HER-2/neu Status Is a Determinant of Mammary Aromatase Activity

In vivo: Evidence for a Cyclooxygenase-2-Dependent Aromatase Mechanism

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Abstract

Cytochrome P450 aromatase (aromatase), a product of the CYP19 gene, catalyzes the synthesis of estrogens from androgens. Given the significance of estrogen synthesis in hormone-dependent breast carcinogenesis, it is important to elucidate the mechanisms that regulate CYP19 expression. The main objective of this study was to define the interrelationship between HER-2/neu, cyclooxygenase-2 (COX-2), and aromatase in mammary tissue. Mammary aromatase activity and prostaglandin E2 (PGE2) levels were increased in mice with mammary-targeted expression of a COX-2 transgene. In vitro, overexpressing COX-2 caused both increased PGE2 production and aromatase activity, effects that were suppressed by celecoxib, a selective COX-2 inhibitor. Previously, we found that overexpression of HER-2/neu was associated with increased levels of COX-2 in human breast cancers. Here, we show that overexpression of HER-2/neu is also associated with increased aromatase activity. These results suggested the possibility that COX-2 was the functional intermediate linking HER-2/neu and aromatase. Consistent with this idea, COX-2 deficiency led to a gene dose-dependent reduction in mammary aromatase activity in a HER-2/neu transgenic mouse model. Complementary in vitro studies showed that HER-2/neu-mediated induction of PGE2 synthesis and aromatase activity were suppressed by inhibiting COX-2. Collectively, our data indicate that COX-2 is the functional intermediate linking HER-2/neu and aromatase and suggest that inhibitors of PGE2 synthesis will suppress estrogen biosynthesis in breast tissue. (Cancer Res 2006; 66(10): 5504-11)

Introduction

Approximately 60% of breast cancer patients have hormone-dependent breast cancers, which express estrogen receptors and require estrogen for tumor growth. Estrogens are synthesized from androgens in a reaction catalyzed by the cytochrome P450 aromatase, encoded by the CYP19 gene (1). In postmenopausal women, peripheral aromatization in adipose tissues is largely responsible for estrogen production, and in particular mammary adipose tissue is considered an important local estrogen source. Aromatase activity is elevated in tumor stroma (2), suggesting that up-regulation of aromatase occurs during carcinogenesis and contributes to tumor growth (3). Thus, estrogen deprivation is a commonly used approach for the prevention and treatment of hormone-dependent breast cancer (4, 5).

Given the significance of estrogen synthesis in hormone-dependent breast carcinogenesis, it is important to elucidate the mechanisms that regulate CYP19 expression. The aromatase CYP19 gene is normally expressed from promoter I.4 in breast adipose tissue, but in breast cancers and cancer-proximal adipose tissue expression occurs predominantly from promoters I.3 and II (6–8). Transcription from promoter II is stimulated by cyclic AMP (cAMP)/protein kinase A (PKA)/cAMP-responsive element binding protein (CREB)–dependent signaling (9, 10). Several findings suggest that cyclooxygenase (COX)–derived prostaglandin E2 (PGE2) may act locally in breast tissue to stimulate CYP19 expression and aromatase activity. PGE2 is a potent inducer of CYP19 transcription and aromatase activity via a CAMP-dependent mechanism in breast adipose stromal cells in vitro (9, 10). Moreover, positive correlations have been detected between COX and aromatase expression in human breast cancer specimens (11, 12). Finally, aspirin, an inhibitor of COX-mediated PGE2 synthesis, was recently found to protect against hormone receptor–positive but not hormone receptor–negative breast cancers (13). Together, these data suggest that COX-derived PGE2 may play a role in hormone-dependent breast carcinogenesis.

