Oncogene (2004) 23, 1631–1635

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Regulation of *HER-2* oncogene expression by cyclooxygenase-2 and prostaglandin E2

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The oncoprotein HER-2/neu is a prosurvival factor and its overexpression has been correlated with adverse prognosis in breast cancers. High levels of the cyclooxygenase-2 (COX-2), a proinflammatory and antiapoptotic enzyme, were detected in HER-2-positive tumors and this observation was linked to an HER-2-mediated induction of COX-2 gene transcription. Here, we report that COX-2 expression, and synthesis of its major enzymatic product, PGE2, leads in turn to an enhanced HER-2 expression. Moreover, COX-2 enzymatic inhibition dramatically reduced HER-2 protein levels, efficiently increased the cancer cells sensitility to chemotherapeutic treatment and acted in synergy with HER-2 inhibitor, trastuzumab. Therefore, we propose an original model where HER-2 and COX-2 transcriptionally regulate each other in a positive loop.
Oncogene (2004) **23,** 1631–1635. doi:10.1038/sj.onc.1207295

Keywords: cyclooxygenase-2; HER-2; erbB-2; prostaglandin; PGE2; cancer

The *HER-2/neu* oncogene encodes a transmembrane receptor protein that is structurally related to the epidermal growth factor receptor (Coussens *et al*., 1985). HER-2 is overexpressed in approximately one third of the primary breast carcinomas (Press *et al*., 1993), and its increased expression is correlated with an adverse prognosis and a decreased overall and disease-free survival (Slamon *et al*., 1987; Ross and Fletcher, 1998). HER-2 overexpression has been demonstrated to enhance proliferative, metastatic and prosurvival signals in breast cancer cell lines (Hung *et al*., 1992; Ignatoski *et al*., 2000) and to induce resistance to hormonal therapy, paclitaxel and TNFα (Hudziak *et al*., 1988; Hung *et al*., 1992; Yu *et al*., 1998). Moreover, an anti-HER-2 monoclonal antibody, trastuzumab, displays clinical activity either alone or in combination with chemotherapy in HER-2-expressing breast cancers (Hudziak *et al*., 1989; Baselga *et al*., 1998, 1999; Pegram *et al*., 1998, 1999).

A link between HER-2 signalling and cyclooxygenase-2 (COX-2) expression has been recently established. Besides a coexpression in different tumor types, several reports showed indeed that HER-2 overexpression or activation increased the COX-2 gene transcription (Vadlamudi *et al*., 1999; Kiguchi *et al*., 2001). Recent data suggested that this regulation occurred through a MAPK-dependent pathway (Subbaramaiah *et al*., 2002).

COX-2 is an inducible enzyme that catalyses the synthesis of prostaglandins, prostacyclins and thromboxanes. COX-2 is expressed in transformed cells (Kutchera *et al*., 1996; Subbaramaiah *et al*., 1996) and in malignant tissues. It plays a major role in carcinogenesis since it has been demonstrated that enhanced synthesis of COX-2-derived prostaglandins (PGs) favors tumor growth by stimulating proliferation (Sheng *et al*., 2001), angiogenesis (Tsujii *et al*., 1998), invasiveness (Tsujii *et al*., 1997) and by inhibition of apoptosis (Tsujii and DuBois, 1995; Sheng *et al*., 1998). Moreover, selective COX-2 inhibitors reduce the formation and growth of experimental tumors (Sheng *et al*., 1997; Kawamori *et al*., 1998; Sawaoka *et al*., 1998; Harris *et al*., 2000) as well as the number of intestinal tumors in Familial Adenomatous Polyposis patients (Steinbach *et al*., 2000). Direct cause-and-effect relationship between COX-2 and tumorigenesis has also been established. COX-2-null mice were protected against development of intestinal and skin tumors (Oshima *et al*., 1996; Tiano *et al*., 2002). Moreover, a recent study demonstrated that COX-2 forced expression in murine mammary gland was sufficient to induce breast cancer in multiparous animals (Liu *et al*., 2001). Finally, COX-2 overexpression was observed in tumors from MMTV/neu mice, and its inhibition with celecoxib reduced the incidence of mammary tumors in this model (Howe *et al*., 2002).

