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Role of COX-2 in tumorspheres derived from a breast cancer cell line

Balraj Singh, Ph.D., Kendra R. Cook, B.S., Laura Vincent, B.S., Carolyn S. Hall, Ph.D., Cecilia Martin, B.S., and Anthony Lucci, M.D.¹

Department of Surgical Oncology and Advanced Research Center for Microscopic Disease, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

Abstract

Background—Cyclooxygenase-2 (COX-2) expression in primary breast cancer predicts tumor cell dissemination to bone marrow, which is a risk factor for recurrence and distant metastasis. “Stem-like” phenotype may be important in cancer metastasis.

Methods—To investigate the role of COX-2 protein in breast cancer stem-like cells we analyzed it by co-immunofluorescence in tumorspheres derived from the MCF7 estrogen receptor-positive breast cancer cell line. To evaluate COX-2 function we utilized a COX-2 inhibitor in a clonogenicity assay performed with tumorspheres-derived cells.

Results—We detected rare cells in tumorspheres (one cell per tumorsphere) with very high COX-2 expression (COX-2^{high}). COX-2 transfected MCF7 cells were able to generate long-term tumorspheres culture, even though transfection efficiency was only one in a million cells. We detected expression of OCT4 in some COX-2^{high} cells, supporting the hypothesis that these cells could be cancer stem-like cells. It is important that COX-2^{high} cells showed less expression of Ki-67 than did neighboring cells, indicating that COX-2^{high} cells may be progenitors of tumorspheres. Celecoxib inhibited the growth of tumorsphere cultures and the ability of tumorsphere-derived cells to form colonies in vitro, indicating an active role of COX-2 in these processes. However, 2 μ M celecoxib failed to eradicate tumorsphere-initiating cells. Finally, we detected rare COX-2^{high} cells among SUM149 inflammatory breast cancer cells growing on plastic in serum-containing medium; the SUM149 cell line produces a very high level of COX-2 protein.

Conclusion—Our results support a role for COX-2 in stem-like breast cancer cells and suggest a mechanism behind a role for COX-2 in disseminated tumor cells, which are known to exhibit characteristic biomarkers and functional properties of stem-like cells.

Keywords

Breast cancer; inflammatory breast cancer; COX-2; tumorsphere; cancer stem-like cell; disseminated tumor cell

¹To whom correspondence and reprint requests should be addressed at the Department of Surgical Oncology, Unit 444, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA. Phone: 713-563-1871; Fax: 713-792-4689; alucci@mdanderson.org.

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INTRODUCTION

Expression of cyclooxygenase-2 (COX-2) occurs early in premalignant epithelial cells of the breast, and this early expression may be linked to epigenetic silencing of the p16 (INK4a) locus [1]. Overexpression of COX-2 induces genomic instability in premalignant breast epithelial cells and in breast cancer cells [2,3]. We have investigated the role of COX-2 in metastasis of breast cancer to bone, which is the major site of metastasis seen in breast cancer. In a preclinical mouse model, we have shown that COX-2 is active in metastasis of breast cancer to bone via production of prostaglandin E₂ (PGE₂), interleukin-8 (IL-8), interleukin-11 (IL-11), and urokinase plasminogen activator (uPA) [4–7]. Furthermore, expression of COX-2 in stages I–III of breast cancer correlates with the presence of disseminated tumor cells (DTC) in the bone marrow [8], which is an independent predictor of clinical metastases [9]. It is interesting that we also detected COX-2 protein in solitary DTCs present in the bone marrow of breast cancer patients, further supporting a role of COX-2 in bone marrow micrometastasis (BMM) [8].

A small fraction of the tumor cells present in a primary tumor (<1%) drives cancer progression [10,11]. These cancer-initiating cells (CIC) can be enriched by several methods including selection for specific cell surface markers, side population (SP) cells, and mammosphere-forming cells in a serum-free medium [11–13]. Different studies have shown that these enrichment techniques select cancer cells that are resistant to radiation and chemotherapy [14,15]. It is interesting that both stem-like cells enriched for either specific cell surface markers CD44^{high}/CD24^{low} and stem-like cells enriched as SP cells have increased ability to form mammospheres [13,16]. DTCs are mostly dormant tumor cells [17] and have higher levels of stem-like phenotype measured by cell surface markers CD44^{high}/CD24^{low} than do primary tumors [18].