There are two isoforms of COX, designated COX-1 and COX-2, which catalyze the first step in the synthesis of PGE2 from arachidonic acid. COX-1 is constitutively expressed in most tissues and seems to fulfill housekeeping functions (14, 15). In contrast, COX-2 is not detected in most normal tissues. However, it is induced by a variety of mitogenic and inflammatory stimuli (16–22), resulting in enhanced amounts of prostaglandins in neoplastic and inflamed tissues (23–25). COX-2 is overexpressed in transformed mammary epithelial cells (20), the preinvasive lesion ductal carcinoma in situ (DCIS; refs. 26–28) and in a significant proportion of breast cancers, particularly in those that overexpress HER-2/neu (26, 29–31). The results of numerous genetic and pharmacologic preclinical studies suggest that COX-2 is a bona fide molecular target for inhibiting breast carcinogenesis, including HER-2/neu–overexpressing cancers (32–36).

Collectively, the above findings suggest that nonsteroidal anti-inflammatory drugs, inhibitors of COX activity, should inhibit PGE2-mediated induction of estrogen biosynthesis in breast tissue. However, no direct causal relationship has been shown between COX expression and aromatase activity in vivo. Therefore, the aim of this study was to analyze the functional relationship between COX-2 and aromatase in vivo. Additionally, because COX-2 expression correlates with HER-2/neu overexpression in human...
Materials and Methods

**Materials.** DMEM, fetal bovine serum (FBS), LipofectAMINE, and hygromycin B were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-human COX-2 antiserum was from Santa Cruz Biotechnology (Santa Cruz, CA). Lowry protein assay kits, and horseradish peroxidase–conjugated secondary antibody, glucose-6-phosphate, glycerol, pepstatin, leupeptin, glucose-6-phosphate dehydrogenase and rotenone were from Sigma (St. Louis, MO). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham Biosciences (Piscataway, NJ). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). [3H]-[3H]androstenedione and [32P]CTP were from Perkin-Elmer Life Science (Boston, MA). Random-priming kits were from Roche Molecular Biochemicals (Indianapolis, IN). Plasmid DNA was isolated using a kit from Promega Corp. (Madison, WI). Luciferase assay reagents were from Analytical Luminescence (San Diego, CA). Celecoxib was from LKT Laboratories, Inc. (St. Paul, MN). The CYP19 cDNA was obtained from Open Biosystem, Inc. (Huntsville, AL).

The 18S rRNA cDNA was purchased from Ambion, Inc. (Austin, TX). siRNA to COX-2, green fluorescent protein (GFP) and DharmaFect 4 reagents were purchased from Dharmacon (Lafayette, CO). PGE2 assay kits were from Cayman Chemical Co. (Ann Arbor, MI).

**Animals.** Generation of the mice used in this study has been described in detail previously (32, 36). In one experiment, multiparous (four to seven pregnancies) nontransgenic and mouse mammary tumor virus (MMTV)–COX-2 transgenic mice were used (32). MMTV/NDL mice express a mutationally activated HER-2/neu allele (NDL, neu deletion mutant; ref. 37). In a second experiment, tissues were used from 20-week-old virgin females that were MMTV/NDL COX-2+/+, MMTV/NDL COX-2+/-, and MMTV/NDL COX-2−/− (36). Mammary tissues were harvested, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

**Human breast cancer samples.** Breast cancer samples were procured in accordance with an Institutional Review Board–approved protocol. Individual cancers were scored for HER-2/neu expression by immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections as a guide. Additionally, fluorescence in situ hybridization analysis was done on approximately half the samples and confirmed the immunohistochemical classification as being HER-2/neu positive or HER-2/neu negative. Purification of frozen tumor tissue was done under direct microscopic guidance using cryostat histologic sections as a guide. Nonneoplastic tissue was sliced into small pieces and then ground in liquid nitrogen before being suspended in buffer (20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 10% glycerol, 5 μmol/L pepstatin, and 5 μmol/L leupeptin). The tissue lysate was then centrifuged for 20 minutes at 800 × g and the pellet containing the nuclear fraction was discarded. The supernant was subjected to ultracentrifugation (1 hour at 100,000 × g) to separate microsomes from cytosol.

