Perioperative cyclooxygenase 2 inhibition to reduce tumor cell adhesion and metastatic potential of circulating tumor cells in non-small cell lung cancer

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Objective: Surgical manipulation of lung cancers may increase circulating tumor cells and contribute to metastatic recurrence after resection. Cyclooxygenase 2 is overexpressed in most non-small cell lung cancer and upregulates the cell adhesion receptor CD44. Our goal was to examine the effects of perioperative cyclooxygenase blockade on the metastatic potential of circulating tumor cells, CD44 expression, and adhesion of cancer cells to extracellular matrix.

Methods: Human non-small cell lung cancer cells (A549) were injected through the lateral tail vein in an in vivo murine model of tumor metastasis with three random treatment groups: no treatment, perioperative selective cyclooxygenase 2 inhibition (celecoxib) only, and continuous celecoxib. Lung metastases were assessed at 6 weeks by a blinded observer. For in vitro experiments, cells were treated with celecoxib, and expression of CD44 was determined by Western blotting. Extracellular matrix adhesion was assessed by Matrigel (BD Labware, Bedford, Mass) assay.

Results: In vivo lung metastases were significantly decreased relative to control by both perioperative and continuous celecoxib ($P = .0135$). There was no significant difference in number of metastases between continuous and perioperative treatment groups. In vitro adhesion to the extracellular matrix was significantly inhibited by celecoxib in a dose-dependent manner ($P < .01$). A549 cells expressed high levels of CD44, upregulated by interleukin 1β and downregulated by celecoxib.

Conclusion: Celecoxib significantly reduced establishment of metastases by circulating tumor cells in a murine model. It also inhibited CD44 expression and extracellular matrix adhesion in vitro. Perioperative modulation of cyclooxygenase 2 may be a novel strategy to minimize metastases from circulating tumor cells during this high-risk period.

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Despite surgical resection of early stage non-small cell lung cancer (NSCLC), the overall 5-year survival remains poor, with many patients having recurrence with distant metastases. Recurrence after surgical resection has typically been thought to be due to micrometastatic disease present at the time of surgery. Recently, there has been a resurgence in interest in the importance of circulating tumor cells (CTCs), the burden of which may be increased by surgical manipulation at the time of resection.^{1,2} It has recently been shown that the presence

Abbreviations and Acronyms

COX	= cyclooxygenase
CTC	= circulating tumor cell
ECM	= extracellular matrix
IL	= interleukin
NSCLC	= non–small cell lung cancer
Tris	= tris(hydroxymethyl)aminomethane

of CTCs portends a poorer prognosis in patients with breast cancer.³ Other studies have reported that approximately 20% to 48% of patients undergoing surgery for early stage lung cancer have CTCs before surgery, and the presence of CTCs has prognostic implications.^{4,6} Although CTCs may be present before surgery, it appears that surgery itself may increase the burden of these cells. Yamashita and colleagues¹ studied patient blood samples both before and after resection for the presence of CTCs and found that 89% of patients without preoperative CTCs had detectable tumor cells in peripheral blood after resection. Furthermore, in a more recent publication, Rolle and colleagues² reported indirect evidence that these surgically induced CTCs may ultimately result in established metastatic disease.

The perioperative period is a time of metastatic vulnerability. Typically, at the time of surgery no active systemic antineoplastic agents are used for fear of interfering with surgical outcomes. Standard chemotherapy regimens, even when given in the neoadjuvant setting, are usually completed well in advance of the planned operation. After surgery, there is a substantial period of recovery before a patient is considered for chemotherapy. Consequently, there is a period of at least a month before surgery and usually 1 to 2 months afterward when the patient is devoid of systemic antineoplastic agents. This perioperative period may in fact be the time when systemic therapies should be used. Surgery and anesthesia have been shown to have immunosuppressive consequences.⁷⁻⁹ Further, many of the inflammatory cytokines incited by the surgical procedure may act as growth factors or tumor promoters. Consequently, any CTCs present before or induced by surgery may have a greater chance of survival in the circulation, and thus a higher potential for establishing metastatic deposits. It seems therefore prudent to consider antimetastatic treatment in this perioperative period with agents that would have little effect on the surgical recovery of the patient.

