoter, expression of COX-2 mRNA, COX-2 protein and accumulation of prostaglandin E2 in the culture medium. Finally, we demonstrated that NDF promotes the ability of colorectal cancer cells to survive in an extracellular matrix milieu, such as Matrigel, and also to invade through a 8 μm porous membrane. These biological activities of NDF and its stimulation of cell proliferation are blocked by a specific inhibitor of COX-2. Taken together, our findings provide the first biochemical evidence of a possible role of the COX-2 pathway in the mitogenic action of NDF in colorectal cancer cells where it may be constitutively upregulated due to the autocrine/paracrine activation of HER2/HER3 heterodimers.

Keywords: NDF; COX-2; colon cancer

Introduction

Accumulating evidence suggests that colorectal tumorigenesis may be regulated by the growth factor-inducible form of cyclooxygenase-2 (COX-2), an enzyme responsible for the conversion of arachidonic acid to prostaglandins (Prescott and White, 1996; Smith *et al*., 1996). PGEs appear to play a variety of roles in the gastrointestinal tract, including participation in physiological secretion and motility, and also in pathologic processes such as neoplasia (Kutchera *et al*., 1996; Eberhart, 1994; DuBois *et al*., 1996). The primary PGEs generated in the colorectum appear to be PGE2 (Smith *et al*., 1996). Recent studies have shown the increased levels of COX-2 and PGE2 in colorectal adenocarcinomas compared to adjacent normal-appearing mucosa (Kutchera *et al*., 1996; Eberhart, 1994; DuBois, *et al*., 1996) and inhibition of COX-2 enzyme by specific COX-2 inhibitors reduces tumor formation, and regresses pre-existing tumors (Boolbol *et al*., 1996; Sheng *et al*., 1997; Chiu *et al*., 1997). The potential neoplastic role of COX-2 expression was demonstrated by Tsujii and DuBois (1995). They demonstrated that cells expressing high levels of COX-2 had increased tumorigenic potential which could be reversed by COX-inhibitors. Using an APC knockout mouse model, Oshima *et al*. (1996) demonstrated that COX-2 expression is induced very early in neoplastic progression, and showed dramatic reductions in the number and size of intestinal polyps by a specific COX-2 inhibitor. The levels of COX-2 expression and PGE2 are also induced by growth factors such as EGF or TGF-α in a number of cell systems, including rat intestinal epithelial cells (DuBois *et al*., 1994) and HCA-7 colon cancer cells (Coffey *et al*., 1997). In brief, these observations suggest a central role of COX-2 in colorectal cancer cells.

Proto-oncogenes are a group of normal genes that play important roles in the regulation of cell proliferation. Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and maintenance of the malignant phenotype. Evidence that the gene products of several activated proto-oncogenes are either growth factor receptors or growth factors has suggested a possible link between proto-oncogenes and growth factor pathways (Ullrich and Schlessinger, 1990). For example, c-*erb*B1 encodes the receptor of *h*uman *E*GF *r*eceptor (HER1) which is overexpressed in a number of epithelial tumor cells (Ullrich and Schlessinger, 1990). The second member of HER family, designated as HER2, shares extensive sequence homology to HER1, predominantly in the tyrosine kinase domain (Hynes and Stern, 1994; Graus-Porta *et al*., 1997; Pinkas-Kramarski *et al*., 1997; Alroy and Yarden, 1997). HER2 has been shown to be overexpressed or amplified, or both, in a number of human malignancies, including breast and ovarian cancer (Hynes and Stern, 1994). Recently, two more

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members, HER3 and HER4, have been added to the HER family as these receptors also share sequence homology with the tyrosine kinase domain of HER1 (Hynes and Stern, 1994; Graus-Porta *et al*., 1997; Pinkas-Kramarski *et al*., 1997). Regulation of the HER family of receptors is complex. These receptors can be transactivated in more than one fashion in a ligand-dependent manner. For example, binding of neu differentiation factor beta-1 (NDF)/heregulin to HER3 or HER4 can activate the HER2 receptor as a result of HER2/HER3 or HER4/HER2 heterodimeric interactions (Hynes and Stern, 1994; Alroy and Yarden, 1997). HER1 and HER2 receptors have been shown to induce transformed properties in recipient cells (Reidel *et al*., 1988), possibly because of excessive activation of signal transduction pathways. In contrast, transformation by HER3 or HER4 receptors requires the presence of HER2 or HER1 (Zhang *et al*., 1996). Although the transforming potential of HER2 is well-established, the mechanism involved remains poorly understood. It has been proposed that this may involve a constitutive activation of the intrinsic tyrosine kinase activity due to either mutations in the HER2 gene and/or transactivation via receptor-dimerization with other members of the HER2 family (Hynes and Stern, 1994). HER3 is unique amongst HER2 family members as it has an impaired tyrosine kinase domain due to the substitution of three amino acids (Guy *et al*., 1994; Sierke *et al*., 1997). In spite of the kinase-dead nature of HER3, NDF has been shown to increase HER3 tyrosine phosphorylation. This is most likely due to formation of a high-affinity co-receptor complex through heterodimeric interaction with HER2 (Carraway and Cantley, 1994; Horan *et al*., 1995). Among the HER family, it is believed the HER2/HER3 complex elicits the most potent mitogenic signal (Carraway *et al*., 1995; Pinkas-Kramarski *et al*., 1996). This may be related to the fact that the c-terminal phosphorylation domain of HER3 contains several consensus sites for the binding of signal-transducing proteins implicated in mitogenic signaling.

Growth factors and their receptors play an essential role in the regulation of the proliferation of colonic epithelial cells. Human colorectal carcinomas and colorectal carcinoma-derived human cell lines are known to express HER1 and TGF-α (Coffey *et al*., 1987; Anzano *et al*., 1989; Untawale *et al*., 1993). Although the significance of HER2 and HER3 receptors is fairly well established in breast cancer, very little is known about their roles in conferring a growth advantage in colorectal neoplasia. In one study, Ciardiello *et al*. (1991) detected HER3 mRNA in 55% primary or metastatic colorectal cancers as compared to 22% in normal colon mucosa. In addition to HER2, gastrointestinal tumors have been found to express intense staining of HER3 protein (Yang *et al*., 1997; Rajkumar *et al*., 1993). Kapitanovic *et al*. (1997) have demonstrated that HER2 is widely overexpressed in colorectal cancers as compared to normal mucosa, and that the amount of overexpression correlates with disease stage and patient survival. Although recent studies have demonstrated the wide occurrence of HER2 expression and COX-2 deregulation in human colorectal cancer cells, it remains unknown whether there is any role of NDF or the HER2/HER3 pathway in the deregulation of COX-2.