**Aromatase activity was determined in microsomes.** Microsomal protein was added to a 0.5 mL reaction mixture containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl2, 5 mmol/L glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase, 2 μmol/L rotenone, and 200 μmol 1/β-[3H]androstenedione. Following preincubation for 3 minutes, the reaction was initiated by the addition of 0.5 μmol/L NADPH and allowed to run for up to several hours at 37°C. Adding 3 mL ice-cold chloroform and applying vigorous shaking and brief centrifugation terminated the reaction. The resulting aqueous layer was further extracted with 3 mL chloroform and treated with 0.5 mL of 5% activated charcoal/0.5% dextran. Following centrifugation of the mixture, the radioactivity in the supernatant was counted. Aromatase activity was quantified by measurement of the tritiated water released from 1/β-[3H]androstenedione. The reaction was also done in the presence of a specific aromatase inhibitor, as a specificity control and without NADPH as a background control. Aromatase activity was normalized to protein concentration.

**Measurements of PGE2.** Mammary gland tissue (∼25 mg wet weight) was homogenized in ice-cold PBS, lipids were acidified and extracted (hexane/ethanol acetate 1:1), and PGE2 levels were determined by liquid chromatographic-electrospray ionization-mass spectrometry as described (38). PGE2 concentrations were calculated by determination of the ratios of peak areas to the internal standards. For analysis of cell lines, PGE2 levels in tissue culture medium were measured by enzyme immunoassay according to the instructions of the manufacturer. PGE2 levels were normalized to protein concentrations.

**Aromatase assays.** Aromatase activity was determined in the microsomal fraction of tissue lysates. Tissue was sliced into small pieces and then ground in liquid nitrogen before being suspended in buffer (20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 10% glycerol, 5 μmol/L pepstatin, and 5 μmol/L leupeptin). The tissue lysate was then centrifuged for 20 minutes at 800 × g and the pellet containing the nuclear fraction was discarded. The supernatant was subjected to ultracentrifugation (1 hour at 100,000 × g) to separate microsomes from cytosol.

**Figure 1.** Levels of PGE2, and aromatase activity are increased in the mammary glands of MMTV/COX-2 transgenic mice. A, PGE2 levels were measured in mammary glands from wild-type (NM) and MMTV/COX-2 (COX-2) multiparous female mice. PGE2 was extracted and assayed as described in Materials and Methods. B, aromatase activity was assayed in the same samples used in A. Enzyme activity is expressed as fmol/mg protein/h. Columns, mean; bars, SD (n = 7); *, P = 0.01; **, P < 0.01. C, total RNA was prepared from mammary glands from wild-type and MMTV/COX-2 mice and analyzed by Northern blotting. The blot was hybridized sequentially with probes for aromatase (CYP19) and 18S rRNA.
To determine cellular aromatase activity, microsomes were prepared from cell lysates by differential centrifugation. Subsequently, the enzyme activity assay was carried out as described above using 12.5 nmol/L 1\(^{1}H\) androstenedione. The activities were normalized to protein concentration.

### Northern blotting
Before RNA extraction, frozen tissues were homogenized using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Total RNA was prepared from mammary tissues using an RNA isolation kit according to the instructions of the manufacturer (Qiagen, Inc., Valencia, CA).

Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen. Fifteen micrograms of total cellular RNA/lane were electrophoresed in a formaldehyde-containing 1% agarose gel and transferred to nylon-supported membranes. COX-2, CYP19, \(\beta\)-actin, and 18S rRNA probes were labeled with \(^{32}\)P[CTP] by random priming. The blot was probed using previously described methods (30).

### Western blotting
Cells were treated with lysis buffer [150 mmol/L NaCl, 100 mmol/L Tris (pH 8.0), 1% Tween 20, 50 mmol/L diethyl dithiocarbamate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 \(\mu\)g/mL aprotinin, 10 \(\mu\)g/mL trypsin inhibitor, and 10 \(\mu\)g/mL leupeptin]. Lysates were sonicated for 20 seconds on ice and centrifuged at 10,000 \(\times\) g for 10 minutes to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (39). SDS-PAGE was done under reducing conditions on 10% polyacrylamide gels as described by Laemmli (40). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (41). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the instructions of the manufacturer.

### Statistics
Comparisons between groups were made by Student’s \(t\) test. A difference between groups of \(P < 0.05\) was considered significant.