The enzyme cyclooxygenase (COX) 2 is upregulated in most lung cancers and has been shown by us and others¹⁰⁻¹⁴ to increase tumor cell migration and invasion in vitro and to increase tumor metastatic behavior in vivo. Recently, this enzyme has also been implicated in modulating adhesion of tumor cells to extracellular matrix (ECM), a critical step in the metastatic process. COX-2 has been shown to upregu-

late expression of CD44, and transfection with antisense COX-2 RNA has been shown to decrease CD44 expression.¹⁵ CD44 is a cell surface receptor for various ECM proteins, including a nonsulfated glucosaminoglycan, hyaluronan, known to be important in tumor cell adhesion and invasion.¹⁶ Hyaluronan is released by invasion of tumor cells into the surrounding matrix and itself has been shown to promote tumor cell motility.^{17,18} Furthermore, some inflammatory cytokines have been implicated as regulators of affinity of CD44 for hyaluronan, and modulation of the cytokine response has the potential to modulate the effects of CD44 on hyaluronan binding.^{19,20} Similarly, COX-2 inhibition was recently shown to downregulate the expression of the CD44 receptor in gastric cancer and to be associated with impaired invasiveness.²¹ Blocking the activity of this enzyme, which is further upregulated by the inflammatory response to surgery, has the possibility of decreasing the metastatic potential of surgically induced CTCs. Although long-term use of many available selective COX-2 inhibitors (especially rofecoxib and valdecoxib) has been associated with cardiovascular side effects, these have only been seen with prolonged duration of treatment. Despite these negative side effects, the antitumor effects of celecoxib are significant, and the drug continues to be used in trials both alone and in combination with other modalities. Further, it appears to be safe when used perioperatively. The medication has been used previously as an analgesic adjuvant in the postoperative setting and has been shown to be safe, not interfering with the recovery of the patient after surgery.²²⁻²⁴ Consequently, if celecoxib can impede the metastatic potential of CTCs, this approach may be relevant as a perioperative treatment for patients undergoing surgical resection of any cancer.

To further understand the effect of COX-2 blockade on the metastatic potential of CTCs, we studied the effects of celecoxib on the ability of CTCs to establish metastatic deposits in vivo in a murine model of lung cancer metastasis and its impact on tumor cell–ECM adhesion and expression of CD44 in vitro.

Methods**Cells and Reagents**

A549 human lung adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, Va). Cells were cultured in Roswell Park Memorial Institute 1640 cell culture medium supplemented with 5% fetal bovine serum (GIBCO, Carlsbad, Calif), 2-mmol/L glutamine, 100-U/mL penicillin, and 100- μ g/mL streptomycin, at 37°C in a 5% carbon dioxide humidified incubator. Before experiments, cell viability greater than 95% was confirmed by trypan blue staining.

In Vivo Animal Model

The University of Southern California Institutional Animal Care and Use Committee approved this animal study. All animals re-

ceived humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” (<http://www.nap.edu/catalog/5140.html>).

The *in vivo* study used a tail vein injection lung metastasis model. Six-week-old severe combined immunodeficient (SCID-bg) male mice (Harlan Sprague Dawley, Inc, Indianapolis, Ind) were housed in polycarbonate cages (5 animals/cage) in a room lit for 12 hours each day and maintained at 27°C for 2 days before treatment. Teklad (4%) diet (Harlan Teklad, Madison, Wis) and tap water were provided *ad libitum*. Treatment diet consisted of Teklad (4%) mixed with celecoxib at 1000 ppm. Mice were randomly assigned to three treatment groups (*n* = 15-16 per group): control, perioperative celecoxib only, and continuous celecoxib. Celecoxib administration began 3 days before tumor cell injection for both perioperative and continuous treatment groups. Treatment was discontinued on postinjection day 3 for perioperative treatment mice and was continued for the duration of the 6-week experiment for the continuous treatment group.