Here we report that human colorectal cancer cell lines express abundant levels of HER3 and HER2 receptors, and that colorectal cancer cells are growth-stimulated by NDF. We observe that HER3 and HER2 receptors are constitutively activated and form HER2/HER3 heterodimeric complexes (in the absence of any exogenous NDF) in colorectal cancer cells, which express and secrete a 40 kDa protein which is immunorecognized by a specific anti-heregulin mAb. Inhibition of HER3 ligand binding site by an anti-HER3 mAb resulted in a decrease in the levels of constitutive HER2/HER3 heterodimers, and an unexpected reduction of COX-2 expression. We also demonstrate that activation of the HER2/HER3 pathway by exogenous NDF induces the expression of COX-2 mRNA, COX-2 protein and the accumulation of PGE2 in culture medium. Finally, we show that NDF promotes the ability of colorectal cancer cells to survive in an extracellular matrix milieu, and to invade. These biological activities of NDF and its stimulation of cell proliferation are blocked by a specific inhibitor of COX-2. Taken together, our findings provide the first biochemical evidence of a possible mediatory role of the COX-2 pathway in NDF-mediated mitogenesis and invasiveness in colorectal cancer cells where it may be constitutively upregulated due to autocrine/paracrine activation of HER2/HER3 heterodimers.

Results

Expression of HER family members in human colorectal cancer cells

To investigate the possible significance of receptor tyrosine kinases in colorectal cancer, we examined the expression of HER family members in a panel of widely used colorectal cancer cell lines. The results presented in Figure 1A demonstrate that HER2 receptor is relatively overexpressed compared to HER1 receptor among the colorectal cancer cell lines used here, and CaC02 and LS174T cells also co-overexpress HER3 receptor in addition to HER2 receptor. Interestingly, co-overexpression of HER2 and HER3 receptors in CaC0-2 and LS174T cells was accompanied by increased expression of c-*myc* as well as tyrosine phosphorylation of protein(s) in the range of 180 kD. To examine the status of HER family member in colorectal tumors, we analysed a small number of residual colorectal specimens. Results in Figure 1B demonstrate that HER2 and HER3 receptors were overexpressed as compared to HER1 in five out of eight tumor specimens, and thus in-part validating the recent reports by Ciardiello *et al*. (1991) and Kapitanovic *et al*. (1997).

To further explore the significance of HER2 and HER3 receptors in colorectal cancer cells, we selected LS174T and CaCo-2 since these cell lines co-express both HER2 and HER3 receptors. We first examined the responsiveness of colon cancer cells to exogenous NDF. As demonstrated in Figure 2, NDF treatment of LS174T and CaCo-2 cells leads to a 2–4-fold growth stimulation over a period of 48 h (Figure 2A), and also rapid (15 min) tyrosine phosphorylation of about 180 kDa protein (Figure 2B). It was interesting to note that the faster migrating 180 kDa tyrosine

phosphorylated band in LS174T and CaCo-2 cells was responsive to exogenous NDF as it became a slower migrating protein band after stimulation of the cells with NDF (Figure 2B).

Next we examined the identity of the HER family members whose tyrosine phosphorylation may be enhanced by NDF stimulation of colorectal cancer LS174T cells. Control and NDF-treated lysates were immunoprecipitated with specific antibodies to HER2, HER3 or HER4 and Western blots were probed with anti-Phos-tyrosine mAb. As shown in Figure 2C, the HER2 receptor had significant baseline tyrosine phosphorylation and NDF treatment further enhanced the tyrosine phosphorylation of HER3 and HER2, but not the HER4 receptor. The low level of HER4 in LS174T cells was also confirmed by immunoblotting the above blot in Figure 2C with an anti-HER4 mAb (data not shown).

Constitutive activation of the HER2/HER3 pathway in colorectal cancer cells

To understand the biochemical basis of the observed baseline tyrosine phosphorylation of HER2 and HER3 receptors (Figure 2C), we hypothesized that colorectal cancer cells may express polypeptide(s) that may activate HER family members in an autocrine/paracrine manner. If this is true, chemical cross-linking of HER receptors should result in the formation of an HER2/HER3 heterodimer in the absence of any exogenous NDF. Data in Figure 3A demonstrate the constitutive formation of a tyrosine phosphorylated HER2/HER3 heterodimeric complex in LS174T cells, and the level of tyrosine phosphorylation of HER2/HER3 complex was further increased by stimulation of cells with exogenous NDF (Figure 3A). We used immunoblotting of total lysates after cross-linking to visualize the dimers to over come the problems of low efficiency of immunoprecipitation. Similar methodology has been used earlier to visualize cross linked complexes. Reprobing of the above blots with anti-EGFR mAb showed a lack of HER1 (HER1/HER2) at this resolution in the HER2/HER3 complex (data not shown).

In order to understand the possible nature of the cellular-derived factor(s) that may regulate the observed constitutive activation of HER2/HER3 receptors, we next explored the possibility of endogenously secreted HRG or HRG-like ligand(s) on the formation of HER2/HER3 dimers in LS174T cells, using an anti-HER3 mAb against the HRG binding site on HER3 receptor (Chen *et al*., 1996). As illustrated in Figure 3B, pretreatment of cells with anti-HER3 mAb resulted in a significant inhibition of heterodimerization between HER3 and HER2 and this was also true when cells were stimulated with NDF.

Colon cells secrete heregulin/heregulin like ligand

We then explored the possibility of expression of heregulin in metabolically-labeled CaCo-2 and LS-174T cells. Cell lysates and serum-free conditioned medium (24 h) were immunoprecipitated with a specific anti-heregulin beta-1 mAb. As can be seen in Figure 4A, colorectal cancer cells express and secrete a protein with an approximate molecular mass of 40 kDa that is immunorecognized by anti-heregulin mAb. To further verify the expression of heregulin in CaCo2 and LS174T cells, we examined the express of HRG transcript(s) by Northern hybridization using a human heregulin 1.8 kb cDNA as a probe. Breast cancer line MDA-MB-231 which express three HRG transcripts (1.7, 2.6, and 6.8 kb) were used as a positive control (Wen *et al*., 1992). As illustrated in the Figure 4B, both CaCo2 and LS174T cell express albeit levels of two smaller HRG transcripts (1.7 and 2.6 kb). Taken together, these observations suggested that CaCO-2 and LS174T cells express and secrete HRG which may be involved in the observed constitutive activation of HER2/HER3 via autocrine/paracrine pathway.

