Cyclooxygenase (COX)-2 and Granulosa Cell Apoptosis In Vitro

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Purpose: *C-myc* was studied in cyclooxygenase (COX)-2 associated granulosa cell apoptosis. **Methods:** Granulosa cells ($N = 5$ cases) were incubated for 24 h in either 1 or 50 μ M COX-2 inhibitor, 1 or 50 μ M COX-1/COX-2 inhibitor, negative or positive controls. Single primer polymerase chain reaction of *c-myc* exon 1 were performed. Bisbenzimidazole-stained control single-stranded (ssDNA) were hybridized to SYBR Gold-stained ssDNA and fluorescent images analyzed.

Results: *C-myc* was disrupted by the high-dose COX-2 inhibitor. Cell viability decreased with COX-1 and COX-2 inhibition. However, cell viability was similar for the positive control and at low-dose COX-2 inhibition.

Conclusion: Inhibition of both COX-1 and COX-2 initiated apoptosis without disrupting *c-myc* suggesting a protective effect on *c-myc*. The low dosage of the COX-2 inhibitor did not disrupt *c-myc* and cell viability. *C-myc* sensitization was not part of apoptosis.

KEY WORDS: Apoptosis; comparative genomic hybridization; cyclooxygenase COX-2; granulosa cells; prostaglandins.

INTRODUCTION

The two isoforms of cyclooxygenase enzymes, COX-1 and COX-2, actively convert arachidonic acid to prostanoids involved in many processes including the rupture of the follicle wall during ovulation (1–5), inhibition of apoptosis or programmed cell death, and stimulation of angiogenesis and tumor growth (6–10). Inhibition of COX-2 by non-steroidal antiinflammatory drugs such as celecoxib induces cell apoptosis (10,11). In contrast, the *c-myc* nuclear proto-oncogene is involved in neoplastic transformation and sensitizes damaged cells to apoptosis (12–14). The null hypothesis was that an inhibi-

tion of COX-2 neither had an effect on granulosa cell apoptosis nor caused disruption of the *c-myc* proto-oncogene. The objectives were to assess *c-myc* integrity and to measure cell viability in cultured granulosa cells after low- and high-dose COX gene inhibitor treatments.

MATERIALS AND METHODS

Collection and Culture of Granulosa Cells

The granulosa cells were from discarded cells in the follicular aspirates of routine in vitro fertilization procedures (15). The study was approved by the Loma Linda University Institutional Review Board. Briefly, ovulation induction was carried out in female patients ($N = 5$ cases) which involved several days of leuprolide acetate treatment followed by injections of recombinant follicle stimulating hormone (average of four ampules in split doses daily, 75 I.U. per ampule)

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for about 9–12 days. Follicular maturation and ovulation were triggered by administering 10,000 units of human chorionic gonadotropin (hCG) generally on the 10th day when the estradiol was over 500 pg/mL and the largest follicular diameter was over 18 mm. At about 34–36 h after hCG administration, the content of each mature follicle was aspirated by means of the transvaginal ultrasound technique and the oocytes separated out and incubated.

The accompanying sheets of granulosa cells that were routinely discarded were pipetted into culture dishes (double-well Falcon 3037, Becton Dickinson and Co., Oxnard, CA) containing a bicarbonate-based human tubal fluid (Irvine Scientific, Santa Ana, CA) supplemented with synthetic serum substitute (Irvine Scientific, Santa Ana, CA). The granulosa cells were pooled and divided into culture dishes containing either 1 or 50 μ M of a COX-2 inhibitor, celecoxib (Celebrex, G.D. Searle and Co., Chicago, IL), 1 or 50 μ M of a COX-1 and COX-2 inhibitor, acetylsalicylate (Aspirin, SmithKline Beecham Inc., Pittsburgh, PA), 100 μ M hydrogen peroxide (negative control), 3.6 mM pentoxifylline (PTX, positive control), or plain culture medium (untreated control). PTX, a methylxanthine phosphodiesterase inhibitor, was chosen as the positive control on the basis of studies showing an inhibition of apoptosis by PTX (16,17). The granulosa cells in the dishes were incubated at 37°C for 24 h in 5% CO₂ in air mixture.

After incubation, the pH of the buffered culture medium in each dish was checked and found to be similar to each other. A small portion of granulosa cells from each dish was removed and stained in 0.5% eosin viability stain (18,19) and the percentage of viable or live cells recorded. Over 100 cells were assessed in each treatment for each patient. The remaining cells were stored frozen at –25°C until assayed. The integrity of the proto-oncogene, *c-myc*, was assessed through a DNA disc chip assay on the basis of comparative genomic hybridization.

