Featured Article

Celecoxib Modulates the Expression of Cyclooxygenase-2, Ki67, Apoptosis-Related Marker, and Microvessel Density in Human Cervical Cancer: A Pilot Study

Gabriella Ferrandina, Franco O. Ranelletti, Francesco Legge, Libero Lauriola, Vanda Salutari, Marco Gessi, Antonia C. Testa, Ulrike Werner, Pierluigi Navarra, Giuseppe Tringali, Alessandra Battaglia, and Giovanni Scambia

Departments of Gynecology/Obstetrics [G. F., F. L., V. S., A. C. T., A. B., G. S.], Histology [F. O. R.], Pathology [L. L., M. G.], and Pharmacology [P. N., G. T.], Catholic University of the Sacred Heart, 00168 Rome, Italy, and Department of Experimental and Clinical Pharmacology and Toxicology, Friedrich Alexander University, Erlangen, D-91051 Nuremberg, Erlangen, Germany [U. W.]

Abstract

Purpose: We investigated whether a short treatment with the cyclooxygenase-2 (COX-2) inhibitor celecoxib could modulate Ki67 antigen and the caspase cleavage product of keratin 18, recognized as a marker of early apoptosis. The activity of celecoxib on microvessel density (MVD) and angiopo-power Doppler sonography-derived indices of tumor vascularization was also assessed. Serum levels of squamous cell carcinoma antigen and the proliferative potential and subsets of peripheral T cells before and after celecoxib treatment were also analyzed.

Experimental Design: Tumor biopsy specimens from 14 patients with cervical cancer were obtained at baseline and after 10 days of celecoxib treatment (400 mg twice daily). Tumor and stroma COX-2 expression, Ki67, apoptosis, and MVD were assessed by immunohistochemistry, whereas prostaglandin E2 levels were measured by RIA.

Results: At baseline, COX-2 integrated density values in tumor compartment ranged from 10.7 to 60.1 (median, 26.5) and were significantly higher than tumor COX-2 integrated density values after celecoxib treatment (range: 4.7–386.6 pg/mg wet tissue in pretreated cases versus 4.8–91.9 pg/mg wet tissue in posttreated cases (P = 0.092).

Conclusions: In cervical cancer, celecoxib treatment decreases tumor COX-2 expression and markers of proliferation and neoangiogenesis, while being ineffective on stroma COX-2 levels, thus suggesting that selective COX-2 inhibitors may be a promising strategy not only for chemopreventive approaches but also for therapeutic approaches in this neoplasia.

Introduction

Much attention has been focused recently on the role of COX-2, one of the three isoforms catalyzing the rate-limiting step in the conversion of arachidonic acid to PGs, in several aspects of tumor biology (1–3). In particular, COX-2 overexpression has been associated with inhibition of apoptosis and increased metastatic potential and neoangiogenesis (3–5). Moreover, it has been also hypothesized that overexpression of COX-2 could impair host immune responses, as suggested by the ability of COX-2 inhibitors or COX-2 antisense constructs to revert tumor-induced immunosuppression (6) and restore monocyte function (7).

In preclinical models, loss of COX-2 activity or COX-2 inhibition has been associated with suppression of intestinal polyp formation (8) and tumor growth (9) as well as with tumor chemoprevention (10).

In humans, evidence has been reported that COX-2 overexpression might be involved in tumor onset and progression. Indeed, COX-2 expression has been shown to increase from normal to preneoplastic and neoplastic lesions (11–13).

Moreover, the vast majority of the data consistently demonstrated an unfavorable prognostic role of high COX-2 levels in several human neoplasias (14–17). We recently showed in cervical tumors that the expression of COX-2 not only in tumor cells but also in stromal compartment may be relevant clinically (18, 19). In particular, an inverse relationship was documented between COX-2 in the tumor and COX-2 in cells of tumor surrounding stroma (18). Moreover,

The abbreviations used are: COX-2, cyclooxygenase-2; PG, prostaglandin; MVD, microvessel density; IDV, integrated density value; SCC, squamous cell carcinoma antigen; PI, proliferation index; MoAb, monoclonal antibody.
both tumor and stroma COX-2 levels were shown to effectively discriminate patients with low versus high chances of response to neoadjuvant chemotherapy and different risk of recurrence/death of disease, suggesting that the evaluation of COX-2 expression both in tumor and stroma areas, rather than in a single compartment, could improve the prognostic characterization of cervical cancer patients (18).

To investigate whether treatment with a selective COX-2 inhibitor may modulate COX-2 expression and other biological factors in cervical cancer, a pilot study was planned in which cervical tumor biopsies were obtained before and after treatment with celecoxib, chosen on the basis of its greater antitumor activity with respect to the other coxibs (20–22).