A549 cells were prepared as a suspension of 10^6 cells in 150 μ L phosphate-buffered saline solution. Cells were injected through a 29-gauge needle into the lateral tail vein under sterile conditions. Mice were killed 6 weeks after injection, and lung metastasis was determined by counting the total number of neoplastic nodules present in the lung, as seen on the surface with a stereomicroscope and verified by immunohistopathologic examination by a blinded observer. Tumors were either snap frozen in Tissue-Tek OCT compound (Miles Inc, Elkhart, Ind) in a beaker of 2-isopropanol on dry ice (−18°C) or fixed in 10% neutral buffered formalin and embedded in paraffin.

Immunohistochemical Testing

Paraffin-embedded tissue sections were prepared on Superfrost Plus slides (Erie Scientific Company, Portsmouth, N.H.) at 4 μ mol/L. Sections were dewaxed in xylene and rehydrated in graded alcohol baths. Citrate at pH 6.0 (Zymed Laboratories Inc, South San Francisco, Calif) was used for antigen retrieval. Slides were left for 20 minutes in this solution at 95°C and allowed to cool to room temperature. Endogenous peroxidase was then blocked by incubation in 3% hydrogen peroxide in methanol. Nonspecific mouse antigen was blocked with rabbit normal serum (Vector Laboratories, Inc, Burlingame, Calif). Endogenous avidin and biotin blocking was done with A/B Blocking reagents (Vector Laboratories). Primary COX-2 antibody (Cayman Chemical Company, Ann Arbor, Mich), was applied at 1:100 dilution in primary antibody diluting buffer (Biomedica, Inc, Foster City, Calif) at 37°C in a humid chamber for 1 hour. Detection was with a biotinylated antirabbit secondary antibody and avidin/biotin complex with horseradish peroxidase (Vector Elite ABC Kit; Vector Laboratories). The substrate used diaminobenzidine (Vector Laboratories). Slides were counterstained with hematoxylin. Diluting buffer was used as a negative control preparation. Sections of human distal vas deferens were used for positive control preparations. Sections were again dehydrated in graded alcohol and coverslipped. Images were viewed with an Olympus BX60 microscope and captured with a cooled charge-coupled device camera (Magnafire; Olympus America Inc, Melville, NY). Images were imported into Adobe Photoshop (Adobe Systems Incorporated, San Jose, Calif) as tagged image format files.

Cell Adhesion Assays

A549 cells were seeded onto 6-well plates at a density of 10^6 cells/well. After attachment overnight, cells were treated with 1% dimethyl sulfoxide or with celecoxib at 50- μ mol/L and 75- μ mol/L concentrations for 24 hours. Twenty-four-well cell culture plates were prepared by adding Matrigel ECM (BD Labware, Bedford, Mass) at 50 g/cm² and allowed to dry overnight. The plates were rehydrated with warm medium without serum at 37°C for 4 hours before adhesion experiments. After treatment, cells were harvested and resuspended in medium containing 5% fetal bovine serum. Cells were then added to the Matrigel-coated plates at 5×10^4 cells/well and allowed to attach for various times. Unattached cells were removed by aspiration and gentle washing with phosphate-buffered saline solution at 15, 30, 60, and 120 minutes. Remaining cells were fixed with formalin and stained with hematoxylin. Attached cells were quantitated with light microscopy in 8 random high-power fields.