Based on findings by others that overexpression of COX-2 is one of the earliest alterations in breast cancer [1] and our findings that expression of COX-2 in primary tumors correlates with BMM [8], we hypothesized that COX-2 may participate in stem-like phenotype. We have provided data supporting our hypothesis that tumorspheres contain cells overexpressing COX-2 with properties expected of “cancer stem-like cells” (e.g., relatively rare and quiescent cells). To clarify our terminology, normal or malignant cells derived from the breast and growing as spheres in a defined medium are referred to as mammospheres, spheres, or tumorspheres. Since we investigated the role of COX-2 in cell lines that have been established from different types of breast cancer, we chose the term tumorspheres.

MATERIALS AND METHODS

Cell lines and tumorsphere culture

The MCF7 Tet-On cell line (MCF7 for brevity), which expresses reverse tetracycline-controlled transactivator for the purpose of inducing gene expression, was obtained from Clontech [Mountain View, CA] and was maintained at 37°C in RPMI 1640 medium supplemented with 10% Tet system-approved fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin in a humidified 5% CO₂ atmosphere. The COX-2 transfected MCF7 Tet On cell line (MCF7-COX2) has been described previously [3,6], and it was maintained similar to the parental cell line. The COX-2 transfected cell line has a significant level of COX-2 expression without the addition of inducer [3,6, this study]; since the COX-2 protein level does not increase significantly by the doxycycline addition, we chose not to add doxycycline. SUM149 inflammatory breast cancer cell line, originally obtained from Dr Stephen Ethier (Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA), was grown in Ham's F-12 medium supplemented with 5% fetal bovine serum, 5 µg/ml of insulin, and 1 µg/mL of hydrocortisone. Both untransfected MDA-MB-231 cell line and COX-2

transfected MDA-MB-231 cell line (with pSG5-COX2 vector) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Tumorsphere cultures were established from adherent cultures dissociated with trypsin or from rare floating cells present in adherent cultures of the parental and COX-2 transfected MCF7 Tet On cell lines. Tumorspheres were cultured in Mammary Epithelium Basal Media (LONZA, Walkersville, MD) supplemented with B27 (Invitrogen), 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 20 ng/ml of basic fibroblast growth factor (bFGF), 20 ng/ml of epidermal growth factor (EGF), and 4 µg/ml of heparin, as described previously [3,12]. The tumorspheres were cultured on low-attachment cell culture dishes or untreated plastic dishes. Tumorsphere cultures were passaged every 14 days. Cells in tumorsphere cultures were collected and centrifuged at 1,500 rpm for 5 minutes at 4°C and then incubated with 0.25% trypsin at 37°C for 5–10 minutes. We removed trypsin by adding 10 ml of growth medium, followed by centrifuging at 1,500 rpm for 5 minutes at 4°C. The cell pellet was resuspended in 3 ml of growth medium and filtered through a pre-wetted 30-µm filter (Miltenyi Biotec, Auburn, CA) to ensure a single-cell suspension. After filtration, the cells were centrifuged, and the pellet was resuspended in 500 µl of growth medium. An aliquot was mixed with an equal volume of trypan blue and was counted on a hemacytometer to determine the number of cells. Cells were plated in a range of 1,000 to 10,000 cells per milliliter.

Analysis of Immunofluorescence

To analyze expression of specific proteins, we spotted tumorspheres onto glass slides by cytospinning them at 400 rpm for 3 minutes, fixing them with 3.7% paraformaldehyde for 15 minutes at room temperature, permeabilizing them with 0.5 % Triton X-100 in phosphate-buffered saline (PBS), blocking them for 2 hours at room temperature in 0.5% Triton X-100 in PBS containing 1% bovine serum albumin, and then incubating them in primary antibody in blocking buffer overnight at 4°C. The primary antibodies were: COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI), COX-2 antipeptide antibody produced in goat, Ki-67 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and OCT4 polyclonal antibody (Cell Signaling Technologies, Danvers, MA). Antibody dilutions were 1:500 for COX-2 antibodies and 1:200 for all other antibodies. Appropriate secondary antibodies were applied for 1 hour at room temperature. The secondary antibodies were: antimouse IgG1 Alexa 488, antimouse IgG1 Alexa 555, anti-rabbit IgG Alexa 488, and anti-goat IgG Alexa 488 (Invitrogen), all used at a dilution of 1:1,000. Samples were incubated with DAPI stain (Invitrogen) at 5 µg/ml in PBS for 10–15 minutes. Coverslips were applied using ProLong Gold mounting medium (Invitrogen), and slides were stored in the dark at 4°C until analysis was performed. We collected indirect immunofluorescence images on a Leica DMLA microscope fitted with Hamamatsu ORCA-ER digital camera using Image-Pro Analyzer 6.1 software [MediaCybernetics, Inc., Bethesda, MD]. We collected selected images on an Olympus IX81 microscope with a Confocal Disc Spinning Unit attachment using 3i Slidebook SB 4.2.0.11 software (Intelligent Imaging Innovations, Inc., Denver, CO).