Among the biologically relevant biochemical parameters, Ki67 antigen was chosen as a marker of proliferation (23), and the caspase cleavage product of keratin 18, recognized by the M30 MoAb, was selected as a marker of early apoptosis (24).

MVD, as assessed by CD31 MoAb, was used for analyzing intratumor angiogenesis. The possible activity of celecoxib on tumor vascularization was also assessed by two-dimensional and three-dimensional angio-power Doppler sonography.

Moreover, serum levels of SCC (25), used in the disease monitoring and prognostic characterization of this neoplasia, were recorded.

Finally, because previous studies have documented the activity of COX-2 inhibitors on human immune functions (6, 7), we investigated whether celecoxib could alter the proliferative response of peripheral T cells to polyclonal activation and influence circulating lymphocyte subsets.

Patients and Methods

Patients and Study Design. This is a pilot study aimed at evaluating the effects of a short-term celecoxib (Solexa Pfizer, 200-mg tablets) treatment on biochemical and ultrasound parameters in cervical tumors.

The protocol was approved by the Institutional Ethic Committee. Inclusion criteria were: women with histological diagnosis of cervical cancer, 18–65 years of age, required to have normal physical examination and normal laboratory values for hepatic and renal functions, and Karnofsky score ≥80.

Subjects were excluded in cases of pregnancy/breast-feeding, history of gastric/duodenal ulcer or inflammatory intestinal disease, use of aspirin or other nonsteroidal anti-inflammatory drugs, known hypersensitivity to these drugs, and history of heart failure or hypertension.

During the screening period, all subjects signed a written informed consent and provided complete medical history. Subjects underwent physical examination, electrocardiographic evaluation, and laboratory tests including hematological and chemical measurements, urinalysis, and serum pregnancy test.

Subjects admitted to the study underwent blood sample collection for baseline assessment of celecoxib concentration, SCC, and hematological/chemical evaluation. Moreover, all subjects underwent two-dimensional and three-dimensional ultrasound evaluation of tumor vascularization and cervical tumor biopsy under colposcopic examination to confirm diagnosis and provide pretreatment tissue samples for immunohistochemical evaluation and PGE2 assessment (day 0).

The duration and dosage of treatment were chosen on the basis of pharmacokinetic data (26). From day 1 to day 10 of treatment, subjects received celecoxib (400 mg twice daily, at 8 a.m. and 8 p.m.). Completion of staging procedures (gynecological examination, imaging, chest X-ray, additional exams) were completed during the treatment period. Two-dimensional and three-dimensional ultrasound assessment of tumor vascularization was performed at the end of treatment, followed by biopsy that was performed in the context of gynecological examination under anesthesia on day 11.

Blood samples for celecoxib concentration assessment were taken on day 1, before starting treatment, and on days 2, 7, and 11. Samples for hematology, biochemistry, and SCC assessment were collected on day 0 and at the end of treatment.

Vital signs were assessed throughout the study. A timeline diagram of the study design is outlined in Fig. 1.

Cervical tumor biopsies were also obtained in five patients not receiving celecoxib and used as control to investigate the possibility that flogistic and reparative responses could affect the modulation of the biochemical factors analyzed.

Immunohistochemical Analysis. Tissue specimens were fixed in 10% neutral-buffered formalin and were paraffin em-
Celecoxib Modulates Biochemical Factors in Cervical Cancer

In tumor specimens were measured by the RIA described previously (27, 28). Four-micrometer-thick sections of representative blocks from each case were deparaffinized in xylene, rehydrated, and treated with 0.3% H2O2 in methanol for 10 min to block endogenous peroxidase activity. All sections were subjected to heat-induced epitope retrieval in a microwave oven. Sections were incubated with anti-COX-2 rabbit polyclonal antibody (1:300; Cayman Chemical Co., Ann Arbor, MI), anti-Ki67 MoAb (clone MIB1; Ylem, Rome, Italy), the anticaspase cleavage product of keratin 18 (clone M-30; Boehringer Mannheim GmbH, Mannheim, Germany), and anti-CD31 (clone JC/70 A; DAKO, Glostrup, Denmark) MoAbs (diluted 1:50) for 1 h at room temperature. Slides from all cases studied were then simultaneously processed for immunohistochemistry on TechMate Horizon automated staining system (DAKO) using the Vectastain avidin-biotin complex method peroxidase kit (Vector Laboratories, Burlingame, CA). Endogenous biotin was saturated by a biotin biotin complex method peroxidase kit (Vector Laboratories, Burlingame, CA). Positive controls for each parameter were always run in the assay (27, 28).