Taken together, there is growing evidence that COX-2 may thus play an important role in mediating HER-2-induced mammary tumor formation. This led us to study the tight interrelations between those two

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Received 19 June 2003; revised 25 September 2003; accepted 3 October 2003

proteins, and to investigate whether HER-2 synthesis could also be regulated by COX-2 in MCF7 A/Z and T47D cells, two breast cancer cell lines.

To test this hypothesis, we first determined the influence of COX-2 transient expression on the HER-2 promoter. Increasing amounts of COX-2 expression vector were transfected in MCF7 A/Z mammary adenocarcinoma cells in the presence of an HER-2 proximal promoter reporter construct, p756LUC (Grooteclaes *et al*., 1994). Luciferase activity was induced in a dose-response manner (Figure 1a). We then assessed HER-2 mRNA levels in response to COX-2 ectopic expression. We established a stably transfected MCF7 A/Z cell line expressing COX-2 protein in the presence of tetracycline. This tetracycline-inducible cell line was named 'MCF7 ind'. HER-2-specific quantitative real-time PCR revealed that inducible COX-2 expression led to increased HER-2 mRNA levels, whereas tetracycline had no effect on HER-2 expression in MCF7 A/Z control cells (Figure 1b). Western blotting demonstrated that transient COX-2 expression in MCF7 A/Z and T47D cell lines enhanced HER-2 protein levels, whereas transfection of an empty vector did not (Figure 1c). These data clearly indicated that COX-2 induced HER-2 mRNA and protein expression.

As prostaglandin E2 (PGE2) is a major product of COX-2 enzymatic activity, we studied the PGE2 effect on HER-2 expression. A luciferase assay demonstrated that increasing amounts of PGE2 induced HER-2 proximal promoter transcriptional activity in a dose-dependent way (Figure 2a). HER-2-specific real-time PCR and western blotting further showed that PGE2 treatment also led to increased endogenous HER-2 mRNA and protein levels (Figure 2b and c). Taken together, these results strongly suggested that COX-2 activity increased HER-2 expression via its enzymatic product PGE2, but cannot rule out that other products are released in response to COX-2 activity and also influence the HER-2 promoter. The PGE2 prostaglandin is known to induce the cAMP signalling pathway. We observed that PGE2-induced HER-2 expression can be specifically blocked after cellular interaction with cAMP response element (CRE) decoys oligonucleotides (Benoit *et al*., unpublished results). However, these experiments remain preliminary and the PGE2-induced signalling pathways leading directly or indirectly to HER-2 gene expression need to be further explored.

To test the biological significance of these observations, the MDA-MB-231 cells were treated with