Effect of constitutive activation of HER2/HER3 receptors on COX-2 expression

Since the HER2/HER3 pathway is constitutively activated in colorectal cancer cells, and COX-2 is widely deregulated in colon cancer, we explored the

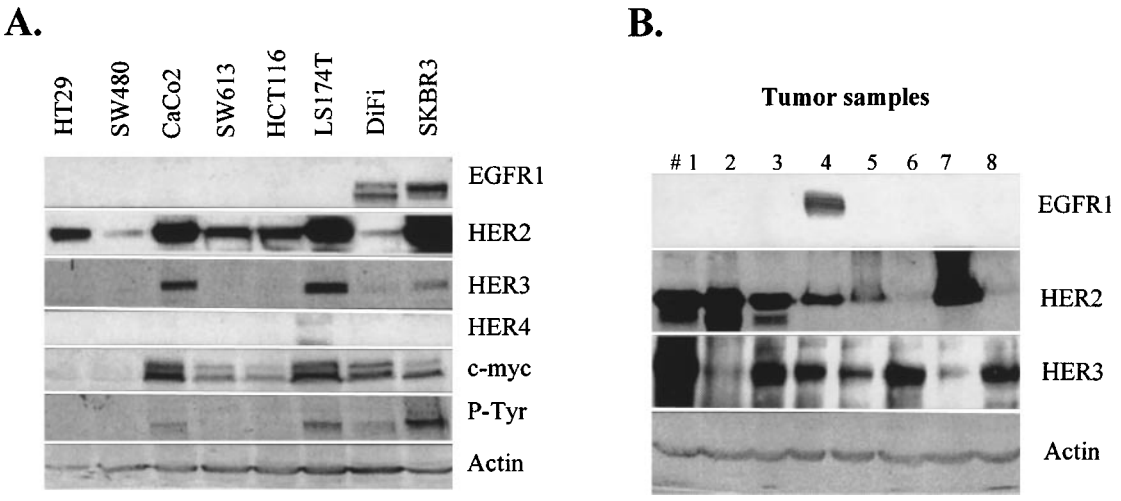

Figure 1 Expression of HER family members in colorectal cancer cell lines and tumor samples. One hundred μg of total protein lysate was separated on a SDS–PAGE gel and blotted with the indicated antibodies. (**A**) Colorectal cancer cell lines. (**B**) Human tumor specimens

potential role of baseline activation of HER3 in the constitutive COX-2 expression in LS174T cells. Results in Figure 5A show that treatment of LS174T cells with mAb against the HER3 ligand binding site was accompanied by a 50% inhibition of COX-2 expression when compared to cells treated with control mouse IgG (compare lane 2 with lane 1). The observed inhibition of COX-2 expression by inclusion of anti-HER3 mAb (lane 2) in complete culture medium (10% serum) was comparable to inhibition of COX-2 expression attained by complete serum-free medium (lane 3). These results suggest that COX-2 expression may be positively regulated by interaction of HER3 receptor with cellular-derived NDF or NDF-like ligands, and also by serum factors. Indeed, data in lane 4 (Figure 5A) confirm that exogenous NDF

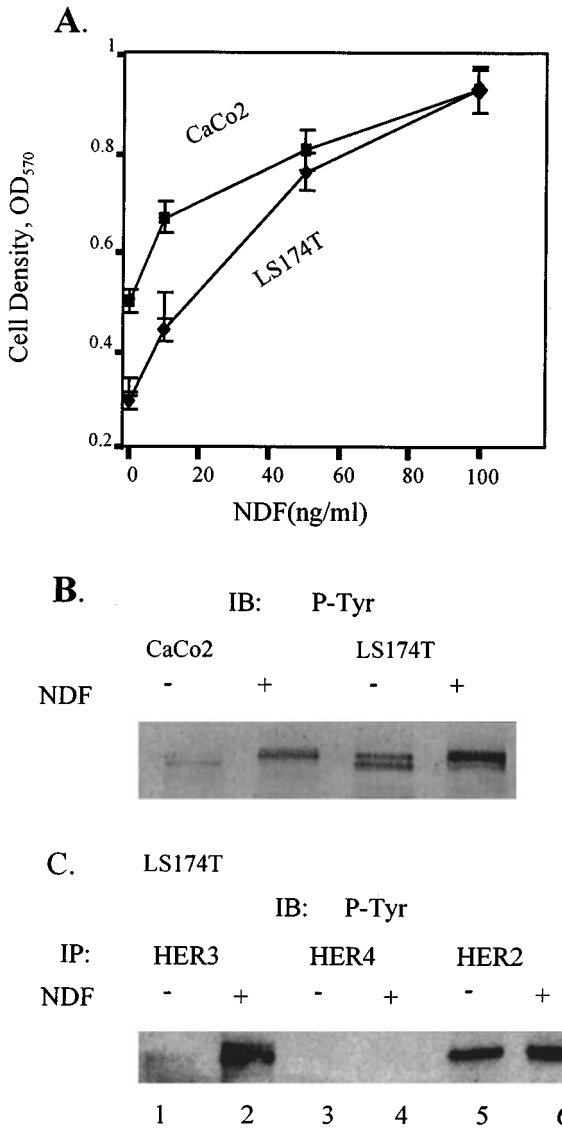

Figure 2 NDF stimulates growth of colon cancer cells. (**A**) Subconfluent cells were incubated with various concentrations of NDF and cell growth was measured after 48 h by MTT-dye uptake method by determining the optical density (OD) at a wavelength of 570 nm. (**B**) Cell extracts (100 μg protein) were analysed for tyrosine phosphorylation by blotting with a Phos-tyrosine mAb. (**C**) LS174T cell lysates were immunoprecipitated with anti-HER2, HER3, HER4, and analysed by SDS–PAGE. Immunoprecipitates were then blotted with a Phos-Tyrosine mAb. Results shown are representative of four times with similar results