The DNA Disc Chip Assay

The frozen granulosa cells in the tubes were thawed at room temperature for 10 min. The cells were centrifuged at 3000 $\times g$ for 1 min to remove the culture media. Each resultant pellet was resuspended in 20 μ L of cold (4°C) alkaline lysis buffer (1% *N*-Lauroylsarcosine, 1.0 M TrisHCl pH 7.5, 0.5 M EDTA, 0.3 M mercaptoethanol, pH adjusted to >10 with sodium hydroxide pellets) for 20 min with periodic mixing (20). A volume (50 μ L) of TE buffer

(1.0 M TrisHCl pH 7.5, 0.5 M EDTA) was added to each tube and followed by heating at 95°C for 5 min to denature the DNA into single strands similar to the “hot start” PCR (polymerase chain reaction) protocol. Single-stranded DNA (ssDNA) were generated using the single-primer PCR method targeting exon 1 of the *c-myc* proto-oncogene (257 base pairs), the antisense primer and DNA from the treated cells (21). Single-stranded antisense DNA were generated using the sense primer and DNA from the untreated cells. The amplified antisense ssDNA from the untreated cells were stained in 9 μ M bisbenzimidazole (Hoechst 33342, Sigma Chemical Co., St. Louis, MO) for 5 min and centrifuge-washed twice to remove excess stain. (3000 $\times g$ for 1 min). The antisense ssDNA derived from the treated cells were stained in 1:10,000 diluted SYBR Gold stock stain (Molecular Probes, Inc., Eugene, OR) for 5 min and excess stain removed by repeated centrifugation. It was unnecessary to adjust the DNA concentration in the tubes because of the matched control-test design and saturation of the positive-charged miniature disc.

The miniature round discs used in the DNA disc chip assay (20) were made using a 2-mm Acupunch biopsy punch pen (Acuderm Inc., Ft. Lauderdale, FL) to cut into the Nytran (Schleicher & Schuell, Inc., Keene, NH) membrane sheet. The discs could be attached into the wells of a microtiter plate to process greater volume of samples similar to a microarray design (Fig. 1). For this study, each disc was first dipped into the tube of control (untreated granulosa cell) antisense ssDNA (stained in Hoechst 33342, blue fluorescence) for a few seconds. Then, the disc was dropped into the tube of sense (treated granulosa cells) ssDNA (stained in Sybr Gold, green–golden fluorescence). This process was repeated several times to obtain replicated data points.

Each tube with the submerged discs was kept at room temperature for a hybridization period of 10–20 s. Several discs were removed using a pair of microforceps, and lined up on a glass slide to form a DNA chip. Each glass slide with the DNA discs was examined using an ultraviolet epi-fluorescent microscope at 50 $\times$ magnification. The fluorescent images were photographed using an inexpensive QuickCam Pro camera (Logitech Inc., Fremont, CA) placed over the microscope eyepiece and saving the 640 $\times$ 480 pixels images to hard disk (20). For comparison purposes, the digital images were pasted on to a photo of a 50-well microtiter plate in a microarray design using the Microsoft Paint program. The images were converted to grey-scale and the pixel intensity

Fig. 1. Cultured granulosa cells were incubated in the presence of pentoxifylline (positive control), a specific COX-2 inhibitor, celecoxib, a COX-1 and COX-2 inhibitor, acetylsalicylate, and hydrogen peroxide (negative control) for 24 h at 37°C in a 5% CO₂ in air mixture. The figure shows a representative portion of the tested cells and the DNA disc chip assay. The eosin viability test was performed to differentiate live (clear color) and dead (red color) cells. The DNA disc chip assay is shown here with individual discs containing fluorescent DNA derived from *c-myc* exon 1 proto-oncogene PCR-synthesized from treated cells. In each row, the discs are the same except that different fluorescent wavelengths were used to first take an image (right column) of the control single-stranded DNA (bisbenzimid-stained, blue color) followed by an image (left column) of the matching single-stranded DNA (Sybr Gold, golden-green color) hybridized together on the same disc. Discs exhibiting lower fluorescence were associated with greater DNA fragmentation and apoptosis.

of each disc analyzed using Paint Shop Pro 6 software (Jasc Software, Inc., Eden Prairie, MN). Briefly, each image was opened in the program and under the color option, grey-scale conversion was selected. A circular tool option was selected to outline the image of the circular disc and the pixel intensity read from the histogram box. Greater damage of ssDNA was observed as a lower pixel intensity of the disc due to lack of pairing or mismatch with the control ssDNA.

Statistical Analysis

For each assay disc, the pixel intensity of the Sybr Gold-stained fluorescent image (treated sense ssDNA) was measured followed by the pixel intensity of the bisbenzimid-stained image (untreated antisense ssDNA). The ratio of the sense to antisense pixel intensities (index of image intensity) was calculated and expressed as mean $\pm$ SEM (standard error of the mean). Analyses of variance followed by significance testing of the means using the Student's *t* test statistic were used. Chi-square test statistic was used to analyze the number of live versus dead granulosa cells in each treatment group. A value of $P < 0.05$ was considered significant.

RESULTS

The *c-myc* exon 1 gene in cultured granulosa cells was disrupted by treatment with the high-dose (50 μ M) COX-2 inhibitor, when compared with the positive control (3.6 mM PTX). In contrast, there were no disruptions in the *c-myc* gene after the low-dose 1 μ M COX-2 inhibitor, 1 and 50 μ M of a combined COX-1 and COX-2 inhibitor, or the 100 μ M hydrogen peroxide treatment (Table I).

