Cyclooxygenase-2-regulated vascular endothelial growth factor release in gastric fibroblasts

Shuhei Miura, Atsushi Tatsuguchi, Ken Wada, Hiroki Takeyama, Yoko Shinji, Tetsuro Hiratsuka, Seiji Futagami, Kazumasa Miyake, Katya Gudis, Yuji Mizokami, Takeshi Matsuoka, and Choitsu Sakamoto

1Fifth Department of Internal Medicine, Tokyo Medical University, Ibaraki-Ken 300-0385; and 2Third Department of Internal Medicine, Nippon Medical School, Tokyo 113-8603, Japan

Submitted 29 December 2003; accepted in final form 22 March 2004

Cyclooxygenase-2 (COX-2) is an inducible enzyme that plays a key role in angiogenesis during gastric ulcer healing. We investigated the role of COX-2 in angiogenesis in gastric fibroblasts stimulated by IL-1β in vitro and that angiogenesis induced by the COX-2-VEGF pathway might be involved in gastric ulcer healing. VEGF and COX-2 expression in surgical resections of gastric ulcer tissue were measured by Western blot analysis. VEGF and COX-2 expression in surgical resections of human gastric ulcer tissue was examined immunohistochemically. IL-1 dose dependently enhanced VEGF release in cultured gastric fibroblasts after a 24-h stimulation. IL-1 also stimulated PGE2 production in gastric fibroblasts via COX-2 induction. NS-398 significantly suppressed VEGF and PGE2 release from IL-1-stimulated gastric fibroblasts; concurrent addition of PGE2 restored NS-398-inhibited VEGF release. COX-2 and VEGF immunoreactivity were colocalized in fibroblast-like cells in the ulcer bed of gastric tissues. These results suggest that COX-2 plays a key role in VEGF production in gastric fibroblasts stimulated by IL-1β in vitro and that angiogenesis induced by the COX-2-VEGF pathway might be involved in gastric ulcer healing.

Address for reprint requests and other correspondence: C. Sakamoto, Third Dept. of Internal Medicine, Nippon Medical School, 1-1-5, Sendagi, Bunkyou-ku, Tokyo 113-8603, Japan (E-mail: choitsu@nms.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
antibodies against vimentin (DAKO, Carpinteria, CA) and COX-2 (Transduction Laboratories, Lexington, KY) were used for immunohistochemical staining (26). A rabbit polyclonal antibody against VEGF (generously provided by Dr. T. Ishiwata, Department of Pathology, Nippon Medical School) was also used for immunohistochemical staining (35).

Preparation of Gastric Fibroblast Supernatant

Gastric fibroblasts (1 X 10^6) were cultured on 24-well plastic plates in 1 ml of RPMI 1640 medium (Nikken, Tokyo, Japan) supplemented with 10% fetal calf serum and penicillin-streptomycin (GIBCO Life Technologies, Gaithersburg, MD) at 37°C in 5% CO₂. Confluent gastric fibroblasts were then washed twice with PBS and cultured in the presence or absence of IL-1α or IL-1β at various concentrations under serum-free condition for 24 h. In some cases, gastric fibroblasts were also cultured in the presence or absence of indomethacin or a selective COX-2 inhibitor, NS-398, at 10 μmol/L. Previous studies (6, 13) have shown that NS-398 at 10 μmol/L selectively inhibits COX-2 activity in vitro. The supernatant was harvested at selected points after stimulation and stored at −20°C. Gastric fibroblasts were also cultured in a similar manner on 6-cm dishes to test COX protein expression by Western blot.

Detection of VEGF and PGE₂

The amount of VEGF-165 in culture supernatant was detected with an ELISA kit (IBL). Briefly, a 100-μl aliquot sample was dispensed into a microtiter plate coated with mouse monoclonal antibody against human VEGF-165. After incubation at 37°C for 1 h, the plate was washed with PBS seven times. Anti-human VEGF rabbit IgG Fab'-peroxidase conjugate was then added, and the plate was incubated at 37°C for 30 min and washed nine times with PBS. A 100-μl aliquot of tetramethyl benzidine solution (0.2 mg/ml) was added as substrate and incubated in the absence of light at room temperature for 30 min. The reaction was stopped by addition of 100 μl of 1 N H₂SO₄. Absorbance at 450 nm was measured by an automatic plate reader. VEGF concentration was determined on a standard curve obtained by a serial dilution of recombinant human VEGF within the range of 0–1,000 pg/ml. The ELISA kit has been shown to specifically detect VEGF-165 among all isoforms, but 6% cross-reactivity was seen with VEGF-121 (47).