Clonogenicity assay

To determine the tumorigenic potential of cancer cells growing as tumorspheres, we performed clonogenicity assay as described previously [3]. To determine the involvement of COX-2 in the ability of tumorsphere cultures to form colonies, MCF7 tumorspheres were dissociated into single cells and plated at 500 cells/mL in RPMI 1640 medium with fetal bovine serum on regular cell culture dishes. These cells were grown for 15 days and then were stained with 0.1% crystal violet in PBS for 5 minutes. The plates were rinsed in water 5–10 times, until no more dye was detected in the rinse. After air drying the colonies, we

counted the colonies with the Bio-Rad Gel Documentation System XR equipped with Quantity One version 4.6.1 software and photographed them with a digital camera.

Western immunoblotting

Expression of COX-2 protein was detected by western blot analysis, as described previously [2]. Samples with equal amounts of protein were separated on 4–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels (BioRad, Hercules, CA) and transferred to a 0.45- μ m nitrocellulose membrane. The COX-2 protein was detected with a monoclonal antibody (Cayman Chemical, Ann Arbor, MI) and with the ECL Advance western blot detection reagents (Amersham Biosciences). The filters were stripped by incubating the membrane in 0.5% Triton X-100 and were re-probed with a monoclonal β -actin antibody (Sigma-Aldrich, St. Louis, MO) to serve as gel-loading controls.

Statistical analysis

Statistical analysis was performed using the two-sample *t* test. $P \leq 0.05$ was considered significant.

RESULTS

Expressions of COX-2 and OCT4 in tumorspheres

We began our study by investigating whether COX-2 protein is expressed in some cells that occur in MCF7-derived tumorspheres. In a previous study, we found that COX-2 is expressed at a relatively lower level in the MCF7 luminal-derived cell line than in the MDA-MB-231 basal-derived cell line [6]. As expected, our immunofluorescence analysis showed that COX-2 protein is expressed in MCF7 cells at a low level when grown as adherent cells in the presence of fetal bovine serum (Fig. 1). In contrast, while most cells present in MCF7-derived tumorspheres lacked expression of COX-2, some rare cells expressed a high level of COX-2 protein (COX-2^{high}) (Fig. 2). We confirmed the specificity of immunofluorescence detection of COX-2^{high} cells with several negative and positive controls, including the use of blocked primary antibody with prior incubation with the peptide antigen, no primary antibody incubation, and detection with a second COX-2 antibody (polyclonal anti-peptide antibody produced in goats).

We conducted the following experiments to determine the relationship of COX-2^{high} cells with other cells present in tumorspheres. To investigate whether COX-2^{high} cells present in tumorspheres express markers of stemness, we performed co-immunofluorescence analysis using COX-2 and OCT4 antibodies. OCT4 is a well-established marker of stemness in embryonic stem cells [19,20]. In this manner, we observed co-expressions of COX-2 and OCT4 in a subpopulation of COX-2^{high} cells (Fig. 2a,b). Strikingly, the OCT4-positive cells were the least abundant in tumorspheres. Any OCT4-positive cell we observed was either COX-2 positive or was immediately adjacent to a COX-2-positive cell (Fig. 2a–d). We could often find a COX-2-positive cell without an OCT4-positive cell in the same vicinity. It is important that both COX-2 and OCT4 proteins from indirect immunofluorescence images were localized to vesicular structures in COX-2^{high} cells (Fig. 2a–d). We confirmed subcellular localization of COX-2 and OCT4 by confocal microscopy, showing that the bulk of COX-2 and OCT4 proteins were localized to vesicular structures (Fig. 2d). The existence of a COX-2^{high} cell adjacent to a COX-2^{low} (cell with a low COX-2 level) or a COX-2-negative cell (Fig. 2) may be explained best by the defining property of stem cells, namely cellular asymmetry, which is regulated by the niche. Stem cells divide asymmetrically and give rise to two distinct daughter cells: one copy of them and one cell programmed to differentiate into another cell type.

