Neoadjuvant Therapy with Celecoxib to Women with Early Stage Breast Cancer

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Received July 24, 2007

Cyclooxygenase-2 (COX-2) is preferentially expressed in breast cancer cells compared to normal breast tissue. COX-2 inhibitors are, therefore, potential therapeutic options for patients with breast cancer.

Women newly diagnosed with non metastatic breast cancer were enrolled into the study after undergoing a diagnostic core needle biopsy. Patients received celecoxib treatment at 400 mg orally twice a day for 14 days, and then underwent surgical excision of their tumor. Core biopsies obtained at the time of initial diagnostic procedure and surgical excision specimens were stained for Ki-67, as well as COX-2 and cleaved poly (ADP-ribose) polymerase (PARP) expression (as an apoptosis marker). Appropriate negative and positive controls were included. We assessed the difference in Ki-67, COX-2 and cleaved PARP expression levels, before and after treatment using the Wilcoxon's matched-pair ranks test and the McNemar's test with continuity correction.

Sixteen patients were enrolled. The median age was 54 years. ER and/or PR expression was present in 81% of tumors; Her-2 neu overexpression was present in 25%. No significant change in COX-2 or cleaved PARP expression was noticed in the post intervention specimen compared to the core biopsies. Surprisingly, there was a significant increase in the Ki-67 expression ($p < 0.009$).

This short term prospective study was conducted to assess the effects of celecoxib, on the proliferative and apoptotic indexes in patients with early stage breast cancer. We have found an increase in the Ki-67 activity, with no significant down regulation of COX-2 or increase in cleaved PARP expression with 14 days of therapy. This could be partly due to the small sample size.

Key words: Breast cancer, Celecoxib, Neoadjuvant therapy

The rate of tumor growth is determined by a balance between pro-mitotic and pro-apoptotic stimuli [1]. An imbalance in favor of cell proliferation may contribute to tumorigenesis and tumor progression [2,3]. Apoptosis is morphologically characterized by nuclear condensation and cytoplasmic shrinkage. Nuclear fragments and cell-surface protuberances then separate to produce apoptotic bodies that are visible microscopically. The distinct morphological features of apoptosis allow semiquantitative apoptotic body assessment using routinely processed and stained histology slides [4,5,6]. This assessment is similar to mitotic figure counting, which is routinely performed in breast cancer evaluation. Analyses of cell turnover (programmed cell death and proliferation) have been reported to provide insight into tumor doubling time, prognosis, and treatment response [7-14]. High apoptotic counts have been associated with high histological grade, a high risk of lymph node metastasis [13], and shortened disease-free survival in limited studies of breast cancer patients [12,15].

Prostaglandins and thromboxanes are the main metabolites of arachidonic acid. They play multiple important physiologic functions such as mediation of signal transduction, cellular adhesion, cellular growth and differentiation [16,17]. Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin and thromboxane synthesis and is available in multiple isoforms (COX-1 and 2). COX-1 is constitutively expressed in most tissues and is involved in normal tissue homeostasis [18]. COX-2 is, on the other hand, an inducible isoenzyme and its expression is mediated by various mitogens including inflammation, cancer, hypoxia, growth factors,

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radiation and carcinogens. COX-2 has been found to be overexpressed in various tumors including colon, head and neck, lung, bladder, prostate, stomach and breast cancer [19-29]. Several studies have evaluated the prevalence of COX-2 expression in breast tissue and COX-2 has been documented to be overexpressed in 20-47% of breast cancer (both in-situ and invasive) and very rarely in normal breast tissue[30-34]. COX-2 overexpression in breast cancer has been associated with increased blood vessel density, higher likelihood of lymph node metastasis, increased apoptotic index, higher tumor grade, and decreased disease free survival [30,32].