The amount of PGE₂ was also detected as described in the instructions of an enzyme immunoassay kit (Assay Design), which included a monoclonal antibody against PGE₂ and alkaline phosphatase molecules covalently attached to PGE₂.

Western Blot Analysis

COX-1 and COX-2 protein expression in gastric fibroblasts was detected by Western blot analysis as previously described (28). Briefly, cultured fibroblasts were harvested in 25 mM Tris-HCl (pH 8.1) buffer containing 0.25 M sucrose, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 μM peptatin A, and 1.0 mM EDTA. The pellet was collected by centrifugation at 10,000 g for 2 min and resuspended in the same buffer. CHAPS was added to 1% (wt/vol), and the mixture was stirred for 2 h at 4°C. After centrifugation at 50,000 g for 20 min, the supernatant was loaded onto an anion-exchange column equilibrated with 20 mMol/L Tris-HCl (pH 8.1) and 0.4% CHAPS.

Samples containing 30 μg of protein were separated on 10% acrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Next, the proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-COX-2 antibody specific for human COX-2 protein. Bound antibody was detected with horseradish peroxidase-conjugated antibodies via an enhanced chemiluminescence detection system. The same nitrocellulose membrane was washed as described previously (28) and then reprobed with anti-COX-1 antibody.

Immunofluorescence Histochemistry

Human gastric ulcer tissue samples with perforation were obtained by surgical resection in nine patients (7 men and 2 women, 44–79 yr old, mean 60.0 yr old). Tissue was fixed with buffered 10% formalin, embedded in paraffin, and sectioned to a thickness of 3 μm.

Single antibody labeling. The tissue sections were rehydrated and immersed in 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. The sections were incubated with 10% normal horse serum for 30 min to block nonspecific binding of the secondary antibody and then incubated overnight with antivimentin antibody (dilution 1:400) at 4°C. Antibody binding was detected by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) and visualized using the diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer’s instructions. Nuclei were counterstained with Mayer’s hematoxylin.

Negative control immunohistochemical procedures included omission of the primary antibody and replacement of the primary antibody by normal mouse IgG.

Double-labeling procedures. Double labeling using immunofluorescence methods and confocal laser scanning microscopy was used to evaluate the colocalization of immunoreactivity for COX-2 (1:40) and VEGF (1:200). The sections were incubated overnight at 4°C with a mixture of the two primary antibodies. The antibody against COX-2 was reacted with a secondary antibody (horse anti-mouse IgG, dilution 1:100; Vector Laboratories) labeled with FITC. The antibody against VEGF was reacted with a secondary antibody (goat anti-rabbit IgG, dilution 1:100; Vector Laboratories) labeled with Texas red; then nuclear counterstaining was carried out with 4',6-diamidino-2-phenylindole (Sigma, St. Louis, MO) for 15 min to facilitate identification of morphological features.

Immunohistochemical control procedures similar to those described for single labeling were employed in conjunction with dual-staining methods. All preparations were examined with a confocal microscope (model TCS4D/DMIRBE, Leica, Heidelberg, Germany) equipped with argon and argon-krypton laser sources.

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by Fisher’s projected least significant difference test as a post hoc test, as appropriate. P < 0.05 was considered statistically significant.

RESULTS

Effects of Proinflammatory Cytokines on VEGF Release in Gastric Fibroblasts

We examined whether human gastric fibroblasts are able to release angiogenic factors when stimulated by inflammatory cytokines such as IL-1α, IL-1β, and TNF-α. IL-1α and IL-1β stimulation increased VEGF release from gastric fibroblasts, whereas TNF-α stimulation for 24 h had no effect on VEGF release (Fig. 1A). IL-1β stimulated increases in VEGF release in a dose-dependent manner within a range of 1.25–10 ng/ml (Fig. 1B).