Lack of expression of Ki-67 in COX-2^{high} cells in tumorspheres

Next, we investigated the lineage relationship between COX-2^{high} cells and other cells present in tumorspheres. We chose to analyze relatively small or young tumorspheres (6 days old) to easily visualize the relative position of COX-2^{high} cells within tumorspheres. This analysis showed clearly that COX-2^{high} cells lacked Ki-67, marker of cell proliferation, or had a much lower level of it than the majority of COX-2–negative cells present in tumorspheres (Fig. 3). In fact, COX-2^{high} cells were observed distally from the Ki-67–positive cells that must have been produced most recently in the tumorspheres (Fig. 3). We confirmed this result with confocal microscopy (not shown). The simplest interpretation of these data is that COX-2^{high} cells are progenitor cells giving rise to tumorspheres.

Effect of celecoxib on tumorsphere culture and colony formation

To determine whether COX-2 function is important in tumorsphere culture, we analyzed the effect of celecoxib—a COX-2–specific inhibitor. The treatment with celecoxib inhibited the growth of MCF7-derived tumorspheres in a concentration-dependent manner, as measured by cell counts (Fig. 4a). Next, we determined whether treatment with celecoxib would inhibit the ability of tumorsphere-derived cells to form colonies *in vitro*. Unlike the tumorsphere culture, the colony-forming assay was performed in a growth medium containing fetal bovine serum that would induce “differentiation”. Results of the *in vitro* colony-forming assay often indicate the cancer cells’ potential for *in vivo* clonogenic activity. We found that, similar to the results of tumorsphere growth in serum-free medium, treatment with celecoxib inhibited the colony-forming ability of tumorsphere-derived cells in a concentration-dependent manner (Fig. 4b). The 10-μM concentration of celecoxib that significantly inhibited both growth of tumorsphere and formation of colonies is in the lower portion of the range of celecoxib concentrations often used for *in vitro* studies. These results indicate that COX-2 function is important for tumorsphere growth and for the colony-forming ability of tumorsphere-derived cells. However, these results do not provide information about the sensitivity of specific subpopulations of cells to celecoxib.

Tumorspheres culture from COX-2 transfected MCF7 cells

We utilized COX-2 transfected MCF7 Tet On cells (abbreviated to MCF7-COX2) to 1) ask whether these cells generate tumorspheres-initiating cells, and 2) determine the relative level of COX-2 protein in 2-dimensional (2-D) culture growing on serum versus 3-D culture growing as tumorspheres (we could not do this with the parental MCF7 Tet On cells because COX-2 protein could not be detected by western blotting). Regarding the first issue, we observed that the MCF7-COX2 cells produced tumorsphere-initiating cells at least as efficiently as the parental MCF7 Tet On cell line. We were able to reproducibly generate long-term tumorspheres cultures (going 16 passages/32 weeks and beyond) from MCF7-COX2 cell line, starting both with rare floating cells in adherent culture and with adherent cells on a culture dish dissociated with trypsin. A representative image of tumorspheres at passage 5, derived from floating cells in MCF7 and MCF7-COX2 cultures, is shown (Fig. 5a). We have shown previously that the tumorspheres express both epithelial and myoepithelial markers [3]. As an important point, generation of MCF7-COX2 clones by transfection was extremely inefficient, approximately one in a million input MCF7 Tet On cells resulting in stably transfected clone, due to cytotoxic and cytostatic nature of COX-2 in these cells [6]. Thus our results, demonstrating the ability of MCF7-COX2 cells to generate tumorsphere-initiating cells, support an involvement of COX-2 in stem-like phenotype.

Our western blotting analysis demonstrated that the COX-2 protein is detected in MCF7-COX2 cell line but not the MCF7 cell line growing as a 2-D culture (Fig. 5b). The 3-D culture conditions caused a decrease in COX-2 protein in the overall cell population (Fig. 5b, compare lane 2 with lanes 6 and 10). We obtained a similar result with tumorspheres

generated from floating cells and those from adherent cells. Interestingly, when the cells from the 3-D culture were dissociated and re-cultured under 2-D culture conditions, the COX-2 protein returned back to a significantly higher level (Fig. 5b, compare lane 5 with lane 6, and lane 9 with lane 10), thus indicating that the 2-D culture conditions favor COX-2 expression at the population level.