### Results
Mammary aromatase activity is regulated by COX-2. We compared levels of PGE\(_2\) and aromatase activity in mammary glands from MMTV/COX-2 and wild-type mice. Because the MMTV promoter is hormonally responsive, maximal expression of the COX-2 transgene requires multiple cycles of pregnancy, lactation, and involution (32). Therefore, we used tissues from multiparous females in our experiments. Levels of PGE\(_2\) and aromatase activity were elevated \(-3\) and \(-4\)-fold, respectively, in mammary glands from MMTV/COX-2 mice relative to those from wild-type controls (Fig. 1A and B). Consistent with the observed increase in aromatase activity, increased amounts of CYP19 aromatase transcripts were detected in tissues from MMTV/COX-2 mice (Fig. 1C).

As a complementary approach, we conducted parallel in vitro studies. Fibroblasts were used as a model system because adipose
stromal fibroblasts are thought to be a major source of locally synthesized estrogen in breast tissues. NIH3T3 fibroblasts were stably transfected with a COX-2 expression vector to generate cell lines that overexpressed COX-2 and consequently exhibited increased PGE2 production (Fig. 2A and B). Aromatase activity was markedly increased in COX-2-overexpressing fibroblasts relative to that in control cells (Fig. 2C). Because PGE2-mediated regulation of aromatase transcription has previously been described (9, 10), it seemed likely that overexpression of COX-2 led to a PGE2-dependent increase in aromatase activity. In support of this notion, treatment of COX-2-overexpressing fibroblasts with the selective COX-2 inhibitor celecoxib caused dose-dependent decreases in both PGE2 production and aromatase activity (Fig. 2D and E). To confirm a direct role for PGE2 in regulating aromatase activity, NIH3T3 fibroblasts were treated with exogenous PGE2 for 24 hours and aromatase activity was then determined. Cellular aromatase activity increased in response to PGE2 in a dose-dependent manner (Fig. 2F). Together, these observations strongly suggest that the observed increase in aromatase activity in mammary glands from MMTV/COX-2 transgenic mice is due to the stimulatory effects of COX-2-derived PGE2 on CYP19 transcription.

**Aromatase activity is increased in HER-2/neu–overexpressing human breast cancers.** Having shown a causal relationship between COX-2 expression and aromatase activity, we next examined their interrelationship in human breast cancers. Previous studies have shown that ~40% of human breast carcinomas express COX-2 (31). Importantly, as mentioned above, COX-2 expression is particularly associated with HER-2/neu overexpression (28, 30, 31). Hence, we postulated that aromatase activity might be increased in human breast cancers that overexpress HER-2/neu. To evaluate this possibility, we compared aromatase activity in two groups of breast cancers stratified according to HER-2/neu status. We had previously used these specimens to interrogate the relationship between HER-2/neu and COX-2 expression, and found a highly significant correlation between COX-2 positivity and HER-2/neu overexpression (30). Remarkably, in the present experiment, we found that the HER-2/neu–overexpressing breast cancers (n = 13) exhibited an almost 200% increase in mean aromatase activity relative to the control group (n = 11; Fig. 3A). To confirm this finding, an experiment was conducted with a second set of 32 breast cancer samples composed of an equal number of tumors that either did or did not overexpress HER-2/neu. Once again, a nearly 2-fold increase in mean aromatase activity was found in HER-2/neu–overexpressing tumors compared with the control group (P < 0.001).

**Genetic or pharmacologic inhibition of COX-2 suppresses aromatase activity in vivo and in vitro.** The data obtained from our analysis of human breast carcinomas suggests a causal relationship between HER-2/neu overexpression, COX-2, and
aromatase activity. Because we had now shown an in vivo correlation between HER-2/neu and COX-2 expression (30), and between COX-2 and aromatase expression (Fig. 1), it seemed likely that COX-2 was the functional intermediate linking HER-2/neu to aromatase activity. Therefore, both whole animal and tissue culture approaches were used to address the involvement of COX-2 in HER-2/neu–mediated induction of aromatase.