Western Blot Analysis of COX-2 Expression Induced by IL-1β in Gastric Fibroblasts

Inflammatory cytokines have been shown to induce COX-2 protein in fibroblasts and macrophages. Therefore, our next step was to examine whether IL-1β induces COX-2 protein production in gastric fibroblasts. Figure 2 shows the result of Western blot analysis with anti-COX-2 antibodies. COX-2 protein expression was remarkably increased by the stimula-
Cells were then stimulated with or without cytokines (IL-1 at 10 ng/ml, IL-1/α, and TNF-α) and cultured for another 24 h. VEGF concentration in the gastric fibroblast supernatant was measured with ELISA.

**Effects of COX Inhibitors on VEGF Release in Gastric Fibroblasts**

Because IL-1β stimulates VEGF release and COX-2 protein production in gastric fibroblasts, we next examined whether IL-1-induced VEGF release is regulated via a COX-dependent pathway. Figure 3 shows that IL-1-stimulated VEGF release was equally inhibited in the presence of 10 μM indomethacin or 10 μM NS-398, although neither inhibitor affected basal VEGF release significantly. We further found that VEGF inhibition by COX inhibitors was restored by the simultaneous addition of PGE2 at 100 μM. These data suggest that PGE2 derived from COX induction by IL-1β stimulation might be involved in VEGF release from gastric fibroblasts.

**Effects of IL-1α and IL-1β on PGE2 Release in Human Gastric Fibroblasts**

To clarify whether gastric fibroblasts can actually release increased amounts of PGE2, we measured PGE2 levels in the supernatant of gastric fibroblasts after IL-1α and IL-1β stimulation. Figure 4 shows that IL-1α and IL-1β induced a significant increase in PGE2 release from gastric fibroblasts at concentrations seen for VEGF production. Because IL-1α and IL-1β did not induce COX-1 protein production in gastric fibroblasts, the increase in PGE2 by the stimulation of these cytokines might be derived from increased COX-2 protein expression. A nonselective COX inhibitor, indomethacin, and a selective COX-2 inhibitor, NS-398, similarly inhibited IL-1β-induced PGE2 production down to control levels, further suggesting that human gastric fibroblasts secrete PGE2 in response to IL-1β via a COX-2-dependent pathway.

**Fig. 1.** Effects of IL-1α, IL-1β, and TNF-α on VEGF release in cultured gastric fibroblasts. A: human gastric fibroblasts (1 × 10^5) were incubated in 24-well culture plates in triplicate without serum at 37°C in 5% CO₂ for 24 h. Cells were then stimulated with or without cytokines (IL-1α at 10 ng/ml, IL-1β at 10 ng/ml, or TNF-α at 10 ng/ml) and cultured for another 24 h. VEGF concentration in the gastric fibroblast supernatant was measured with ELISA. B: dose response of VEGF release induced by IL-1β in gastric fibroblasts. IL-1β induced VEGF release in a dose-dependent manner within a range of 1.25–10 ng/ml. Results (means ± SE) are representative of 5 similar experiments. Significantly different from control: *P < 0.05; **P < 0.01.

**Fig. 2.** Western blot analysis of cyclooxygenase-2 (COX-2) in cultured gastric fibroblast lysate. A, sheep COX-2 protein (10 ng); B, lysate of gastric fibroblasts cultured in serum-free medium; C, lysate of gastric fibroblasts cultured with IL-1β (10 ng/ml); D, lysate of gastric fibroblasts cultured with IL-1β (10 ng/ml) and indomethacin (10 μM); E, lysate of gastric fibroblasts cultured with IL-1β (10 ng/ml) and NS-398 (10 μM).