For COX-2, the intensity of immunohistochemical staining was evaluated as described previously (15, 18). The evaluation of MVD was performed as described by West et al. (29), with minor modifications. Briefly, tumor sections were analyzed at low-power magnification to assess areas of neoangiogenesis. Within these areas, individual microvessels were counted in two or three separate random fields within a superimposed grid (area, 0.0625 mm2). A single countable microvessel was defined as any endothelial CD31-positive cell or group of cells that was clearly separated from other vessels, stroma, or tumor cells without the necessity of a vessel lumen or RBCs within the lumen. Areas of diffuse hemorrhage or necrosis were avoided. Ki67 and M30 positivities were expressed as the percentage of tumor cells with nuclear staining counted in three separate ×40 microscopic fields (at least 200 cells per field were counted). Samples were considered M30 positive if >3% immunostained cells were detected. The analysis of all tissue sections were performed by two different pathologists (F. O. R. and M. G.) without any prior knowledge of the clinical and biological parameters. Disagreement between the pathologists was noted in two (14%) cases for COX-2, one (7%) for Ki67, two (14%) for MVD, and two (14%) for M30 staining, and consensus was achieved by joint reevaluation.

The heterogeneity of COX-2 expression was assessed in three biopsies from different areas of the tumor and was concluded to be less than 20% both in tumor and stromal compartment.

**PGE2 Assay.** Tissue samples for PGE2 assay were obtained at the time of biopsies by cutting in half the same tissue specimen used for immunohistochemical analysis. PGE2 levels in tumor specimens were measured by the RIA described previously (30), with slight modifications. At the time of assay, tissues were thawed, weighed, and placed in 50 mM Tris-HCl (pH 7.4) containing indomethacin (1 μg/ml). The w/v ratio was 100 mg of wet tissue/1 ml of medium. Tissues were then homogenized with an Ultra-Turrax TR50 homogenizer (two bursts of 30 s at 4°C). Homogenized tissues were centrifuged at 20,000 rpm for 25 min at 4°C.

One hundred microliters of supranatant were diluted to 250 μl with 0.025 M phosphate buffer (pH 7.5) and mixed with 2, 500, and 3000 cpm of [3H] and appropriately diluted (1:120,000) antiserum to give a final volume of 1.5 ml. A duplicate standard curve (ranging from 2 to 400 pg per tube, with an EC50 of 28 pg/tube) was run with each assay. The intra-assay and interassay variability were 5 and 10%, respectively. Separation of antibody-bound PGE2 was obtained with activated charcoal (Sigma Chemical Co., St. Louis, MO), which absorbs 95–98% of free PGE2. After centrifugation for 10 min at 4°C, supernatant solutions were decanted directly into 10 ml of liquid scintillation fluid. Radioactivity was measured by liquid scintillation counting. Results are expressed as picograms of PGE2 per milligram of wet tissue.

**Assessment of Celecoxib Plasma Concentrations.** EDTA blood samples (5 ml) taken before starting treatment and on days 2, 7, and 11 were immediately centrifuged, and the plasma samples were stored at −80°C until assay. An aliquot of the plasma sample (1.00 ml) was mixed with 100 μl of acetonitrile:water (50:50, v/v) and 150 μl of internal standard stock solution for 1 min. Afterward, 1 ml of 0.1 M Na-acetate buffer (pH 5) and 4 ml of dichloromethane:hexane (50:50, v/v) were added. The tubes were shaken for 10 min and centrifuged at 4000 × g for 10 min. The organic layer (3.5 ml) was transferred, and the solvent was evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 140 μl and injected in 100-ml aliquots onto the columns. Plasma calibration standards were prepared in the same manner in 100 μl of acetonitrile:water containing increasing amounts of celecoxib to achieve final concentrations from 5 to 2000 μg/ml.

Quantification of celecoxib was performed by using a sensitive, rapid, and selective method based on reversed-phase high-performance liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry as described previously (31).

**SCC Determination.** SCC assay was performed as described previously (25) with the commercially available RIA kit (SCC-RIA kit; Abbott Laboratories Diagnostic Division, Abbott Park, IL).

**Two-Dimensional and Three-Dimensional Ultrasound Evaluation.** All patients underwent ultrasound examination including gray scale, color, spectral Doppler and angio-power three-dimensional techniques available on a commercial Technos MP ultrasound unit (ESAOTE S.p.A., Genova, Italy).