COX-2 inhibitors have been shown to sensitize tumor cells to death receptor-induced apoptosis[35]. The higher the COX-2 expression, the higher is the response to COX-2 inhibitors [36]. Celecoxib (celebrex) is a selective COX-2 inhibitor and is FDA-approved for the treatment of osteoarthritis, rheumatoid arthritis and acute pain among others. It has a favorable side effect profile compared to nonselective NSAIDs, in particular, gastrointestinal ulcers. A placebo-controlled randomized study on the efficacy of celecoxib in reducing the incidence of colorectal polyps in patients with familial adenomatous polyposis (FAP) was recently reported[37]. In that study, celecoxib was administered at either 100 mg orally twice a day or at 400 mg orally twice a day for 6 months. The medication was well tolerated at both dose levels, the most common toxicities were diarrhea (placebo 13%, 100 mg 19%, and 400 mg 13%) and abdominal pain (placebo 13%, 100 mg 3%, 400 mg 7%). Celecoxib at 400 mg orally twice a day caused a 28% reduction in the number of colorectal polyps compared to a 4.5% reduction for placebo. Based on that study, the FDA has approved celecoxib at 400 mg orally twice a day for patients with FAP.

Materials and methods

The average waiting time between the day a diagnostic biopsy for breast cancer and undergoing excision of the tumor (lumpectomy or modified radical mastectomy) was around 2 weeks. During this 2-week period, women with histologically confirmed breast cancer were given celecoxib at 400 mg orally twice a day, free of charge. The aim of the study was to assess if the administration of celecoxib at 400 mg orally twice a day as preoperative therapy to women with early stage breast cancer affects the proliferative index of their tumors, as measured by Ki-67. We hypothesized that the administration of celecoxib for 2 weeks decreases the proliferative index of breast cancers. Tissue samples obtained at the time of original diagnostic biopsy and from the tumor resected after therapy with celecoxib, were tested for COX-2 overexpression and cleaved PARP by Immunohistochemistry (IHC) and stained for Ki-67. At the same time, routine staining for various other markers like estrogen and progesterone receptor (ER/PR) expression, her-2/neu overexpression, lymphovascular invasion, and tumor grade were done. The study was approved by the University of Oklahoma IRB and all patients signed an informed consent prior to enrollment on the study.

IHC for COX-2. Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 30 minutes at room temperature. Sections were then microwaved in PBS (pH 7.4) for 4 minutes for antigen retrieval. Nonspecific binding was blocked with avidin then biotin for 15 minutes each. The primary antibody against human COX-2 (Cayman Chemical, Ann Arbor, MI) recognizes a 19-amino acid sequence at the COOH terminus that is absent in COX-1. This antibody was applied at a dilution of 1:500 for 2 hours at room temperature. After rinsing with PBS, the biotinylated secondary IgG antibody was applied for 30 minutes at room temperature. Slides were then rinsed in PBS, and avidin conjugated to horseradish peroxydase (ABC reagent) was applied for 45 minutes at room temperature. The chromogen 3,3c-diaminobenzidine (Research Genetics, Huntsville, AL) was subsequently added, and the color reaction was evaluated with light microscopy. The reaction was stopped by immersing slides in deionized water. Slides were then counterstained, dehydrated, and a coverslip was attached. As a negative control, the primary Mab antibody was omitted and PBS was applied. The HCA-7 colon cancer cell line was used as a positive control for COX-2.

IHC Scoring. For COX-2, weighted score was computed that represents the product of percentage of tumor cell positivity and intensity as previously described [34]. Percent tumor cell positivity was categorized as follows: 0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; 4, >75%. Expression was categorized as negative (score 0-2), or positive (score 3-4).

IHC for cleaved PARP. Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 30 minutes at room temperature. Sections were then microwaved in PBS (pH 7.4) for 4 minutes for antigen retrieval. Nonspecific binding was blocked with avidin then biotin for 15 minutes each. The primary antibody that was used is directed against the cleaved (89 kDa) portion of PARP (Cell Signaling Technology; Beverly, MA). After rinsing with PBS, the biotinylated secondary IgG antibody was applied for 30 minutes at room temperature. Slides were then rinsed in PBS, and avidin conjugated to horseradish peroxydase (ABC reagent) was applied for 45 minutes at room temperature. The chromogen 3,3c-diaminobenzidine (Research Genetics, Huntsville, AL) was subsequently added, and the color reaction was observed with light microscopy. As a negative control, the primary Mab antibody was omitted and PBS was applied. Staining for cleaved PARP was reported as absent, weak, moderate or strong and the tumor was considered negative to cleaved PARP expression if the staining was absent or weak, and positive if the staining was moderate or strong.