**Fig. 3.** Effect of indomethacin (IND) and NS-398 on VEGF release in cultured gastric fibroblasts. Human gastric fibroblasts (1 × 10^5) were incubated in 24-well culture plates in triplicate without serum at 37°C in 5% CO₂ for 24 h. Cells were then stimulated with or without IL-1β (10 ng/ml) in the presence of IND, NS-398, or NS-398 + PGE2 (100 μM) for 24 h. As control, cells were also cultured in the presence of IND or NS-398 without IL-1β stimulation. Results (means ± SE) are representative of 5 similar experiments. *Significantly different from respective control, P < 0.05.
Effects of PGE<sub>2</sub> on VEGF Release in Gastric Fibroblasts

To confirm whether gastric fibroblasts release VEGF in response to PGE<sub>2</sub> stimulation, we examined the effect of graded concentrations of PGE<sub>2</sub> on VEGF release in fibroblasts. There was a dose-dependent stimulation of VEGF production after treatment with PGE<sub>2</sub> at 0.1–100 μmol/l. Maximum VEGF release was about three times more than control levels 24 h after PGE<sub>2</sub> stimulation (Fig. 5). Because the concentration of PGE<sub>2</sub> detected in the supernatant after IL-1β stimulation was ~0.1–1 μmol/l, these results, taken together, suggest that the amount of PGE<sub>2</sub> secreted by gastric fibroblasts is enough to induce VEGF release by gastric fibroblasts in an autocrine manner in vitro.

Kinetics of VEGF and PGE<sub>2</sub> Release Induced by IL-1β

To clarify the kinetics of IL-1β-stimulated VEGF and PGE<sub>2</sub> release, we examined IL-1β-induced increases in VEGF and PGE<sub>2</sub> release in the course of 24 h of stimulation. After IL-1β stimulation, PGE<sub>2</sub> was first detected at 3 h; thereafter, PGE<sub>2</sub> levels continued to rise until levels peaked at 24 h. In a similar pattern, VEGF was detected initially 6 h after IL-1β stimulation and continued to increase for 24 h (Fig. 6). The accumulation pattern of VEGF was likely to follow that of PGE<sub>2</sub>. Taken together, these data imply that human gastric fibroblasts release PGE<sub>2</sub> via upregulation of COX-2, and, thereafter, PGE<sub>2</sub> stimulates VEGF release in an autocrine or paracrine manner.

VEGF and COX-2 Expression in Gastric Mesenchymal Cells in Human Gastric Ulcer Tissue

Our previous report showed that COX-2 expression was exclusively localized in gastric mesenchymal cells of the gastric ulcer bed in surgically resected gastric tissue or biopsy specimens of the gastric ulcer margin, as shown immunohistochemically (44). The rate of COX-2-positive cells was also found to significantly increase in the active and healing stages of ulcers, compared with Helicobacter pylori-negative normal gastric mucosa. To analyze the location of VEGF and COX-2 protein in gastric ulcer tissue, we performed double-staining analysis of ulcerated regions by using immunofluorescence-conjugating antibodies.

Numerous inflammatory cells and spindle-shaped cells were found beneath necrotic tissue of the ulcer bed in resected sections (Fig. 7A). Reaction against vimentin was found in all spindle-shaped cells, indicating that these cells are fibroblasts or myofibroblasts (Fig. 7B). COX-2 was expressed in these spindle-shaped mesenchymal cells and also in spherical inflammatory cells of the granulation tissue (Fig. 7C). Strong VEGF immunoreactivity was also observed in the same gastric tissue section of the ulcer bed (Fig. 7D). Double staining of COX-2 and VEGF with immunofluorescence-conjugating antibodies revealed that VEGF and COX-2 were coexpressed in these spindle-shaped and spherical cells of the ulcer bed (Fig. 7, E and F).

DISCUSSION

In the present study, we have shown that COX-2-dependent PGE<sub>2</sub> release in turn stimulates autocrine or paracrine VEGF release in gastric fibroblasts. Although another study showed that gastric fibroblasts secrete VEGF in response to TNF-α (40), we have shown for the first time a COX-2-PGE<sub>2</sub> auto-

![Fig. 4. Effect of IL-1α and IL-1β on PGE<sub>2</sub> release in gastric fibroblasts. Gastric fibroblasts were stimulated as described in Fig. 2 legend. Results are representative of 5 similar experiments. Vertical bar indicates a logarithmic scale, so SE bars were deleted. *Significantly different from control, P < 0.05. **Significantly different from IL-1β alone, P < 0.05.](#)