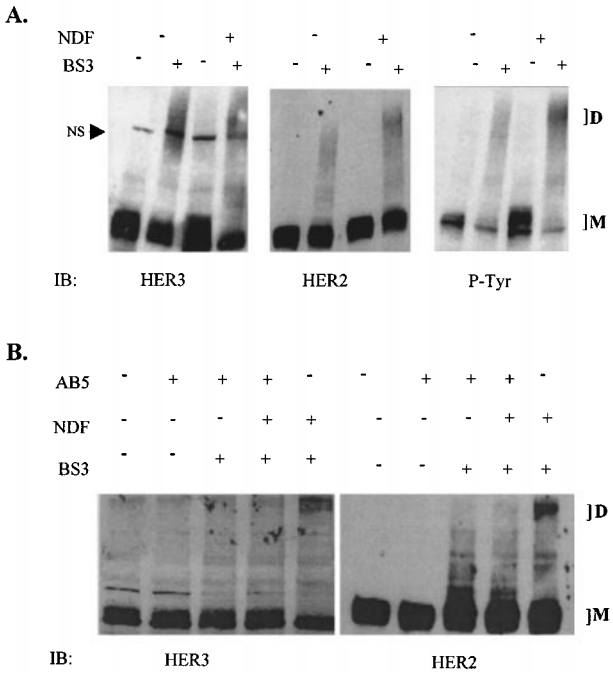

Figure 3 HER2/HER3 pathway is constitutively activated in colon cells. (**A**) Constitutive formation of HER2/HER3 dimer. Monolayers of LS174T cells were incubated with or without NDF and cross-linking was performed with 3 mM BS3 for 45 min. Total lysates (300 μg) were subjected to SDS–PAGE blotted with HER2, HER3 and Phos-tyrosine mAbs. M, monomer; D, dimer; and IB, immunoblotting. How ever we observed a non specific band in HER3 probed blot present in all the four lanes, and shown by NS. (**B**) Blockade of constitutive HER2/HER3 dimer formation with an anti-HER3 mAb (Ab5). LS174T cells were pretreated with anti-HER3 mAb and cross-linking was performed with 3 mM BS3 and then electrophoresed and immunoblotted as described above

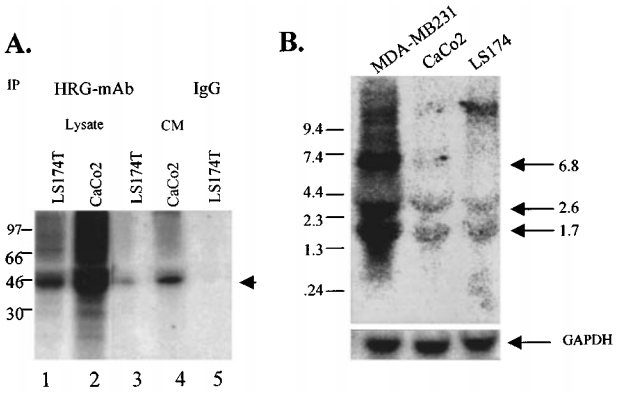

Figure 4 Expression of HRG in colorectal cancer cells. (**A**) LS174T and CaCo-2 cells were metabolically labeled with ^{35}S-methionine for 16 h. Cell lysates (Lys) and conditioned medium (CM) were immunoprecipitated with an anti-heregulin beta-1 mAb (HRG) and analysed by SDS–PAGE followed by fluorography. Lane 5, CM from LS174T cells was immunoprecipitated with control IgG. Results shown are representative of three experiments with similar results. (**B**) Total RNA (75 μg) from the indicated cell lines was analysed by Northern hybridization. The blot was stripped and reprobed with GAPDH probe as a control. Three known isoforms present in positive control MDA-MB-231 cells are shown by arrows. Size of RNA markers (BRL) are indicated on the left

induces the expression of COX-2 protein by threefold in LS174T cells (compare lane 4 with lane 3). Similarly, serum starvation of LS174T cells resulted in very low or undetectable levels of COX-2 mRNA (Figure 5B, compare lane 2 with lane 1). Furthermore, addition of exogenous NDF in serum-free medium restored mRNA levels comparable to those of normal cells growing in 10% serum (compare lane 3 with lanes 1 and 2). These results suggest that expression of COX-2 is possibly regulated by constitutively activated the HER2/HER3 pathway.

NDF induces COX-2 expression and PGE2 biosynthesis

In the next series of experiments we characterized the regulation of the COX-2 pathway by NDF. Cell extracts from NDF-treated and control LS174T cells were analysed for the activation of HER3 and the expression of down-stream target gene products such as c-*myc*, and COX-2 protein. As illustrated in Figure 6A, the kinetics of NDF-mediated activation of HER3 receptors coincided with the stimulation of the expression of c-*myc* and COX-2 protein. Similarly, in CaCo-2 cells, NDF treatment also stimulated the expression of COX-2 protein (Figure 6B) and COX-2 mRNA (Figure 7A). The observed NDF-mediated stimulation of COX-2 expression in colorectal cancer cells was functional, as there was a significant increase in the levels of accumulated PGE2 in the culture medium in NDF-treated CaCo-2 cells compared to the PGE2 levels in the control culture medium (Figure 6C). However the observed increase in the COX-2 protein was not accompanied by concomitant increase in the accumulation of PGE2 in the medium but a delay was observed with maximum levels reaching at 24 h. The delay in the accumulation of PGE2 in the medium could be due to culturing of the cells in the low serum conditions and such conditions are known to reduce the activity of the other component enzymes in the PGE2 pathway and delay PGE2 secretion (Chen *et al*., 1997). The observed stimulation of PGE2 secretion in NDF-treated colorectal cancer cells was mediated through COX-2, as it could be effectively blocked by a specific COX-2 inhibitor, NS-398 (Ciardiello *et al*., 1991) (see Figure 9D).

To determine whether this increase in the level of COX-2 protein in NDF-treated colorectal cancer cells is associated with an increased expression of COX-2 mRNA Northern blot analysis was performed. Figure 7A shows that NDF treatment increased the steady-state levels of 4.6 kilobase mRNA of COX-2 by 2–4-fold in CaCo-2 cells (compare lane 1 with lanes 2 and 3). Co-treatment of cultures with cycloheximide, a protein synthesis inhibitor, superinduced the COX-2 mRNA by twofold when compared to cycloheximide-mediated stabilization of COX-2 mRNA (Figure 7A; compare lane 5 with lane 6). Pretreatment of cells with actinomycin D (transcription initiation inhibitor) also prevented NDF-mediated increased COX-2 expression (compare lanes 3 and 4 with lane 6). These results indicate that COX-2 mRNA expression is regulated by NDF at the pretranslational level.