Detection of COX-2^{high} cells in SUM149 cell line growing as adherent culture

Next, we investigated whether we could detect COX-2^{high} cells among breast cancer cells growing as adherent cells under conditions that favor differentiation (culture medium that contained FBS). To address this question, we chose an aggressive breast cancer cell line SUM149 that has been established from the primary tumor of a patient with inflammatory breast cancer. Among various breast cancer cell lines tested thus far (including MCF7, MDA-MB-231, Hs578T, MDA-MB-435S, SK-BR-3, MDA-MB-157, DU4475, and MDA-MB-453), we found that the SUM149 cell line produces the highest level of COX-2 protein. To illustrate this point, Fig. 6 presents a western blot with a significantly higher level of COX-2 protein in the SUM149 cell line than in a MDA-231 cell line transfected with COX-2. After performing co-immunofluorescence analysis with two COX-2 antibodies, a monoclonal antibody and an anti-peptide polyclonal antibody produced in goat, we clearly detected intensely labeled COX-2^{high} cells among a uniformly labeled cell population of SUM149 cells (Fig. 6a–d). It is noteworthy that the presence of COX-2^{high} cells may not be restricted to SUM149 cell line; such cells can be detected in MDA-MB-231 cell line and occasionally even in MCF7 cell line (Fig. 1).

Next, we investigated whether OCT4 is expressed in COX-2^{high} cells or their vicinity in SUM149-derived tumorspheres. We performed a COX-2/OCT4 co-immunofluorescence analysis on 7-days old first generation tumorspheres (Fig. 7). We did not detect any OCT4-positive cells in this experiment. One interpretation of these data is that molecular profile that governs tumorsphere formation is different between MCF7 and SUM149 cell lines. As mentioned above, the tumorspheres from different cell lines differ greatly in properties.

DISCUSSION

Relevance of the tumorspheres culture model

There is significant interest in the hypothesis that genetic alterations in a stem cell drive the progression of cancer. In breast cancer, the cancer stem cell would be an abnormal progenitor cell that could result from genetic or epigenetic misregulation. However, although indirect evidence supports the cancer stem-cell hypothesis, direct evidence that demonstrates tumor formation from a single cell of breast cancer is lacking. In this study, we investigated the mechanism and role of COX-2 in tumorsphere culture derived from MCF7 cells. We chose the MCF7 cell line with positive estrogen receptors because this cell line allowed us to culture well-formed tumorspheres for multiple passages. In a study by Cariati et al. [21], cultured tumorspheres from the MCF7 cell line without any prior enrichment displayed resistance to proapoptotic agents and significantly greater tumorigenicity than its parental line displays, with as few as 1,000 cells able to form tumors in immunodeficient mice. We have previously presented preliminary characterization of the tumorsphere culture, including the demonstration of both luminal epithelial and myoepithelial cell lineages present in the tumorspheres [3]. According to Dontu et al. [12], tumorspheres contain a heterogeneous cell population representing different degrees of lineage development and differentiation.

In this study, we chose tumorsphere culture to enrich the stem-like phenotype, since this culture shows the key defining features of stem cells, namely self-renewal and pluripotency.

Spheres can be cultured from the progenitor cells present in normal breast tissue, breast tumors, or some breast cancer cell lines [12,16,22]. Spheres contain both luminal epithelial and myoepithelial cells, indicating a bipotent nature of progenitors [3,12]. Additionally some degree of differentiation occurs in mammospheres culture, including the formation of extracellular matrix [12] that may aid the structural organization of mammospheres. None of the past studies have identified the progenitor cells that give rise to mammospheres, although tumorsphere culture conditions enrich for CD44^{high}/CD24^{low} cells and cells with increased resistance to chemotherapy [16].