Statistical Design. Ki-67 expression was reported as percentage of tumor cells expressing the antigen. COX2 and cleaved PARP expression were reported as negative or positive. Pre and post-treatment levels of Ki-67 expression were compared using Wilcoxon's matched-pairs signed-ranks test. Pre and post-treatment levels of COX2 and cleaved PARP

expression were compared using McNemar test with continuity correction. Two-tailed p values ≤0.05 were considered statistically significant.

Results

A total of 16 patients were enrolled into the study. Patient and tumor characteristics are summarized in table-1. The median patient age was 54 years (34 to 74 years). All patients had an ECOG performance status of 0 or 1. Eighty one percent of tumors expressed estrogen and/or progesterone receptors and 25% had her-2/neu overexpression. The tumors were well differentiated in 5 patients (31%), moderately differentiated in 7 patients (44%) and poorly differentiated in 4 patients (25%). Six patients had pathologic stage I (38%), seven had stage IIA (44%) and three had stage IIB (18%).

Table 1. Patient and tumor characteristics.

Number of patients	16
Median age in years (range)	54 (34-74)
Estrogen receptor positive	13 (81%)
Progesterone receptor positive	10 (62.5%)
Her-2/neu overexpression	4 (25%)
Tumor differentiation:	
Well differentiated	5 (31%)
Moderately differentiated	7 (44%)
Poorly differentiated	4 (25%)
Tumor stage:	
Stage I	6 (38%)
Stage IIA	7 (44%)
Stage IIB	3 (18%)

Intervention effect results are summarized in table 2. There was wide variation in the baseline Ki-67 expression among tumors with a range between 3 and 48%, with a mean expression of 20.5%. Surprisingly, the percentage of Ki-67 expression increased after treatment with celecoxib to a mean of 25.3% (p=0.009). Only 5 patients had a minor reduction in the ki-67 expression (the biggest reduction was 5%), while 11 patients had an increase in the expression.

There was little to no change in COX-2 staining after 2 weeks of treatment with celecoxib (p=0.4795). The majority of patients maintained the same staining intensity with no change at all; only 4 patients had a small reduction in the staining intensity (2 patients from 1 to 0 and 2 patients from 2 to 1). There was no correlation between the chance of having a drop in COX-2 expression and the tumor stage, grade, hormone receptor status or her-2/neu status.

Similarly, cleaved PARP expression did not change significantly between the pretreatment and post treatment specimens (p=0.6171). Only one patient had an increase in cleaved PARP staining (from 0 to moderate). Eight patients had a surprising increase in cleaved PARP staining. Like in COX-2 staining, no patient or tumor feature was more or less associated with a change in cleaved PARP staining.

Therapy with celecoxib was generally well tolerated and only one patient had epigastric discomfort and did not complete the 2 week course of celecoxib.

Discussion

Neoadjuvant therapy of breast cancer allows investigators to analyze the effect of various interventions on the breast cancer cells as the surgical excision can be done following the intervention and a good piece of tissue is thus available

Table 2. Change in Ki-67, COX-2 and cleaved PARP expression with therapy.

Patient	Age	Ki Pre-treatment (%)	Ki Post-treatment (%)	Ki Change	COX Pre-treatment	COX Post-treatment	PARP Pre-treatment*	PARP post-treatment
1	51	45	50	5	2	2	W	W
2	62	28	23	-5	1	1	W	W
3	47	48	60	12	2	3	M	W
4	36	37	65	28	1	0	W	W
5	66	8	6	-2	0	0	W	W
6	62	5	10	5	1	0	W	0
7	63	9	10	1	1	1	W	0
8	46	45	55	10	2	2	M	W
9	54	3	7	4	2	2	W	0
10	60	6	5	-1	1	1	W	W
11	74	11	9	-2	2	2	0	M
12	58	12	20	8	2	1	M	W
13	50	17	15	-2	1	1	W	W
14	34	11	18	7	2	1	0	0
15	57	20	22	2	1	1	W	0
16	54	22	30	8	3	3	W	0

*W: Weak; M: Moderate; S: Strong

for various testing modalities. In this study, we tested the effect a two week administration of high dose celecoxib, 400 mg orally twice a day, will have on the Ki-67 expression of breast cancers. We also studied the effect of this intervention on COX-2 expression and cleaved PARP as an apoptosis marker. The surgical excision was done within 24 hours of finishing the 2 week course of celecoxib.