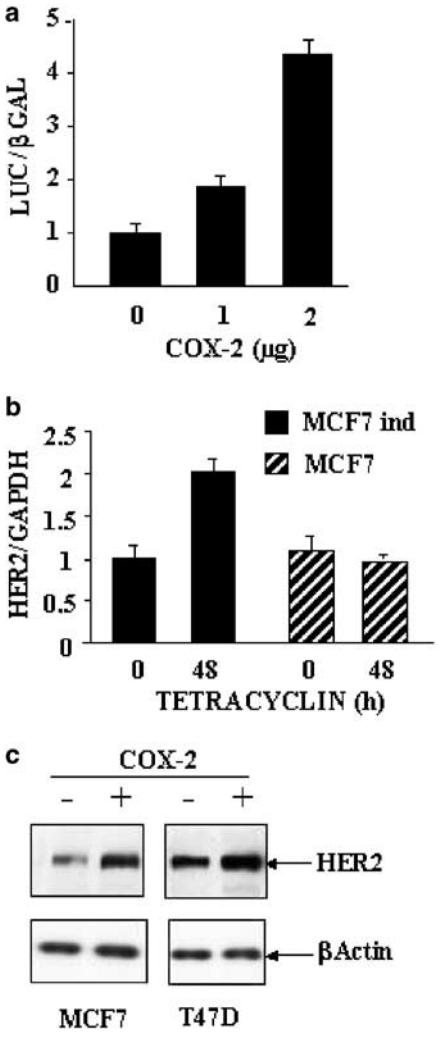

Figure 1 COX-2 expression induces HER-2 gene expression. (**a**) Breast adenocarcinoma MCF7 A/Z cells (a gift from Prof Mareel, university of Gent, Belgium) were transfected with an HER-2 proximal promoter luciferase reporter construct (0.5 μg), an RSV-βGAL plasmid (0.4 μg) and indicated amounts of COX-2 expression plasmid (a generous gift of Prof KKWu, Houston, TX, USA). Total amount of DNA was kept constant by addition of an empty pcDNA3 plasmid. At 6 h after transfection, arachidonic acid (10μM), the limitative COX-2 enzymatic substrate, was added in the medium. After 24 h, cells were lyzed, luciferase activity was determined with the Luciferase Reporter Gene Assay kit and βGalactosidase activity was assessed with the chemoluminescent βGal Reporter Gene Assay Kit (Roche, Mannheim, Germany). Luciferase activities were standardized with βGal activities and expressed as fold induction relative to the activity observed with the reporter plasmid alone. All the transfections were realized with FuGENE (Roche). (**b**) Control (hatched boxes) or COX-2 inducible (black boxes) MCF7 A/Z cells were treated for 48 h with tetracycline (1 μg/ml) in the presence of arachidonic acid (10 μM). Quantitative real-time PCR was performed using HER-2-specific oligonucleotides and GAPDH as internal control. The quantitative PCR reaction samples involved 2 μl of 20 $\times$ diluted cDNAs, 2 μl of 10 $\times$ SYBR Green PCR mix buffer, 1.6 μl $MgCl_2$, 25 mM and 7 μM of each primer. Quantification was performed with the Light Cycler PCR Technology (Roche, Mannheim, Germany). The primer sequences were as follow: *GAPDH*: 5′-ATGGGGAAGGTGAAGGTGGTC-3′ and 5′-TGATGGCATGGACTGTGG-3′; *HER-2*: 5′-AGACGAAGCATACGTGA-3′ and 5′-GTACGAGCCGCACATC-3′. (**c**) MCF7 A/Z and T47D breast adenocarcinoma cells were transfected either with pcDNA3 (−) or COX-2 expression (+) plasmid. At 24 h after transfection, cells were lyzed in SDS 1%, and 10 μg of total cellular extract were analysed by Western blotting using HER-2 (Upstate Biology, Lake Placid, UK) and βActin (Sigma, Bornem, Belgium)-specific antibodies. Arrows indicate HER2- and βActin-specific bands. The T47D cells were obtained from ATCC