Western Blot

A549 cells were seeded at density of 10^6 cells/well in 12-well plates with medium containing 5% fetal bovine serum and allowed to attach overnight. Cells were then washed in phosphate-buffered saline solution and subjected to the following treatments for 24 hours: 1% dimethyl sulfoxide, interleukin (IL) 1 β (10 ng/mL), celecoxib at 50 μ mol/L, celecoxib at 75 μ mol/L, IL-1 β plus celecoxib at 50 μ mol/L, and IL-1 β plus celecoxib at 75 μ mol/L. Cell pellets were lysed, and a protein assay was performed with Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc, Hercules, Calif). Protein samples were mixed with equal volumes of tris(hydroxymethyl)aminomethane (Tris)–glycine sodium dodecylsulfate loading buffer (Invitrogen Corporation, Carlsbad, Calif), heated at 90°C for 2 minutes, and loaded onto 8% to 16% Tris-glycine ready gels (Invitrogen) at 15 μ g/lane. Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed at 125 V for 90 minutes, and protein was transferred onto polyvinylidene fluoride membranes at 25 V for 90 minutes. Membranes were washed and blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline solution with 0.1% polysorbate. Mouse antihuman CD44 antibody (Abcam Inc, Cambridge, Mass) at 1:1000 and anti- β -actin antibody at 1:5000 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) were applied to the membranes for 1 hour at 37°C. After washing, blots were incubated with horseradish peroxidase–linked antimouse IgG conjugate (GE Healthcare Technologies, Waukesha, Wis) at 1:5000 for 1 hour at room temperature. Antibody complexes were visualized with enhanced chemiluminescence (GE Healthcare Technologies). Blots were scanned with Fluorochem 8900 (Alpha Innotech Corporation, San Leandro, Calif), AlphaEase imaging software (version 5.0; Alpha Innotech), and Adobe Photoshop software (version 6.0; Adobe).

Statistical Analysis

In vitro cell adhesion experiments were performed in duplicate. Data for both *in vitro* and *in vivo* experiments are expressed as mean $\pm$ SD unless otherwise indicated. Analysis of variance with one between factor and repeated measures followed by the Bonferroni multiple comparison adjustment was performed for

Figure 1. Tumor metastasis in murine model. A, Means (bars) and SDs (error bars) of number of tumor foci, as counted by a blinded observer, for each group. Asterisk indicates $P < .05$ versus control. B, Gross specimens depicting surface tumor nodules for control (A1), perioperative treatment (A2), and continuous treatment (A3) groups at 6 weeks from injection. C, Histologic examination (hematoxylin and eosin) with COX-2 immunohistochemical counterstain shows tumor nodules within lung parenchyma for control (A1), perioperative treatment (A2), and continuous treatment (A3) groups. COX-2 stains are shown to illustrate tumor nodules more clearly and to show continued expression of protein despite COX-2 inhibition. COX-2 inhibitors are enzyme blockers and do not predictably affect either messenger RNA or transcription of protein.

both in vitro and in vivo experiments. The significance of the interaction between time and treatment group was used to determine the statistical significance with time between treatments in the cell adhesion experiment. We also tested the significance of the slope with time within each group by means of regression analysis.

Results

Experimental Tumor Metastasis

Mice treated with continuous celecoxib demonstrated a mean of 33 ± 60 tumor metastases, whereas control mice had a mean of 188 ± 220 tumor metastases. Mice treated

Figure 2. Graph representing tumor cell adhesion to Matrigel ECM depicts decreased ability of cells to attach when treated with celecoxib. *DMSO*, Dimethyl sulfoxide; *hpf*, high-power field.

with perioperative celecoxib also demonstrated decreased tumor nodules, with a mean of 55 ± 87 foci (Figure 1, A and B). This difference treatment and control groups was statistically significant ($P = .0135$). The clinical difference between treatment groups did not reach statistical significance, however, when examined by single treatment group comparisons. The results of gross examination were confirmed by immunohistochemical staining for COX-2 on serial sections from selected tumor samples. The sizes of the tumor metastases in both the perioperative and continuous groups were also smaller than that in the control group (Figure 1, C).

In Vitro Assays

Adhesion assays were performed to assess effects of celecoxib on cell-ECM adhesion. After transient exposure of cells to celecoxib at 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$, cells demonstrated markedly decreased cell-ECM adhesion relative to untreated control cells ($P < .01$; Figure 2). The effects on cell adhesion did not improve with time during the course of the experiment.