The patient characteristics were typical of patients with early stage breast cancer. The tumor characteristics were also unremarkable. The study showed a surprising increase in the Ki-67 staining after treatment with celecoxib from 20.5% to 25.3% (p=0.009). This indicates a lack of significant reduction in the Ki-67 staining after treatment with celecoxib. Several factors might have contributed to this finding. First, the variability in Ki-67 staining is significant and there is a good degree of inter-observer variation. Second, the core biopsy procedure itself could have had an effect on the Ki-67 staining in the surgical excision specimen and therefore masking any potential reduction in the Ki-67 staining from celecoxib therapy. Third, the 2 week duration of therapy we used was chosen randomly and maybe it is not enough to have a significant effect on the study endpoints. Fourth, the ptients number of the study is small. This study was conducted around the time when data about the cardiac side effects of COX-2 inhibitors emerged and this significantly affected our ability to enroll more patients on the trial.

Similarly, the 2 week administration of celecoxib had no significant effect on the COX-2 expression in the cancer cells and on cleaved PARP expression. These findings will need to be validated in larger studies as this study is providing preliminary results on a small sample size of cases and is by no way large enough to address the question it tried to answer to begin with.

References

[1] STELLER H. Mechanisms and genes of cellular suicide. Science (Wash. DC) 1995; 267: 1445–1462

[2] MERLINO G. Regulatory imbalances in cell proliferation and cell death during oncogenesis in transgenic mice. Semin. Cancer Biol. 1995; 5: 13–20

[3] McDONNELL T. J. Cell division versus cell death: a functional model of multistep neoplasia. Mol. Carcinog. 1993; 8: 209–213

[4] van de SCHEPOP H. A. M., de JONG J. S., van DIEST P. J.,et al. Counting of apoptotic cells: a methodological study in invasive breast cancer. J. Clin. Pathol.: Mol. Pathol. 1996; 49: M214–M217

[5] HARRISON D. J. Counting apoptosis—why and how?. J. Clin. Pathol.: Mol. Pathol. 1996; 49: M245–M246

[6] CUMMINGS M. C., WINTERFORD C. M., WALKER N. I. Apoptosis. Am. J. Surg. Pathol. 1997; 2: 88–101

[7] TATEBE S., ISHIDA M., KASAGI N., et al. Apoptosis occurs more frequently in metastatic foci than in primary lesions of human colorectal carcinomas: analysis by terminal- deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling. Int. J. Cancer 1996; 65: 173–177

[8] SUGAMURA K., MAKINO M., KAIBARA N. Apoptosis as a prognostic factor in colorectal carcinoma. Surg. Today (Tokyo) 1998; 28: 145–150

[9] HEATLEY M. K. Association between the apoptotic index and established prognostic parameters in endometrial adenocarcinoma. Histopathology (Oxf.) 1995; 27: 469–472

[10] AIHARA M., TRUONG L. D., DUNN J. K., et al. Frequency of apoptotic bodies positively correlates with Gleason grade in prostate cancer. Hum. Pathol. 1994; 25: 797–801

[11] MAGI-GALLUZZI C. M., MURPHY M., CANGI M. G., et al. Proliferation, apoptosis, and cell cycle regulation in prostatic carcinogenesis. Anal. Quant. Cytol. Histol. 1998; 20: 343–350

[12] LIPPONEN P., AALTOMAA S., KOSMA V-M., et al. Apoptosis in breast cancer as related to histopathological characteristics and prognosis. Eur. J. Cancer 1994; 30A: 2068–2073

[13] FRANKFURT O. S., ROBB J. A., SUGARBAKER E. V., et al. Apoptosis in breast carcinomas detected with monoclonal antibody to single-stranded DNA: relation to bcl-2 expression, hormone receptors, and lymph node metastases. Clin. Cancer Res. 1997; 3: 465–471