Interestingly, while the *c-myc* gene remained intact in all but the high-dose COX-2 inhibitor, the percentages of viable granulosa cells (Fig. 1) decreased in all groups except in the positive control group (PTX) and in the low-dose COX-2 inhibitor group. The two groups with the lowest percentages of live cells were the hydrogen peroxide (negative control) and the high-dose COX-2 inhibitor treatment groups.

DISCUSSION

The COX-2 enzyme is active in granulosa cells converting arachidonic acid to prostanoids implicated in

Table I. Viability and *c-myc* Exon 1 Integrity in Cultured Human Granulosa Cells After 24-h Exposure to Either a COX-2 Inhibitor or a COX-1 and COX-2 Inhibitor

Treatment	Eosin stain (N)	Live (%)	No. of <i>c-myc</i> assays (N)	Index of image pixel intensities
3.6 mM Pentoxifylline	400	76.0 $\pm$ 2.2	14	0.47 $\pm$ 0.04
50 μ M COX-2 inhibitor	400	27.0 $\pm$ 1.0*	18	0.39 $\pm$ 0.04*
1 μ M COX-2 inhibitor	400	62.3 $\pm$ 1.4	20	0.47 $\pm$ 0.06
50 μ M COX-1 and -2 inhibitor	400	36.5 $\pm$ 0.6*	20	0.44 $\pm$ 0.05
1 μ M COX-1 and -2 inhibitor	400	42.5 $\pm$ 0.6*	20	0.42 $\pm$ 0.04
100 μ M H ₂ O ₂	400	10.8 $\pm$ 0.4*	18	0.42 $\pm$ 0.02

Note. Cell viability was determined using the Eosin stain while *c-myc* integrity was assessed using the DNA disc chip assay. Values are presented as Mean $\pm$ SEM.

* $P < 0.05$ (Different from positive control, pentoxifylline).

the rupture of the follicular wall during ovulation (4,5). In this study, inhibition of COX-2 using a high dose of celecoxib was associated with *c-myc* gene disruption and apoptosis as shown by decreased granulosa cell viability (22). Previous reports link an inhibition of COX-2 to blocked interleukin-1 β -induced synthesis of prostanoids (5) and the release of arachidonic acid (23) which were associated with the induction of apoptosis (24), uncoupling of oxidative phosphorylation and downregulation of *bcl-2* (10,12). In contrast, inhibition of both COX-1 and COX-2 with acetylsalicylate caused apoptosis without disruption of the *c-myc* proto-oncogene, similar to the hydrogen peroxide treatment. This suggested that COX-2 inhibitor induction of granulosa cell apoptosis in the presence of COX-1 and prostanoids synthesis did not involve the *c-myc* proto-oncogene unlike the apoptosis process induced by acetylsalicylate. This study involved only low and high doses of the COX-2 inhibitor. Intermediate doses would probably have intermediate responses with an increasing disruption of the *c-myc* function.

The *c-myc* nuclear proto-oncogene is located in the long arm of chromosome 8 and is involved in neoplastic transformation and modulating RNA-turnover rates (25,26). Its function in DNA-damaged cells has been reported to be the sensitization of cells to apoptosis or programmed cell death (12–14). *C-myc* encodes for a transcription factor involved in regulating G2 phase apoptosis (13), in addition to regulating cell proliferation and differentiation activities (25,26). Increased expression of the *c-myc* protein in DNA-damaged cells leads to apoptosis in contrast to cancer-related transformation in DNA-intact cells (27). The interesting question to ask is whether or not *c-myc* is part of the damaged DNA during apoptosis. Obviously a damaged *c-myc* does not participate in the apoptotic process. In the acetylsalicylate-treated cell, an intact *c-myc* gene was present in the apoptotic cell. This suggested that inhibition of COX-1 and COX-2 was associated with *c-myc* function, apoptosis, and loss of cell viability.

In contrast to the higher dosage of the COX-2 inhibitor, the lower dosage did not disrupt the *c-myc* gene and cell viability was high suggesting that the lower dosage was not cytotoxic and did not cause granulosa cell apoptosis. A recent study also reported on the lack of cytotoxicity in cultured HCA-7 cells at low doses of the COX-2 inhibitor, celecoxib (22). In this study, only the higher dosage of the COX-2 inhibitor was associated with granulosa cell apoptosis. In vivo, the granulosa cells are responsible for

the aromatase conversion of androgens to estrogens. Apoptotic granulosa cells may result in symptoms associated with androgen excess.

In this study, the DNA disc chip assay (20) was used to assess the integrity of the *c-myc* DNA fragments produced by PCR amplification. The DNA disc chip assay was simple in design and cost-effective for gene analysis and permits small laboratories to take part in the exciting fields of comparative genomic hybridization (28) and microarray analysis (29). The principle behind this fluorescent assay was based on undamaged treated DNA hybridizing with control DNA located on a membrane disc similar to the Southern blot or dot-blot (30). Intact DNA exhibits maximal fluorescence in contrast to damaged or mutated DNA (31) that fails to hybridize and thus exhibits reduced fluorescence.

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