Initially, we chose to evaluate the consequences of COX-2 deficiency on aromatase activity in mammary glands in a HER-2/neu transgenic mouse model. To achieve this goal, we generated MMTV/NDL females that were COX-2+/+, COX-2+/−, and COX-2−/−. Expression of the NDL transgene was previously found to be similar in mammary glands from MMTV/NDL mice of all three COX-2 genotypes (36). COX-2 deficiency led to a reduction in intramammary PGE2 (36) and a corresponding decrease in aromatase activity (Fig. 3). These results suggest that COX-2-derived PGE2 plays a key role in regulating aromatase activity in HER-2/neu–overexpressing mammary tissue. Complementary in vitro studies support this conclusion. Although overexpression of HER-2/neu occurs in tumor cells in vivo, we stably overexpressed HER-2/neu in NIH3T3 cells in an effort to model this phenomenon. NIH3T3 cells were chosen because this cell line has higher levels of aromatase activity and is easier to transfect than most breast cancer cell lines. Overexpression of HER-2/neu induced COX-2 and stimulated increases in PGE2 synthesis and aromatase expression and activity (Fig. 4). HER-2/neu–mediated induction of both PGE2 synthesis and aromatase activity was abrogated by treatment with celecoxib (Fig. 5A and B). To confirm this pharmacologic observation, we determined the effect of suppressing COX-2 expression on levels of PGE2 production and aromatase activity in fibroblasts that overexpressed HER-2/neu. Transfection of siRNA to COX-2 led to a >75% reduction in amounts of COX-2 compared with parental cells or cells that received siRNA to GFP (Fig. 5C). Notably, siRNA-mediated suppression of COX-2 levels was associated with a significant decrease in PGE2 production and aromatase activity in HER-2/neu–overexpressing cells (Fig. 5D and E). Taken together, our in vivo and in vitro data strongly support a model in which HER-2/neu stimulates aromatase activity via COX-2 up-regulation and consequent increases in PGE2 production.

Discussion

A positive correlation between COX and aromatase (CYP19) expression in human breast cancers has previously been reported (11, 12). This correlation has been suggested to reflect a functional relationship based on the knowledge that PGE2 can stimulate CYP19 transcription, and in particular drive expression

Figure 5. HER-2/neu–mediated induction of PGE2 biosynthesis and aromatase activity is suppressed by inhibiting COX-2. Control and HER-2/neu–expressing NIH3T3 fibroblast cell lines were generated by stable transfection with empty vector (control) or HER-2/neu expression vector, respectively. A and B, cells were treated with vehicle or 1.0 μmol/L celecoxib for 24 hours. A, cell culture medium was harvested and PGE2 levels were determined by enzyme immunoassay. B, aromatase activity was determined in cell lysates. Enzyme activity is expressed as fmol/μg protein/min. C to E, HER-2/neu–overexpressing NIH3T3 fibroblasts were transfected as indicated with siRNA to COX-2 or GFP. C, 100 μg cell lysate protein was subjected to immunoblotting. The immunoblot was probed with antibodies specific for COX-2 and β-actin. D, amount of PGE2 in medium was determined by enzyme immunoassay. E, aromatase activity was determined as described in Materials and Methods and expressed as fmol/μg protein/min. Columns, mean (n = 6); bars, SD. *, P < 0.001.
levels of aromatase activity were increased (Fig. 3A). A striking correlation between HER-2/neu and aromatase activity was observed (Fig. 3A) in human breast cancers stratified according to HER-2/neu status. Therefore, we compared aromatase activity in HER-2/neu-associated with reduced in mammary tissue from mice engineered to be deficient (Fig. 3B). Furthermore, overexpression of COX-2 in fibroblasts led to increased aromatase activity (Fig. 2). Consistent with a role for COX-2-derived PGE2 in regulating CYP19 transcription, celecoxib caused dose-dependent suppression of PGE2 synthesis and a corresponding reduction in aromatase activity in this cell system (Fig. 2D and E). Additionally, exogenous PGE2 caused aromatase activity to increase in aromatase activity (Fig. 2F). Together, these data provide the first demonstration that COX-2 expression regulates aromatase expression and activity both in vivo and in vitro.