![Fig. 5. Effect of PGE<sub>2</sub> on VEGF release in cultured gastric fibroblasts. Human gastric fibroblasts (1 × 10<sup>5</sup>) were incubated in 24-well culture plates in triplicate without serum at 37°C in 5% CO<sub>2</sub> for 24 h. Cells were then stimulated with PGE<sub>2</sub> (0.1–100 μM) for 24 h. Results (means ± SE) are representative of 5 similar experiments. *Significantly different from control, P < 0.05.](#)

![Fig. 6. Time course analysis of VEGF and PGE<sub>2</sub> release in cultured fibroblasts with or without IL-1β. Human gastric fibroblasts (1 × 10<sup>5</sup>) were incubated in a 10-cm culture dish without serum at 37°C in 5% CO<sub>2</sub> for 24 h. Cells were then stimulated with or without IL-1β (10 ng/ml) in 10 ml of medium. An aliquot of supernatant (400 μl) was taken at 3, 6, 12, and 24 h after stimulation, and VEGF and PGE<sub>2</sub> concentrations were determined. Values are means ± SE from 5 similar experiments. • PGE<sub>2</sub> stimulated by IL-1β; □ PGE<sub>2</sub> without stimulation; ○ VEGF stimulated by IL-1β; ◊ VEGF without stimulation.](#)
In the context of COX-2 regulation, VEGF release by gastric fibroblasts in vitro. Our results suggest that, as seen in granulation tissue of gastric ulcers in vivo, PGE2-induced VEGF production in COX-2-expressing fibroblasts may play a key role in angiogenesis. A sequence of responses including COX-2 expression, PGE2 production, and VEGF release was triggered by IL-1β and IL-1α stimulation, suggesting the possible involvement of inflammatory cytokines in VEGF production through COX-2 induction in the stomach. However, it should be noted that, in the present study, NS-398- and indomethacin-induced inhibition of VEGF release was not complete, in contrast to their near-total inhibition of PGE2 production, suggesting that some other factor being stimulated by IL-1β may also be involved in VEGF release from gastric fibroblasts. The human VEGF that we focused on in the present study is a basic, heparin-binding, and homodimeric 45-kDa...
glycoprotein that includes at least five different splicing variants: VEGF-121, VEGF-145, VEGF-165, VEGF-189, and VEGF-206 (10, 32). Among these proteins, we selected VEGF-165 to measure the amount of VEGF released from fibroblasts; VEGF-165 is the most abundant and widespread isoform in vivo and has the most potent biological activity, in contrast to VEGF-121, which is considered a soluble protein, and VEGF-189 and VEGF-206, which have been shown to be almost completely sequestered in the extracellular matrix (31). Our results therefore suggest that biologically active VEGF secretion in gastric fibroblasts is probably induced via the COX-2-PGE2 pathway.

We previously showed that increased COX-2 expression in gastric tissue of experimentally induced ulcers is involved in the ulcer repair process in mice (28). In addition, we have found strong COX-2 protein induction in fibroblasts and macrophages between necrotic and granulation tissues of the ulcer bed in surgically resected human gastric tissue (44). Nevertheless, we still do not know the role of mesenchymal COX-2 expression in the ulcer repair process in the human stomach. However, our present data raise the possibility that VEGF released from COX-2-expressing fibroblasts might be a key to this repair process, because VEGF has been recently shown to stimulate angiogenesis in the wound repair process: keratinocytes were shown to express VEGF mRNA in the healing process of wounds, and decreased VEGF mRNA expression was shown to impair the healing of skin wounds in genetically diabetic db/db mice (8, 9, 12). Furthermore, in the wound repair process, VEGF responses in vivo might be tied to COX-2 expression, given that celecoxib, a selective COX-2 inhibitor, impaired angiogenesis and delayed gastric ulcer healing, with concomitant decrease in serum VEGF-to-endothelin ratios (24). In addition to our finding in vivo, COX-2-dependent hepatocyte growth factor (HGF) release has also been shown in gastric fibroblasts after IL-1β stimulation (3, 41). HGF is well known as a powerful stimulator of epithelial cell migration and proliferation (42) and, therefore, is considered one of the factors involved in the ulcer repair process. Thus gastric fibroblasts expressing COX-2 at the ulcer margin might play a key role in the ulcer repair process by releasing not only growth factors such as VEGF and HGF, but also PGE2, which has been involved in various defense systems of the gastric mucosa (21, 33).