COX-2^{high} cells as progenitors of tumorspheres

We took advantage of the tumorsphere culture system to determine the relative position of COX-2^{high} cells in the “developmental” hierarchical organization of cells within tumorspheres by co-analyzing markers of proliferation (Ki-67) and stemness (OCT4). Our results presented here are consistent with COX-2^{high} cells being progenitor cells of tumorspheres. These results include: 1) COX-2^{high} cells being a small fraction of the overall cell population in a tumorsphere, 2) evidence from Ki-67 immunofluorescence analysis indicating that COX-2^{high} cells may divide early and do not proliferate, and 3) further evidence from co-immunofluorescence of COX-2 and OCT4 that a subpopulation of COX-2^{high} cells are stem-like cells (Figs. 1–3). Our results support a model in which OCT4-positive COX-2^{high} cells (smallest subpopulation, e.g. those cells staining both red and green in Fig. 2) are cancer stem cells, while OCT4-negative COX-2^{high} cells are transit-amplifying cells with relatively diminished self-renewal potential and increased potential for cell proliferation compared with OCT4-positive COX-2^{high} cells; the remaining COX-2 negative/COX-2^{low} cells could represent “differentiated” cells that are nontumorigenic (see Fig. 2). The concept that COX-2 is involved in a stem-like phenotype is supported by recent studies that support a role of PGE₂ in both embryonic stem cells [23,24] and adult stem cells, e.g., hematopoietic stem cells [25]. In a recent study investigating the organ regeneration in zebrafish, Goessling et al. [26] provided evidence that Wnt activation in stem cells requires PGE₂ and suggested that the PGE₂/Wnt interaction is a master regulator of vertebrate regeneration and recovery.

We hypothesized that if COX-2^{high} cells were progenitors of tumorspheres or were their early progeny, COX-2^{high} cells would have to proliferate early and thus would lack a Ki-67 biomarker of proliferation at the time of analysis. In other words, determination of relative amounts of COX-2 and Ki-67 in individual cells and the relative physical positions of these cells in tumorspheres using COX-2 and Ki-67 co-immunofluorescence would shed light on the lineage relationship between COX-2^{high} cells and the recently produced Ki-67-positive cells. Our results showing that the COX-2^{high} cell is positioned distal to the Ki-67-positive cells within a tumorsphere (Fig. 3) support the hypothesis.

Although our results from the immunofluorescence analysis and from the inhibitory effects of celecoxib provide indirect evidence for a role of COX-2 in tumorspheres, we do not yet understand the biochemical pathways that are involved in such a role. The signaling studies in breast cancer cell lines indicate that COX-2 signaling can be linked with several pathways involving PI3K pathway, IL-8, IL-11, BCL2, uPA, Wnt signaling, EGFR, and Id-1, which could potentially explain the role of COX-2 in stem-like phenotype [3–7,27,28]. Additional indirect evidence supporting the role of COX-2 in stem-like phenotypes comes from the studies showing that 1) selection of a subpopulation resistant to doxorubicin within the MCF7 cell line accompanies selection for overexpression of COX-2 [29], and 2) in a complimentary approach, selection of subpopulation of cells that overexpresses COX-2 in a transfection experiment selects for a doxorubicin-resistant subpopulation [3].

We have not yet determined the relationship of COX-2^{high} cells with CD44^{high}/CD24^{low}, CD133-positive, or aldehyde dehydrogenase (ALDH)-positive stem-like cells [11,30,31]. However, indirect evidence shows that the COX-2 promoter may be active in CD44+/CD24- stem-like cells isolated from pleural effusions of breast cancer patients [32]. In the future, it will be important to show that COX-2^{high}/OCT4+ cells and COX-2^{high} cells can colonize in SCID-NOD mice more efficiently than in the mixed-cell population.

It is important to consider our results in the context of significant heterogeneity that exists in tumorsphere cultures with regard to the signal for the relative amount of COX-2 protein among COX-2^{high} cells. Many factors could contribute to this heterogeneity including 1) heterogeneity in progenitor cells and 2) dynamic nature of protein turnover. This dynamic nature could result in varying amounts of COX-2 protein that would depend on when a given COX-2^{high} cell was produced with reference to the time of analysis. It is possible that both cancer stem cells and transit-amplifying cells (heterogeneous themselves) could generate tumorspheres. Some differences could emerge when culturing the tumorsphere for multiple passages. For example, we often observe an increased tendency to aggregate in late-passage tumorsphere cultures. Most importantly, we do not yet know whether a lineage relationship exists between the tumorsphere with or without COX-2^{high} cells.