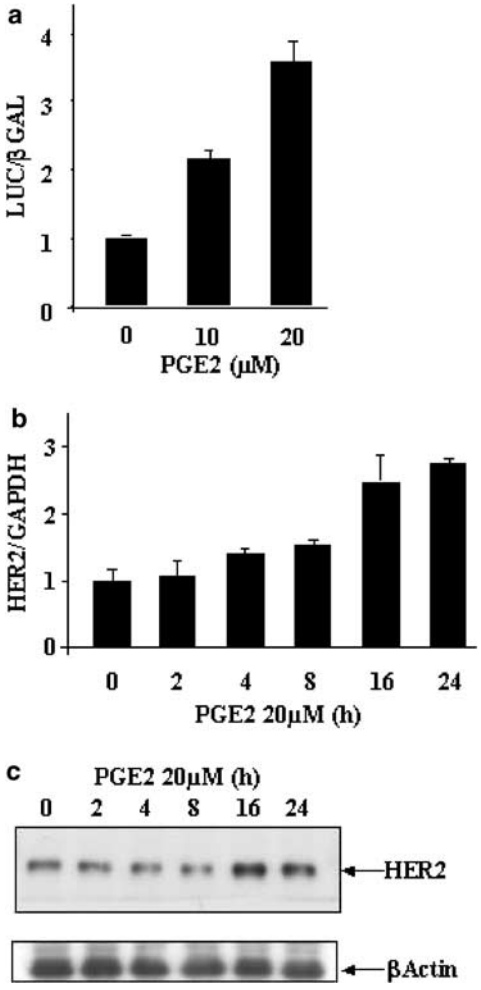

Figure 2 PGE2 induces the HER-2 gene expression. (**a**) MCF7 A/Z cells were transfected with an HER-2 proximal promoter luciferase construct (0.5 μg) and an RSV-βGAL plasmid (0.4 μg). At 6 h after transfection, cells were treated with PGE2 at the indicated concentrations for additional 24 h. LUC and βGal activities were evaluated as described in Figure 1. (**b**) MCF7 A/Z cells were stimulated with PGE2 (20 μM) for the indicated times. Quantitative real-time PCR was performed as in Figure 1. (**c**) MCF7 cells were treated with PGE2 (20 μM) for the indicated times and lyzed in SDS 1%. In all, 10 μg of total cellular extract was analysed by Western blotting as described in Figure 1. Arrows indicate HER-2- and βActin-specific bands

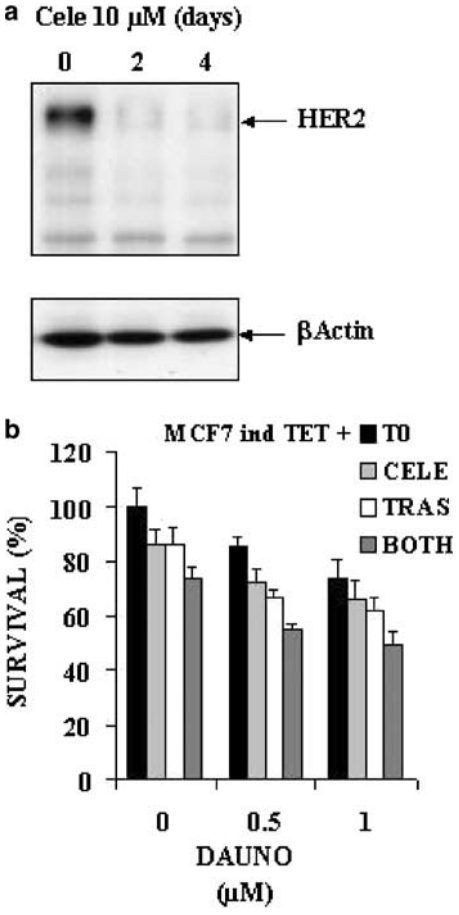

Figure 3 COX-2 inhibition decreases HER-2 expression and sensitizes cancer cells to daunomycin. (**a**) MDA-MB-231 cells were treated with celecoxib (10 μM) for the indicated times and lyzed in SDS 1%. In total, 10 μg of total cellular extract was analysed by Western blotting as described in Figure 1. Arrows indicate HER-2- and βActin-specific bands. (**b**) MCF7 ind cells, where COX-2 expression was induced by tetracyclin treatment, were left untreated or were pretreated for 48 h with trastuzumab (1 μM), celecoxib (10 μM) or both before a 24 h daunomycin stimulation. Cell viability was assessed by a colorimetric assay (WST-1, Roche) and expressed as percentage of the control

celecoxib, a specific COX-2 inhibitor, widely used in arthritis treatment. Western blotting experiment was then carried out and revealed that COX-2 inhibition completely blocked HER-2 expression (Figure 3a).