Because a fundamental step in the metastatic process is tumor cell attachment to a secondary site and subsequent invasion, we examined the CD44 cell surface receptor, which is implicated in tumor cell adhesion to the extracellular ligand hyaluronidate and increased invasion in vitro. Untreated A549 cells demonstrated strong expression of CD44 receptor according to Western blot. Exposure of cells to celecoxib resulted in decreased CD44 expression, whereas treatment with the inflammatory cytokine IL-1 β led to CD44 upregulation. This stimulatory effect of IL-1 β was abrogated by the addition of celecoxib (Figure 3).

Discussion

Although surgical resection of NSCLC is intended to cure, the resultant increase in inflammatory cytokines, decrease in immunocompetence, and potential for “stirring up” CTCs

Figure 3. Western blot for CD44. IL-1 β increased expression of CD44. Celecoxib (*Cel*) decreased expression of CD44, even after IL-1 β stimulation. *DMSO*, Dimethyl sulfoxide.

may all play a role in the subsequent appearance of metastatic deposits. Recent adjuvant strategies with chemotherapy for early stage disease have resulted in an improved survival, suggesting that early treatment of micrometastatic disease is advantageous. However, the perioperative period is typically a time when no systemic therapies are given, and often chemotherapy is delayed for a number of months while the patient recovers from surgery. By that time, the initial events in the metastatic cascade presumably would have already taken place. It is prudent, therefore, to reexamine the perioperative period as a time of vulnerability for metastasis and an opportunity to affect metastatic progression in patients undergoing surgery for NSCLC.

The metastatic process is complex but can be looked at in terms of multiple steps. Initially, tumor cells invade into a lymphovascular channel and detach from the main tumor mass. These cells then enter the circulation as CTCs and must avoid immune surveillance to survive long enough to arrive at a favorable distant site, a process that may involve a chemokine gradient. The concept of surgical manipulation increasing CTCs is at least 4 decades old. The no-touch technique of colorectal cancer resection, popularized by Turnbull in the 1960s and 1970s, was aimed at decreasing the shedding of tumor cells resulting from mechanical manipulation at the time of surgery.^{26,27} Although techniques for detecting CTCs were not available 40 years ago, newer techniques of isolating rare cells in peripheral blood are now available and are being used to address the relevance of CTCs.

It has been surmised that only certain CTC (such as tumor stem cells) have the potential to become metastatic deposits.²⁸ Nonetheless, recent evidence obtained with newer technology to detect rare cells in the circulation supports the view that detection of significant numbers of CTCs is relevant because it is associated with a poorer prognosis.^{4-6,29} Rolle and colleagues² found increased CTCs in patients undergoing limited resection (lobectomy) versus those undergoing pneumonectomy, suggesting that the level of CTCs is correlated with the extent of parenchymal manipulation. Ya-

mashita and coworkers² found that the increased surgical manipulation associated with video-assisted thoracoscopic resection resulted in increased CTCs after surgery. Consequently a treatment that can be given around the time of surgery that has the possibility of reducing the metastatic potential of circulating cells may reduce the risk of recurrence in patients with early stage disease.

The anti-inflammatory and antineoplastic effects of celecoxib, combined with a favorable side effect profile, make it an ideal candidate for perioperative therapy. Celecoxib has been used perioperatively as an adjuvant analgesic without detectable side effects.²²⁻²⁴ In the short term, it has not been associated with increased cardiovascular side effects, and in contrast to nonspecific COX inhibitors (nonsteroidal anti-inflammatory agents), it has little effect on either renal or platelet function. In this study, we aimed to expand on previous reports that COX-2 inhibition decreases tumor growth and metastases in vivo^{10,11,14} by examining the potential benefit of perioperative exposure. Specifically, we hypothesized that downregulation of the activity of the COX-2 enzyme may limit the ability of CTCs to establish metastatic deposits, perhaps through a mechanism that involves tumor cell to distant site attachment and early invasion.