To further confirm the role of NDF in the transcriptional regulation of COX-2 gene, we transiently transfected chimeric luciferase gene fused with 5′ region of COX-2 promoter (Kutchera *et al*., 1996) and

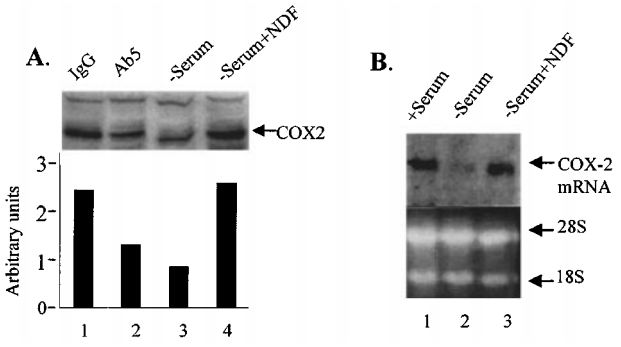

Figure 5 Constitutive activation of the HER2/HER3 pathway upregulates the expression of COX-2. (**A**) Subconfluent LS174T cells, cultured in the presence of 10% serum (lanes 1 and 2) or 0% serum (lanes 3 and 4), were treated with control IgG (lane 1), anti-HER3 mAb (lane 2) or NDF (lane 4). Total lysates were subjected to SDS–PAGE and blotted with anti-COX2 mAb. Quantitation of the COX-2 band is shown in the bottom panel. (**B**) Regular (lane 1) and 24 h serum-starved (lanes 2–3) LS174T cells were treated with or without NDF (50 ng/ml); COX-2 mRNA was analysed by Northern blotting

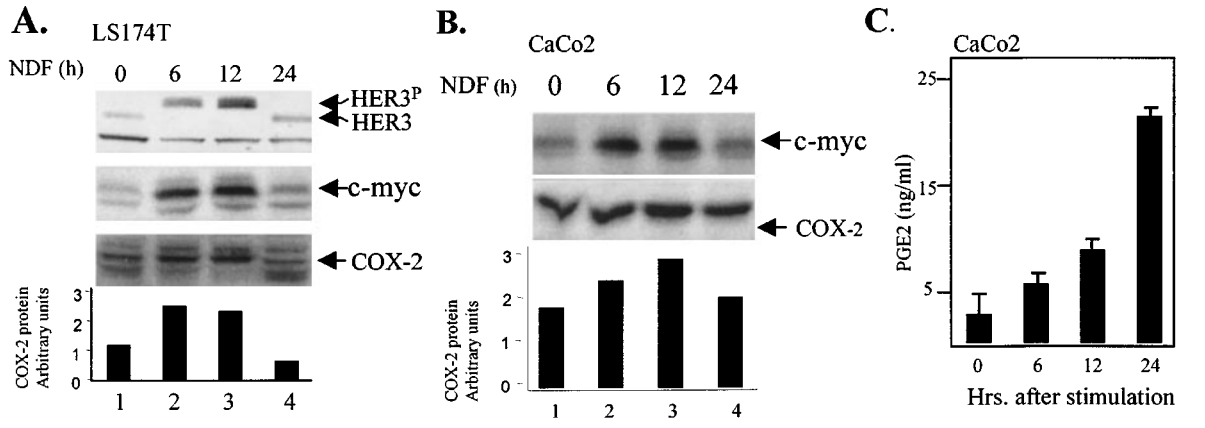

Figure 6 NDF induces COX-2 expression and PGE2 biosynthesis. (**A**) LS174T and (**B**) CaCo-2 cells were incubated with NDF (50 ng/ml). Total lysates (100 μg protein) were subjected to SDS–PAGE and blotted with HER3, COX-2 and c-*myc* mAb. (**C**) CaCo-2 cells (80% confluent) were treated with NDF (50 ng/ml) for the indicated times and PGE2 levels were assayed by RIA

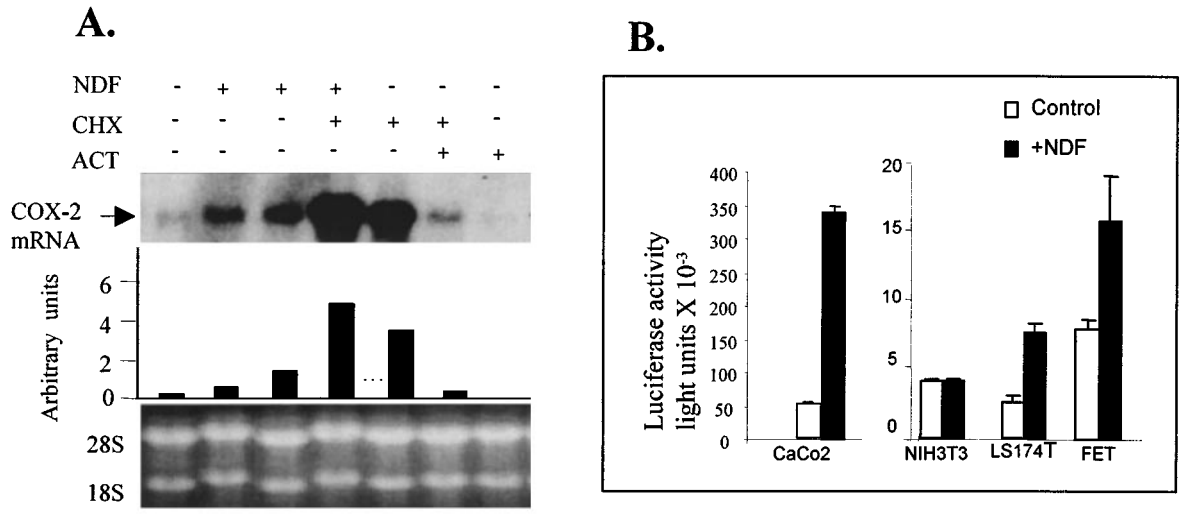

Figure 7 NDF regulates COX-2 gene at the transcriptional level. (**A**) CaCo-2 cells were treated with cycloheximide (50 μg/ml) or actinomycin-D (20 μg/ml) in the presence or absence of NDF (100 ng/ml) for 6 h, except lane 1 (3 h). Total RNA was isolated as described in the Materials and methods section and levels of COX-2 mRNA was detected by Northern blotting. Quantitation of COX-2 mRNA is shown in the bottom panel. (**B**) Luciferase reporter gene containing 5′ region of COX-2 gene was transiently transfected in to various cell lines and luciferase activity was measured after 36 h of transfection. Cell were treated for 6 h with NDF where indicated before lysis. Luciferase activity was normalized with b-gal activity. Data are means of two transfections and error bars represent standard error of means. Experiments with LS174, FET and NIH3T3 are repeated three times with similar results

the activity of the promoter was assayed in the presence or absence of NDF. In these studies, we used murine fibroblast NIH3T3 cells which express no or undetectable levels of HER3 were used as a negative control (Carraway *et al*., 1995). As illustrated in the Figure 7B, CaCo-2 cells showed high basal COX-2 promoter activity even in the absence of exogenous ligand and its activity was further stimulated by NDF treatment. However, in control NIH3T3 cells, the COX-2 promoter activity was not stimulated by NDF. Since CaC0-2 cells have very high levels of the basal activity, we examined the effect of NDF on COX-2 promoter activity in two other colorectal cancer cell lines FET and LS174T showed four- and tenfold less basal activity respectively, as compared to CaCo-2 cells, and NDF can effectively stimulate the level of COX-2 promoter activity to 2–3-fold (Figure 7B). The NDF treatment (4 h) of FET cells which express both HER2 and HER3 receptors also resulted in induction of expression of COX-2 mRNA (data not shown). The higher COX-2 promoter basal activity in CaCo-2 also correlated well with higher COX protein levels. These results show that NDF/NDF like factors can up regulate COX-2 promoter activity in colorectal cancer cells and thus confirms the regulation of COX-2 by transcriptional mechanism. In brief, our results demonstrate that the HER2/HER3 pathway induces COX-2 mRNA, protein expression and PGE2 biosynthesis in human colorectal cancer cells.