Based on these data, an important prediction would be that aromatase activity should be higher in human breast cancers that overexpress COX-2. Previous studies from our laboratory and others have reported that COX-2 expression is particularly associated with HER-2/neu overexpression in human breast cancers (30, 31). Therefore, we compared aromatase activity in human breast cancers stratified according to HER-2/neu status. A striking correlation between HER-2/neu expression and aromatase activity was observed (Fig. 3A). More specifically, levels of aromatase activity were increased ~2-fold in HER-2/neu-overexpressing human tumors. To our knowledge, this is the first time a molecular determinant of aromatase activity has been identified (i.e., HER-2/neu status). Importantly, these data may provide a rational explanation for the previous observation by Ellis et al. (42) that ER-positive, HER-1/2-overexpressing breast cancers showed an improved response rate to the aromatase inhibitor letrozole compared with tamoxifen in this setting.

Because overexpression of COX-2 induced aromatase, we hypothesize that the correlation we observed between overexpression of HER-2/neu and enhanced aromatase activity reflects the increase in levels of COX-2 in HER-2/neu-overexpressing breast cancers (30, 31). Consistent with this interpretation, COX-2 deficiency led to a gene dose-dependent reduction in aromatase activity in mammary tissue from HER-2/neu–transgenic mice (Fig. 3B). Furthermore, overexpressing HER-2/neu in vitro induced COX-2 and aromatase (Fig. 4). In this instance, the selective COX-2 inhibitor celecoxib or siRNA to COX-2 suppressed HER-2/neu-mediated induction of PGE2 synthesis and aromatase activity (Fig. 5).

The current results help to refine and validate previous models that have attempted to explain the stimulatory effects of PGE2 on estrogen biosynthesis (43, 44). Overexpression of COX-2 in breast cancer cells leads to increased PGE2 synthesis (Fig. 6). PGE2, in turn, stimulates expression of the gene encoding aromatase via increased production of cAMP in stromal or tumor cells. Thus, estrogen synthesis is increased, which leads to increased proliferation of neoplastic cells. Our data suggest that overexpression of HER-2/neu will be the primary determinant of increased COX-2 expression and PGE2 synthesis in tumor cells, leading, in turn, to enhanced aromatase activity. COX-1 is also expressed in breast tissue and can contribute to PGE2 production (45). Additional studies are needed to determine the significance of COX-1-derived PGE2 as a determinant of aromatase activity and to identify the PGE receptors that are responsible for regulating CYP19 expression in vivo.

The demonstration that suppressing levels of COX-2 can inhibit mammary aromatase activity (Fig. 3B) has potentially significant clinical implications, because it suggests that inhibition of COX activity may be a viable strategy for decreasing estrogen biosynthesis and hence estrogen-dependent breast tumorigenesis in postmenopausal women (46). Indeed, because prostaglandins...
activate aromatase transcription from promoter II, which is predominantly active in breast cancer and cancer-proximal adipose tissue, COX inhibitors may specifically target aromatase in mammary tissue. Importantly, ~80% of DCIS express COX-2 (28), and these preinvasive tumors also tend to express estrogen receptor. Thus, the current results suggest that inhibiting COX-2-derived PGE\(_2\) production could represent a useful chemopreventive approach for retarding DCIS progression to invasive cancer. Indeed, the findings in this study provide a mechanism that may explain, at least in part, why aspirin protects against hormone receptor–positive breast cancer more effectively than hormone receptor–negative breast cancer (13).

Because an inverse relationship between HER-2/neu overexpression and estrogen receptor levels has been described (47), one might speculate that COX-2 inhibition, despite inhibiting aromatase, would have an attenuated effect on breast cancer development and growth. However, various hormonal agents are active even when low levels of estrogen receptor are expressed (48). Hence, even among patients with HER-2/neu–overexpressing tumors and low levels of estrogen receptor, our results suggest that there could be a significant benefit resulting from inhibiting PGE\(_2\) biosynthesis and thereby aromatase activity.

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References


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