Our in vivo experiment showed a decrease in the number of established pulmonary metastases in both perioperatively and continuously treated mice when A549 NSCLC cells were injected into the peripheral circulation. This finding is perhaps most striking when placed within the context of the doses administered. The effects were seen at doses of 1000 ppm of chow, which have been shown to correspond to serum levels in the range of 9 to 11 $\mu\text{mol/L}$ in animal studies.^{14,32} This concentration range is well below the inhibitory concentration of 50% for celecoxib, as well as below levels needed for induction of apoptosis.^{14,33,34} Although one must be cautious in extrapolating directly from in vitro data to the in vivo situation and vice versa, the difference is significant. Despite the lack of a statistically significant difference between mean number of lung metastases seen in the continuous and perioperative treatment groups, histologic examination revealed that the size of established metastases was smaller in the continuous treatment group than in the perioperative group, suggesting a role for ongoing tumor growth inhibition with celecoxib, presumably through other mechanisms.

A critical step in the metastatic process involves the attachment of the CTC to a distant site, a process that requires tumor cell receptors such as the CD44 receptor. This work was influenced by previous studies, which have shown a strong association between COX-2 and CD44 expression. In the lung cancer cell line A549, upregulation of COX-2 led to an increase in CD44 expression, and treatment with the exogenous downstream product of COX-2, 16,16-dimethyl prostaglandin E₂, and also to increased CD44 expression and tumor cell

invasion.^{15,35} Moreover, CD44 has been found to be a fundamental requirement for tumor metastasis. Weber and colleagues³⁶ found that in CD44 knockout experiments with mice that normally produce spontaneous colon cancers and metastases, the lack of CD44 expression in CD44^{-/-} mutants does not inhibit primary tumor formation but rather prevents the formation of spontaneous secondary metastases. We demonstrated that celecoxib decreases CD44 expression in tumor cells in vitro. Additionally, it is able to abrogate the stimulatory effects of the inflammatory cytokine IL-1 β , which is of particular importance in examining the efficacy of a treatment administered during the proinflammatory state of the perioperative period. Similarly, in the adhesion assays, exposure of celecoxib decreased the ability of these cells to adhere to Matrigel, a surrogate for ECM.

We have inferred that one of the possible mechanisms for the decrease in established metastases may be related to downregulation of the CD44 receptor, with resultant decrease in both adhesion and subsequent invasion at a distant site. The metastatic process is complex, however, and many other mechanisms may play roles. Celecoxib has been shown to affect the immune response, which may play a role in the persistent viability of circulating cells.³⁷ Further, changes in the metastatic site to tumor cell chemokine gradient and in stromal-tumoral interaction are also possibly affected, and studies addressing these issues are currently underway. We and others have shown that the effects of celecoxib also interfere with the production of matrix metalloproteinases, and this probably plays a role in the invasion of metastatic cells.³⁷ The well-established effects on apoptosis and angiogenesis may play a role in the continued growth of established implants, possibly explaining the size difference of tumors in the perioperative and continuous treatment groups. However, they probably do not play a role in the initial adhesion and invasion and subsequent establishment of a metastatic deposit, as discussed previously.

There are notable shortcomings in this study. The artificially induced CTCs with a large number of cancer cells in an immunocompromised mouse present a very different condition from the rare CTCs present in the clinical setting. Although artificial, this model does give some insight into the ability of celecoxib to inhibit establishment of metastatic implants, which may be relevant when discussing the metastatic potential of CTCs in the perioperative period. This result was profound even at relatively low doses of celecoxib, well below any tumoral cytotoxic effect. It would be closer to ideal to manipulate an established mouse lung tumor surgically and then assess metastatic implants, but the size of the animal and our inability as yet to detect CTCs in very small volumes of blood limit our ability to do such an experiment.

In conclusion, celecoxib significantly reduced establishment of lung cancer metastases in an artificial model of CTCs in vivo. In vitro, celecoxib was shown to inhibit

CD44 expression and tumor cell adhesion to ECM. Perioperative modulation of COX-2 may be a novel treatment strategy to minimize metastases from CTCs during this high-risk period. Continuous therapy, at least until standard chemotherapy is initiated, may provide the additional benefit of suppressing ongoing tumor growth in those cells that have eluded other host tumor clearance mechanisms.^{25,30,31}

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