Expression of COX-2 in different cell-types treated with HRG

To examine if NDF also induces COX-2 in cells other than colon, we treated breast (MCF-7, MDA-453) and ovarian (SKOV-3) cell lines with exogenous HRG. As

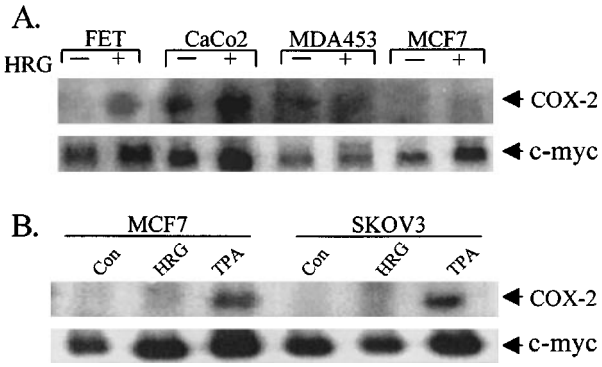

Figure 8 NDF specifically induces COX-2 mRNA in colorectal cancer cells. Indicated cell lines were treated with or with out NDF or TPA (phorbol ester) for 4 h. Total RNA (25 μg) was analysed by Northern analysis using COX-2 cDNA as probe as described in Figure 7. Blots were stripped and reprobed with c-*myc* cDNA (bottom panels in **A** and **B**)

shown in Figure 8, HRG treatment did not induce the COX-2 mRNA expression in breast and ovarian cells but induced the stimulation of c-*myc* mRNA expression, another potential target of HRG action. However, TPA (phorbol ester) a known inducer of COX-2 expression induced COX-2 mRNA in breast and ovarian cell lines suggesting presence of functional COX-2 gene in these cell lines. These results suggest that HRG mediated COX-2 accumulation in colon cells may be a tissue specific effect and this may be related to either stabilization of COX-2 mRNA or potential contribution of tissue-specific transcription factors in colon cancer cells. These possibilities are being investigated as a part of a separate study.

Inhibition of NDF-mediated mitogenesis and invasiveness by a COX-2 inhibitor

To investigate the significance of COX-2 in the action of NDF in colorectal cancer cells we examined the effect of a specific COX-2 inhibitor, NS-398, on NDF-mediated growth stimulation of LS174T cells. We have used NS-398 at a concentration of 20 μM in our experiments based on the earlier studies showing specificity and concentration required for specific inhibition of COX-2 (Coffey *et al*., 1997). The results shown in Figure 9A demonstrate that co-treatment (4 h) with NS-398 inhibited NDF-induced increased cell growth in LS174T cells. Since transformed colonic epithelial cells are known to survive in an extracellular matrix milieu such as Matrigel™, we also evaluated the effect of NS-398 on the ability of LS174T cells to form colonies in Matrigel™. As shown in Figure 9B, treatment with NS-398 resulted in a significant inhibition of NDF-mediated increase both in the number and size of colonies. Treatment with NDF also promoted the ability of LS174T cells to invade through 8 μm Matrigel™-coated filters and this phenotypic effect of NDF was suppressed by NS-398 (Figure 9C). The observed blocking effect of NS-398 on the biological action of NDF was due to the inhibition of COX-2 enzyme in NDF-treated cells, as cotreatment with NS-398 suppressed the stimulation of PGE2 release by NDF (Figure 9D). Taken together, these observations suggest a possible mediatory role of the COX-2 pathway in NDF-stimulated mitogenic responses in colorectal cancer cells.

Discussion

Activation of kinase activity associated with receptor tyrosine kinases by ligand binding is thought to be the common mechanism by which transmembrane receptors for growth factors transduce signals that activate cell proliferation. Growth factor receptors such as HER1 and HER2 play an essential role in the regulation of the proliferation of colonic epithelial cells. Emerging lines of evidence suggest that in addition to growth factors, the process of colorectal tumorigenesis may also be driven by the upregulation of the inducible form of cyclooxygenase-2 (COX-2), an enzyme responsible for the conversion of arachidonic acid to PGEs. The present study was undertaken to investigate the expression and activation of the HER family members, and to explore the regulation of COX-2 expression by the heregulin/HER3 pathway in human colorectal cancer cells.

The results presented here indicate that NDF is very potent mitogen for colorectal CaCO-2 and LS174 cells which express both HER2 and HER3 receptors. Treatment with exogenous NDF enhance the levels of tyrosine phosphorylation of HER3 and HER2 receptors