In the present study, we have further demonstrated that VEGF localized in fibroblast-like spindle cells in surgically resected gastric tissue samples of perforated ulcers. As we previously showed, COX-2 was also found strongly expressed in fibroblasts of the ulcer bed in the human stomach. VEGF has been shown to be colocalized in COX-2-expressing fibroblasts, suggesting that VEGF release from gastric fibroblasts in vivo could also depend on COX-2 expression, as seen in our isolated cultured gastric fibroblasts. Our in vivo immunohistochemical colocalization of COX-2 and VEGF in gastric fibroblasts of the ulcer bed is consistent with other in vivo studies showing a relationship between COX-2 expression and angiogenesis. In chronic and proliferating granuloma of the rat, COX-2 mRNA has been shown to increase with neovascularization in parallel with VEGF mRNA (25). Indomethacin and SC-236, a selective COX-2 inhibitor, have been shown to inhibit neovascularization in a dose-dependent manner in a mouse corneal model of angiogenesis (26). In carrageenin-induced granulation tissue in rats, COX-2-derived PGE2 has been shown to play a significant role in angiogenesis through VEGF formation (16). However, these in vivo studies have failed to show where COX-2 and VEGF expressions are localized. In the present study, we have clearly shown that COX-2 and VEGF are expressed in mesenchymal cells, such as fibroblasts of the ulcer bed, and might interact as we have shown in vitro.

VEGF-induced angiogenesis has been mostly shown in connection with cancer development. One recent study clearly showed that colon cancer cells release a variety of COX-2-dependent proangiogenic growth factors, whereas endothelial cell migration and tubular formation were observed only in COX-2-expressing cancer cells cocultured with endothelial cells (45). In a recent study, we also observed the immunohistochemical colocalization of VEGF and COX-2 in cancer cells of human gastric carcinoma (43). Many studies in addition to ours show VEGF expression in cancer cells (15, 29). Here, however, we show COX-2 and VEGF localized mainly in mesenchymal cells scattered in inflammatory granulation tissue of the ulcer bed. Only faint COX-2 expression can be observed in regenerated epithelial cells of the ulcer margin, as we previously showed (44). Recently, another study (1) also showed that COX-2 and VEGF are expressed in fibroblasts of granulation tissue induced by sponge implants in rats. Therefore, although COX-2 might play important roles in VEGF expression in cancer tissue and inflammatory granulation, the expression site for this key factor might differ in each tissue.

Regarding NSAID-caused gastric mucosal injury, COX-2, in addition to COX-1, has been found to be a target enzyme for NSAIDs (46). Conventional NSAIDs inhibiting COX-1 and COX-2 have been shown to cause serious and significant ulcer complications, including bleeding and perforation (7, 37). Although it is clear that selective COX-2 inhibitors such as celecoxib and rofecoxib cause less gastric damage than conventional NSAIDs (18, 38), these selective COX-2 inhibitors have still been shown to occasionally cause serious gastrointestinal complications (17). Although we do not know whether gastric fibroblasts express COX-2 in NSAID-induced gastric ulcers, if COX-2 were expressed, its biological activity would be inhibited. Thus we must consider the possibility that NSAID-induced inhibition of the COX-2-dependent ulcer repair process might ultimately lead to the serious complications of gastric mucosal damage attributed to NSAIDs.

In conclusion, COX-2 plays a key role in regulating VEGF production in gastric fibroblasts. Gastric fibroblasts strongly expressing COX-2 and VEGF at the ulcer margin might be involved in the ulcer repair process.

REFERENCES

COX-2 REGULATES VEGF RELEASE BY GASTRIC FIBROBLASTS

Gallo O, Franchi A, Magnelli L, Sardi I, Vannacci A, Boddi V, Ferrara N and Davis-Smyth T. 
Ferrara N, Houck K, Jakeman L, and Leung DW. 
Gabriel SE, Jaakkimainen L, and Bombardier C. 
Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, and Otomo. 
Hawkey CJ, Laine L, Simon T, Quan H, Shingo S, Evans J, and Sharma S. 