We do not yet know the basis of differential subcellular localization of COX-2 in tumorspheres. We observed COX-2 as perinuclear and in vesicular structures (Figs. 2 and 3). COX-2 localizes to endoplasmic reticulum, nuclear envelope, and caveolae-like structures in the plasma membrane in differentiated cells [33–35]. Interestingly, the OCT4 transcription factor that must function in the nucleus was also observed outside the nucleus in COX-2^{high} cells (Fig. 2). Although OCT4 has been detected in nonnuclear locations in previous studies on embryonic stem cells [36], its functional significance is not known. One distinct possibility would be that OCT4 represents a mechanism of sequestration from the site of function.

We believe that our observations may be generally applicable to other breast cancer cell lines, although the COX-2 network in tumorsphere-initiating cells may differ based on specific genetic and epigenetic contexts. Given that the MCF7 cell line is estrogen receptor (ER)-positive, even if our findings were applicable to only ER-positive breast cancers, it would be highly significant since the majority of breast cancers are ER-positive. MCF7 cell line exhibits a phenotype that is predominantly luminal when it is grown in 2-D culture conditions [16], although tumorsphere culture also shows the existence of bipotent progenitors [3,16]. In contrast, the SUM149 cell line exhibits a phenotype of mixed lineage comprising both luminal- and basal-like cell lineages [16]. Thus, it is possible that the nature of cancer stem cells in these two cell lines is different. Xiao et al. [37] have shown that increased stemness may be a characteristic of inflammatory breast cancers. In this regard, our results showing the presence of rare COX-2^{high} cells among SUM149 cells growing as adherent cells in the presence of fetal bovine serum (that contains differentiation stimuli) may be important. Further studies are needed to compare COX-2^{high} SUM149 cells with COX-2^{high} cells present in MCF7-derived tumorspheres and COX-2-positive cells present as DTCs in breast cancer patients. We hope that these investigations will lead to therapeutic targeting of COX-2 networks in DTCs, thus reducing the risk of recurrence in BMM-positive breast cancer patients.

Does celecoxib treatment eradicate tumorsphere-initiating cells?

Our interpretation of the experiments involving celecoxib is that it is unable to eradicate tumorsphere-initiating cells at a low dose. 2 μ M celecoxib, which inhibits more than 50% COX-2 activity in most cell lines [6, B Singh and A Lucci, unpublished data] did not significantly affect tumorspheres or clonogenicity of cells grown as tumorspheres (Fig. 4).

We were successful in culturing tumorspheres in the continuous presence of 2 μ M celecoxib for three passages without any significant inhibitory effect (data not shown). More recently we have obtained data that the variant cells expressing a high level of COX-2 protein exist in aggressive breast cancer cell lines, which are responsible for resistance against celecoxib treatment [B Singh, LR Irving, K Tai, and A Lucci, manuscript submitted for publication]. Our results have implications for the development of rational therapy against COX-2.

In conclusion, we have provided evidence for the first time that cells growing as breast cancer cell lines are heterogeneous with regard to expression of COX-2. We identified rare COX-2^{high} cells that appear to give rise to tumorspheres. These results provide important new insights into the role of COX-2 in breast cancer and in disseminated tumor cells present in the bone marrow of breast cancer patients.

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FIG. 1.

Low expression of COX-2 in MCF7 cells being grown as adherent culture. **a**, MCF7 cells growing in serum-supplemented RPMI medium were dissociated with trypsin and subjected to immunofluorescence detection of COX-2 using a monoclonal antibody and Alexa 488-conjugated secondary antibody as described in Materials and methods. MDA-231 cells, which are known to express a higher level of COX-2 than MCF7 cells, were subjected to COX-2 immunofluorescence detection in parallel to MCF7 cells. **b**, MCF7 cells growing in serum-supplemented RPMI medium were grown on a chamber slide and subjected to immunofluorescence detection of COX-2. **c**, MDA-MB-231 cells, grown on a chamber slide, were detected by COX-2 immunofluorescence in parallel to MCF7 cells. Immunofluorescence images both with (right) and without DAPI stain (left) are shown. All images were captured at 20X magnification.

FIG. 2.