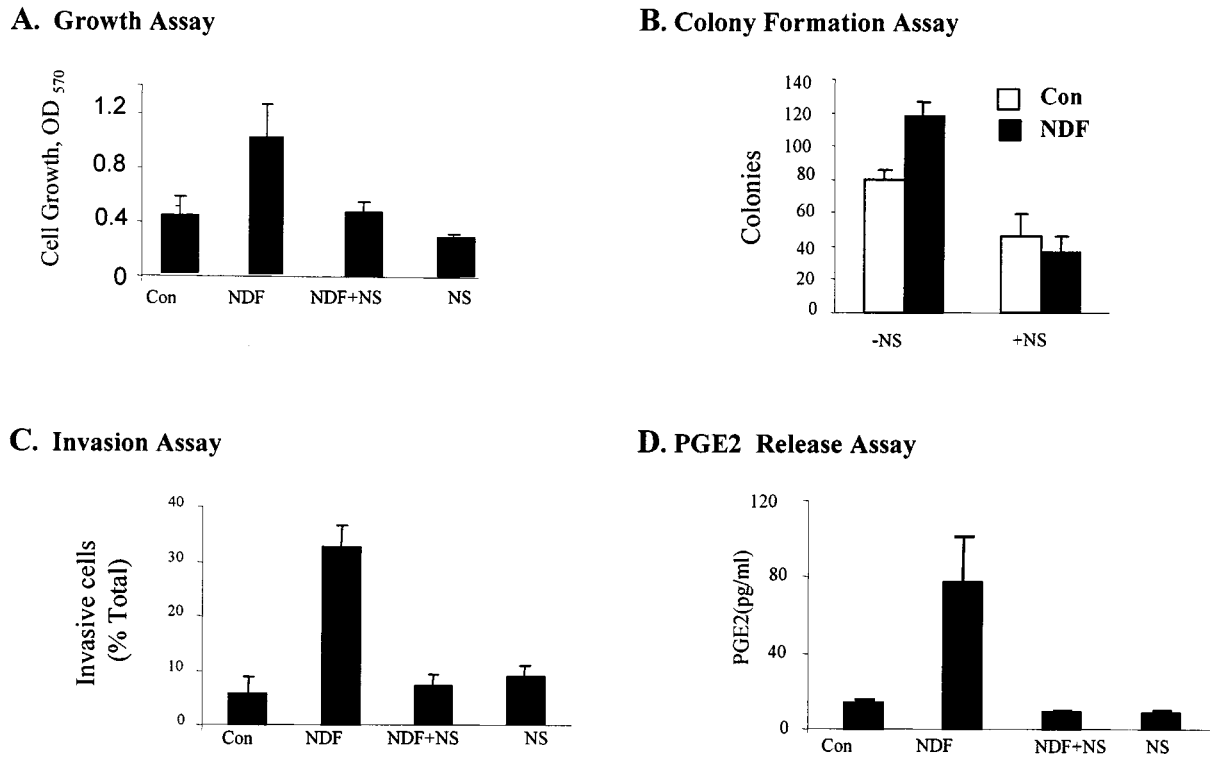

Figure 9 Inhibition of the biological effects of NDF in colorectal cancer cells by a COX-2 inhibitor. (**A**) Serum-starved LS174T cells were treated with NDF (50 ng/ml) for 4 h in the presence or absence of COX-2 inhibitor NS398, (20 μM, suboptimal concentration, ref. Tsujii and Dubois, 1995). NS-398 was added to the medium 30 min before the addition of NDF for and untreated cells were treated with DMSO control. Cell growth was measured by MTT-dye uptake method by determining the optical density (OD) at a wavelength of 570 nm as described in Figure 2. (**B**) Inhibition of NDF-stimulated cell survival by NS-398 in Matrigel™ assay. Cells were plated in Matrigel™ and colony formation was counted after 48 h. (**C**) Inhibition of NDF-mediated invasion by NS-398. After 48 h, cells which migrated through the Matrigel™ layer and the 0.8 μm membrane were counted. (**D**) Inhibition of NDF-mediated PGE2 release by NS-398. The amount of PGE2 in the medium was measured after 24 h of treatment. Each data point is a mean of two independent experiments performed in duplicate except in **B** where $n=6$

over the easily detectable baseline levels of endogenous receptor activation. Our conclusion that colorectal cancer cells may secrete factors that activate HER2/HER3 receptors in an autocrine/paracrine manner is supported by the following evidence: (i) constitutive activation of HER2 and HER3 receptors on tyrosine residue (Figure 2B and C); (ii) formation of HER2/HER3 heterodimers in the absence of any exogenous HRG (Figure 3A); (iii) a reduction in the levels of baseline HER2/HER3 heterodimers, tyrosine phosphorylation of HER3 upon treatment with anti HER3 mAb against ligand binding site (Figure 3B); and (iv) CaCo-2 and LS174T express and secrete of a protein with an approximate molecular mass of 40 kDa immune-recognized by a specific anti-HRG beta-1 mAb (Figure 4). Taken together, these findings provide new evidence in support of constitutive activation of the HER2/HER3 pathway and growth-stimulation of colorectal cancer cells by autocrine/paracrine heregulin or related factors. In the past, there were examples of enhanced expression of heregulin in mouse and human mammary epithelial cells transformed by HER2 (Martin *et al*., 1987), but constitutive formation of HER2/HER3 dimers has not been previously described. At the moment, we do not know the basis of the enhanced expression of heregulin in colorectal cancer cells, and whether there is any contribution of other pathways that are commonly deregulated in colorectal cancer, e.g. mutations in the APC gene and activation of Src (Prescott and White, 1996).

The finding that incubation of colorectal cancer cells with an anti-HER3 mAb against the NDF binding site results in a significant suppression of baseline expression of COX-2 expression is important, as it suggests that autocrine/paracrine activation of HER2/HER3 by cellular-derived factors such as heregulin may be involved in the regulation of COX-2 in colorectal cancer cells. We have demonstrated that the activation of HER3 receptor by recombinant HRG was accompanied by in-parallel stimulation of expression of COX-2 mRNA and COX-2 protein, as well as, PGE2 synthesis. The regulation of COX-2 mRNA by NDF appears to be transcriptional, as it is super-induced by cycloheximide and partially inhibited by actinomycin D. This view is further supported by the observation that NDF can enhance induction of COX-2 promoter activity in three colon cells. The induction of COX-2 pathway by NDF may have functional implications in colorectal cancer cells, as both the growth-stimulatory and invasiveness effects of NDF were completely blocked by a specific COX-2 inhibitor. The observation that the constitutive expression of COX-2 by endogenous NDF, or related factors which also regulate the growth of colorectal cancer cells, raises the possibility that the reported variations of basal COX-2 expression among different colorectal cancer cells could be due, in part, to variable degrees of interaction between endogenous NDF and HER2/HER3 receptors. It is possible that the endogenous NDFs and/or overexpressed HER2 family members could act as one potential cellular factor(s) that controls the action of the COX-2 protein. This is important because HER2 and HER3 have been shown to be widely overexpressed in human colorectal cancer, and this fact may have functional implications in the deregulation of the COX-2 pathway (Yang *et al*., 1997; Rajkumar *et al*., 1993; Kapitanovic *et al*., 1997 and this study).

Data from the literature suggest that COX-2 may also be positively regulated in some cells with mutated APC gene and/or activated Src family members, as both of these events are common in colorectal cancer (Prescott and White, 1996). In this context, it is significant to note that inclusion of anti-HER3 mAb completely prevented the formation of HER2/HER3 heterodimers, but only inhibited the levels of COX-2 protein by 50% compared to COX-2 expression in control cells. Therefore, it is possible that factors other than heregulin, i.e., activation of Src family members, may play a role in the observed COX-2 expression in anti-HER3 mAb-treated colorectal cancer cells either directly or indirectly via interaction with the carboxyl-region of the HER3 receptor. Alternately, it is also possible that overexpressed HER2/HER3 receptors may be partially activated intracellularly by endogenous ligands like the binding of v-*sis* to the PDGF-receptor in v-*sis*-transformed NRK cells (Keating and Williams, 1988), and thus by-pass the inhibition by exogenous anti-HER3 mAb. Further investigations are needed to determine the possible contribution of other pathways, and also signal-transduction pathways utilized by heregulin to regulate COX-2 expression.