[14] ZHANG G-J., KIMIJIMA I., ABE R., et al. Apoptotic index correlates to bcl-2 and p53 protein expression, histological grade, and prognosis in invasive breast cancers. Anticancer Res. 1998; 18: 1989–1997

[15] VAKKALA M., LÄHTEENMÄKI K., RAUNIO H.,et al. Apoptosis during breast carcinoma progression. Clin. Cancer Res. 199; 5: 319–324

[16] XIE WL. Mitogen-inducible prostaglandin G/H synthase: a target for nonsteroidal anti-inflammatory drugs. Drug Dev Res 1991; 25: 249–265

[17] TSUJII M, DUBOIS RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxidase synthase 2. Cell 1995; 83: 493–501

[18] O'NEILL G, HUTCHINSON AF. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS Lett 1993; 330: 156–160

[19] LIM HY, JOO HJ, CHOI JH, et al. Increased expression of cyclooxygenase-2 protein in human gastric carcinoma. Clin Cancer Res 2000; 6: 519–525

[20] BENNETT A, CHARLIER EM, McDONALD AM, et al. Prostaglandins and breast cancer. Lancet 1977; 2: 624–626

[21] WOLFF H, SAUKKONEN K, ANTTILA S, et al. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res 1998; 58: 4997–5001

[22] YIP-SCHNEIDER MT, BARNARD DS, BILLINGS SD, et al. Cyclooxygenase-2 expression in human pancreatic adenocarcinomas. Carcinogenesis 2000; 21: 139–146

[23] MOHAMMED SI, KNAPP DW, BOSTWICK DG, et al. Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. Cancer Res 1999; 59: 5647–5650

[24] HIXSON L, ALBERTS D, KRUTZSCH M, et al. Antiproliferative effect of nonsteroidal anti-inflammatory drugs (NSAIDs) against human colon cancer cells. Cancer Epidemiol Biomarkers Prev 1994; 3: 433–438

[25] CHAN G, BOYLE JO, YANG EK, et al. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 1999; 59: 991–994

[26] HIDA T, YATABE Y, ACHIWA H, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 1998; 58: 3761–3764

[27] WOLFF H, SAUKKONEN K, ANTTILA S, et al. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res 1998; 58: 4997–5001

[28] TUCKER ON, DANNENBERG AJ, YANG EK, et al. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res 1999; 59: 987–990

[29] TANJI N, KIKUGAWA T, YOKOYAMA M. Immunohistochemical study of cyclooxygenases in prostatic adenocarcinoma; relationship to apoptosis and Bcl-2 protein expression. Anticancer Res 2000; 20: 2313–2319

[30] COSTA C, SOARES R, REIS-FILHO JS, et al. Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. J Clin Pathol 2002; 55: 429–434

[31] HALF E, TANG XM, GWYN K, et al. Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. Cancer Res 2002; 62: 1676–1681

[32] DENKERT C, WINZER KJ, MULLER BM, et al. Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma. Cancer 2003; 97: 2978–87

[33] RISTIMÄKI A, SIVULA A, LUNDIN J, et al. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 2002; 63: 632–635

[34] WATANABE O, SHIMIZU T, IMAMURA H. et al. Expression of cyclooxygenase-2 in malignant and benign breast tumors. Anticancer Research 2003; 23: 3215–21

[35] TOTZKE G, SCHULZE-OSTHOFF K, JANICKE RU. Cyclooxygenase-2 (COX-2) inhibitors sensitize tumor cells specifically to death receptor-induced apoptosis independently of COX-2 inhibition. Oncogene 2003; 22: 8021–30

[36] HIDA T, KOZAKI K, MURAMATSU H, et al. Cyclooxygenase-2 inhibitor induces apoptosis and enhances cytotoxicity of various anticancer agents in non-small cell lung cancer cell lines. Clin Canc Resear 2000; 6: 2006–2011

[37] PHILLIPS RKS, WALLACE MH, LYNCH PM, et al. A randomized, double blind, placebo controlled study of celecoxib, a selective cyclooxygenase 2 inhibitor, on duodenal polyposis in familial adenomatos polyposis. Gut 2002; 50: 857–860