In order to test the putative pharmacological applications of these observations, the MCF7 A/Z breast cancer cells stably transfected with an inducible COX-2 expression vector (MCF7 ind) were pretreated *in vitro* with celecoxib, trastuzumab or a combination of both compounds before incubation with the cytotoxic agent daunomycine, a topoisomerase II inhibitor commonly used in the treatment of various human cancers. MCF7 A/Z cells that do not express COX-2 respond poorly to celecoxib either alone or in combination with daunomycin or daunomycin plus trastuzumab (data not shown). However, when COX-2 expression is induced in the MCF7 A/Z ind clone, celecoxib or trastuzumab reproducibly increased the daunomycin cytotoxic effect (Figure 3b). Moreover, when both inhibitors were simultaneously used to pretreat the cells before daunomycin addition, an additive effect was observed and the three drug combination therefore displayed the most efficient cytotoxicity. The same experiment was performed in MDA-MB-231 breast cancer cells that express COX-2 as well as moderate levels of HER-2. In these cells, similar data were obtained as celecoxib and trastuzumab individually increased daunomycin cytotoxicity, while a better effect was observed when both inhibitors were combined (data not shown).

Our data thus clearly demonstrate for the first time that COX-2 positively regulates HER-2 expression, through PGE2 production, and that COX-2 inhibition leads to a decreased HER-2 expression and to an

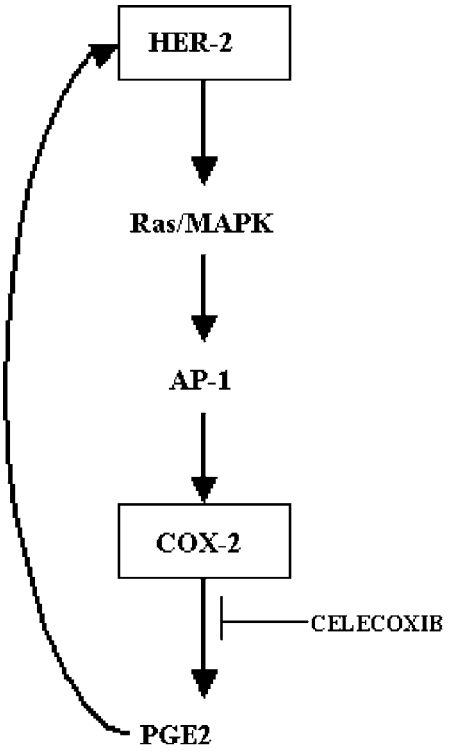

Figure 4 A model for HER-2 and COX-2 interactions. HER-2 expression is known to induce COX-2 expression through the Ras/MAPK/AP-1 pathway. In this report, we demonstrate that COX-2 can in turn stimulate HER-2 expression through its major product, PGE2. Selective COX-2 inhibitors, such as celecoxib, can disrupt this positive loop

improved cell sensitivity to daunomycin. As it has been previously demonstrated that HER-2 activated COX-2 synthesis (Vadlamudi *et al*., 1999; Kiguchi *et al*., 2001; Subbaramaiah *et al*., 2002), we, therefore, propose an original model where HER-2 and COX-2 transcriptionally regulate each other in a positive loop (Figure 4). This interrelation could be a new mechanism explaining the marked resistance of HER-2-overexpressing cells to antineoplasic therapies. Based on the expression of both proteins in some cancers, previous reports already suggested a benefit for combined HER-2 and COX-2 inhibition in the treatment of cholangiocarcinoma (Sirica *et al*., 2002) and colon cancers (Mann *et al*., 2001). We provide here additional support for coupling classic chemotherapy with COX-2 and HER-2 inhibitors.

It would also be most interesting to determine whether a similar effect could be observed with other receptors belonging to the EGFR family. Recent data showed that PGE2 activated EGF receptor by phosphorylation and triggers the subsequent mitogenic signalling pathway (Pai *et al*., 2002), but further experiments are required to establish the role of COX-2 and PGs on EGFR.

Acknowledgements
V Benoit is Research Assistant, A Chariot and M-P Merville are Research Associates at the National Fund for Scientific Research (Belgium). This research was supported by the 'Leon Fredericq Foundation', the 'Centre Anticancéreux près l'Ulg' (Liège, Belgium) and by grants from Télévie and the National Fund for Scientific Research (Belgium).

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