Co-immunofluorescence of COX-2 and OCT4. **(a, b)** MCF7-derived tumorspheres (at passage 5, 8 days after cells in tumorspheres were dissociated) were subjected to immunofluorescence analysis as described in Materials and Methods. COX-2 (red) and OCT4 (green) could be detected on the same cell (see arrows in **a**) or adjacent cells (arrows in **b**). Note that COX-2^{high} cells are rare and OCT4-positive cells are even less abundant. **(c)** Expressions of COX-2 and OCT4 in adjacent cells. We dissociated tumorspheres (passage 5) with trypsin on day 8 before subjecting them to immunofluorescence analysis. The image shows a core of 4 cells present within a tumorsphere that resisted cell dissociation. One of these cells is positive for COX-2, and an adjacent cell is positive for OCT4. **(d)** Confocal microscopy showing membrane localization of COX-2 and OCT4 in a COX-2^{high} cell. The slide used for **a** and **b** was viewed under confocal microscope to better visualize the subcellular localization. The image presented is a single optical section of a z-series. Note the bright staining of COX-2 and OCT4 in the upper cell.

FIG. 3.

Lack of Ki-67 in COX-2^{high} cells. MCF7-derived tumorsphere (passage 4, 6 days after dissociation) were subjected to co-immunofluorescence analysis of COX-2 and Ki-67. Two small tumorspheres are shown (**a** and **c**, without DAPI to visualize green color better; **b** and **d**, with DAPI to visualize nuclei), each showing that COX-2^{high} cell (red) had relatively less Ki-67 (green) than other cells had in the vicinity, indicating that the COX-2-positive cell must be the first cell to divide among the group of cells present within a tumorsphere.

FIG. 4.

Inhibition of tumorspheres culture and its colony-forming ability with celecoxib. **(a)** Growth inhibition in tumorspheres culture (3-D culture). We dissociated MCF7 tumorspheres at passage 5 with trypsin and plated them at 15,000 cells per 15 ml in 100-mm dishes with different concentrations of celecoxib. Fourteen days later, tumorspheres were dissociated and stained with trypan blue, and viable cells were counted with a hemocytometer. The Y-axis shows total number of cells per dish. **(b)** Clonogenicity inhibition. MCF7 tumorspheres at culture passage 6 were dissociated into single cells and plated at 7500 cells/15 ml in RPMI 1640 medium containing fetal bovine serum (2-D culture). Colonies were allowed to grow for 15 days before staining with crystal violet. The number of colonies per dish is shown in a table on the right side of the figure. The experiments shown here are representative of at least three similar experiments at different passages.

FIG. 5.

Analysis of tumorspheres culture from MCF7-COX2 cell line. Tumorspheres were derived from the COX-2 transfected MCF7 Tet On cell line as described in Materials and Methods. **(a)** Representative bright field images of tumorspheres at passage 5 are shown. **(b)** Detection of COX-2 protein by western blotting. 5 μ g total protein was subjected to western blotting. Lanes 1 and 2, original cell lines at passage 11; lanes 3–6 (ts1), 3-D cultures derived from floating cells at passage 7; lanes 7–10 (ts2), 3-D cultures derived from adherent cells at passage 7; lanes 3,5,7,9, 3-D cultures grown in parallel to those shown in lanes 4,6,8,10, respectively, were dissociated with trypsin and cultured under 2-D conditions until they reached subconfluency before preparing lysates for blots. SK-BR-3 and SUM149 cell lysates from 2-D cultures are included as positive controls.

FIG. 6. Detection of COX-2^{high} cells among SUM149 cells growing as adherent culture. (a–d) SUM149 cells growing as adherent culture in a regular growth medium were dissociated with trypsin, cytopun onto coverslips, and subjected to co-immunofluorescence analysis using two different COX-2 antibodies. Brightly stained and rare COX-2^{high} cells were detected by both antibodies, a monoclonal antibody (red) and a polyclonal antibody (green), which confirmed their identities. The images show single fluorochromes (a, b) and merged versions of 2 and 3 fluorochromes, respectively (c, d). (e) A western blot showing a relatively high expression of COX-2 in SUM149 cell line.

FIG. 7.

Lack of OCT4 expression in SUM149-derived tumorspheres. We analyzed 7-days old first generation tumorspheres derived from SUM149 cell line by co-immunofluorescence of COX-2 and OCT4 as described in Materials and Methods. We detected COX-2 with a monoclonal antibody followed by an Alexa 488-conjugated secondary antibody, and OCT4 with a rabbit polyclonal antibody followed by an Alexa 594-conjugated secondary antibody. The right panel shows a bright field image. Images were captured using 63Xoil. Scale Bar=20μm.