Another significant finding of this study is the suppression of the mitogenic and invasive action of heregulin in colorectal cancer cells by a specific inhibitor of COX-2. Since inclusion of NS-398 to the culture of LS174T cells also prevented the stimulatory effect of heregulin on the release of PGE2 in the culture medium our results would suggest that COX-2 possibly may play a mediatory role in the action of heregulin in colorectal cancer cells. There is precedence for the role of COX-2 in the mitogenic effect of another growth factor, EGF (Coffey *et al*., 1997). Although overexpression of HER2 has been linked to cellular transformation and proposed to have a role in tumor progression in breast cancer cells (Lupu *et al*., 1995), our finding is the first to demonstrate both the mitogenic and invasive actions of heregulin in colorectal cancer cells, and its blockade by a specific inhibitor of COX-2, a well identified target in colorectal cancer. Taken together, our results raise new questions: (i) about the nature of signaling pathway(s) that may be involved in the regulation of COX-2; (ii) the possible molecular basis of cell type-specific regulation of COX-2 by NDF in colorectal cancer cells; and (iii) at what point in colorectal tumorigenesis the HER2/HER3 pathway is activated and its relationship to the upregulation of COX-2 and development of malignant phenotype.

Materials and methods

Cell cultures and reagents

Human colorectal carcinoma cell lines DiFi (Mandal *et al*., 1997; LS174T, CaCo-2, SW480, HT-29, SW613 and HCT116 (Mandal *et al*., 1997), FET (Brattain *et al*., 1984; Mandal *et al*., 1998), and breast cancer SKBR3 (Kumar *et al*., 1991) cells were maintained in DMEM-F12 (1 : 1) supplemented with 10% fetal calf serum. Antibodies against EGFR (# MS320-P1), HER2 (# MS-301-P1), HER3 (# MS262-P), HER4 (# MS-270-P1), c-*myc* (#MS-

130-P0), heregulin beta 1 (# RB-276-P0, original source Dr Yosef Yarden) and HER3 ligand binding site (# MS303PA) were obtained from Neomarkers Inc., Recombinant NDF-beta 1 (Kumar *et al*., 1996) was obtained from the Amgen. MAbs against COX-2 (#C22420), and Pho-tyrosine (# 4G10) were obtained from Signal Transduction and UBI, respectively.

Cell extracts

For preparation of cell extracts, cells were washed 3× with phosphate buffered saline and lysed in buffer (50 mM Tris-HCl, pH 7.5; 120 mM NaCl; 0.4% NP-40; 100 mM NaF; 200 μM $NaVO_5$; 1 mM PMSF; 10 μg/ml leupeptin; 10 μg/ml apotinin) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates containing equal amounts of protein were resolved on a 7% SDS–PAGE, transferred to nitrocellulose, and probed with the appropriate antibodies by using an ECL method or alkaline phosphatasebased color reaction method (Kumar *et al*., 1991; Kumar and Atlas, 1992).

Metabolic labeling and immunoprecipitation

An equal number of cells were metabolically labeled for 16 h with 100 μCi/ml ^{35}S-methionine in methionine-free medium containing 2% FBS in the absence or presence of mAb 225 and treated as indicated. Cells extracts were prepared as described above. Cell extracts containing an equal amount of total trichloroacetic acid perceptible counts were immunoprecipitated with the desired or control Ab, resolved on a SDS–PAGE gel, and analysed by autoradiography (Kumar and Atlas, 1992).

Matrigel^{TM} and invasion assays

Cell survival and the ability to form colonies in an extracellular matrix (Matrigel™; Becton Dickinson) were assayed according to Sheng *et al*. (1997). Cells were suspended in a DMEM:Matrigel™ (1 : 1) solution and poured on top of the thin gel layer in a 48 well plate. Cells were grown for 48 h at 37°C and the colonies formed were counted using an inverted microscope.

Cell invasion assays were performed as described previously (Adam *et al*., 1994), with few modifications and by using modified Boyden diffusion chambers containing a 8 μm porous membrane (Becton Dickinson). Conditioned medium obtained from NIH3T3 cells was used as a chemoattractant and was placed in the lower chamber. LS174T cells were serum starved for 24 h, trypsinized and placed in the upper chamber and treated with NDF in the presence or absence of COX-2 inhibitor. After 48 h the membranes were fixed and stained with DAPi. Cells that invaded and migrated to the lower surface were counted by using a Leica fluorescent microscope.

Chemical cross-linking

Cross-linking was performed according to Chen *et al*. (1996) and Fanger *et al*. (1989) with some modifications. Monolayer cells were incubated at 4°C with or without NDF (100 ng/ml) for 2 h. Cross-linking was performed at 4°C with 3 mM BS3 (Pierce). After 45 min of incubation, the reaction was quenched with the addition of ammonium acetate (20 mM final concentration), washed with PBS and lysed with lysis buffer. Total protein lysate (300 μg) was separated on a 6% SDS–PAGE and immunoblotting was performed with HER3, HER2 and Phos-tyrosine mAb.

Northern hybridization and COX-2 promoter-luciferase assays

Total cytoplasmic RNA was analysed by Northern hybridization using a complementary DNA probe (Oxford Biomedical Research) for human COX-2 mRNA as described (Kumar and Atlas, 1992). For HRG expression, a 1.8 kb human NDF α2b cDNA insert was used as a probe (Wen *et al*., 1992). 28S and 18S RNA were used to assess the integrity of the RNA and for RNA-loading control. COX-2 luciferase construct was obtained from Dr Steve Prescott and Luciferase assay were performed as described (Kutchera *et al*., 1996) with some modifications. Colon cells were serum starved in low serum medium (DMEM containing 0.1% serum) for 48 h before transfection. Serum starved colon cells were transiently co-transfected with COX-2 luciferase plasmid and control pSV b-Gal vector (Promega) using Lipofectamine (GIBCO). The transfection efficiency was normalized by cotransfection with pSV b-Gal vector (Promega). Luciferase activity was measured 36 h after transfection using luciferase assay kit (Promega). Where indicated cells were treated with (NDF 100 ng/ml) for 6 h before lysis.

PGE2 synthesis

Cells were grown in 60 mm plates to 80% confluence and serum starved for 48 h. Fresh medium (5 ml) containing 0.2% BSA was added and cells were treated with NDF (100 ng/ml). At indicated times, medium was collected and the amount of PGE2 was determined by PGE2 [^{125}I] RIA kit (Perspective Diagnostics) (Granstrom and Kindahl 1978). PGE2 concentration was normalized to total protein.

Abbreviations

NDF, recombinant neu-differentiation factor-beta 1; COX-2, cyclooxygenase-2; mAb, monoclonal antibody; PGE2, prostaglandin E2.

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