Tumor dimensions, the amount of vascularity within the tumor (vascular score), the highest peak systolic velocity, and the highest time-averaged maximum velocity were calculated according to the method described previously (32).

After completing B-mode evaluation and color Doppler analysis, power Doppler was activated using a standardized pulse repetition frequency set at 750 Hz. The acquisition data set for three-dimensional volumetric reconstruction was obtained performing a scan, covering the tumor mass. The following three-dimensional parameters describing the tumor vasculature were calculated: (a) “relative color,” which is equal to the color volume measurement, divided by the total volume measurement; (b) “average color,” which corresponds to the sum of the color voxel intensities, divided by the color volume; and (c)
“flow intensity,” which corresponds to the sum of the intensity of each color voxel, divided by the total volume.

Lymphocyte Mitogenic Response and Lymphocyte Subsets. The response of peripheral T cells to the polyclonal stimulator phytohemagglutinin was assessed by using the intracellular covalent coupling dye carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) as described (33). The number of cell divisions were computed by ModFit/Cell Proliferation Model software (Sigma Chemical Co.) and expressed as PI.

For the analysis of lymphocyte subsets, blood samples obtained before and after celecoxib administration were stained by the whole blood technique using a lyse-then-wash step (33). MoAbs to CD3, CD4, CD8, CD25, CD62L, and HLA-DR (all from Becton Dickinson Biosciences, San Jose, CA) and CD45RB (PharMingen, San Diego, CA) were used. MoAbs were labeled with the fluorescent dye FITC, phycoerythrin, and PerCP and combined to assess the lymphocyte subsets. Cells were measured by a FACScan flow cytometer (Becton Dickinson) using forward and side scatter signals to establish the lymphocyte gate and exclude unwanted events (i.e., not viable cells, debris, and cell clumps) from cell evaluation. Fluorescence signals were collected in log mode. A minimum of 5000 cells of interest were acquired for each sample.

Statistical Analysis. Wilcoxon’s signed rank sum test for paired samples were used to analyze the statistical significance of the differences of tumor and stroma COX-2 IDV, Ki67, M30, MVD, and PGE2 in pretreatment versus posttreatment tissue biopsies. The same tests were used to analyze the color and power Doppler ultrasound indices and SCC levels in pre- and posttreatment cases.

Statistical analysis was performed using SOLO (BMDP Statistical Software, Los Angeles, CA).

Results

Patient Enrollment, Compliance, and Toxicity. From January 2002 to December 2002, 14 subjects entered the study at the Department of Gynecologic Oncology at Catholic University of Rome. Clinicopathological characteristics of patients are listed in Table 1. Median age was 56 years (range, 32–72). Ten (71.4%) patients were stage IIB, two (14.3%) were stage IIIa, and two (14.3%) were stage IIIb of disease, according to the International Federation of Gynecologists and Obstetricians classification. Histologically, all tumors were SCCs. Seven (50%) cases were moderately (G2) differentiated tumors, whereas seven (50%) were poorly (G3) differentiated tumors according to WHO classification.

The compliance rate to celecoxib was 100% because all patients completed the planned celecoxib assumption. No adverse events were registered, with the exception of one patient experiencing a mild cutaneous rash that spontaneously resolved.

There were no treatment-related modifications of chemistry and hematology values (data not shown).

Immunohistochemistry. Fig. 2 shows the values of tumor and stroma COX-2 IDV, the percentage of Ki67, and MVD levels in pre- and post-celecoxib cervical tumor biopsies.

Specific immunoreaction of M30-recognized antigen was faintly detectable in two pretreatment biopsies, and this pattern did not show any modification related to treatment.

There were no relationships among the basal levels of the three markers (data not shown). Moreover, no differences according to clinicopathological characteristics were observed.

At baseline, COX-2 IDV in tumor compartment ranged from 10.7 to 60.1 (median, 26.5) and were significantly higher than tumor COX-2 IDV after celecoxib treatment (range, 0.6–42.3; median,12.6; $P = 0.0043$). Only one case (7%) showed an increase in tumor COX-2 IDV levels in posttreatment biopsy. In contrast, there was no difference in stroma COX-2 IDV in tissue samples before (median, 17.9; range, 8.5–33.8) and after celecoxib treatment (median, 17.5; range, 5.7–46.1; $P = 0.22$). In five cases (35.7%), we observed an increase of stroma COX-2 IDV in posttreatment samples.

The percentages of Ki67-positive tumor cells in pre-celecoxib cases ranged from 39.3 to 87.4 (median, 50.8) and were

### Table 1 Characteristics of the patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment</th>
<th>Age (yr)</th>
<th>FIGO stage</th>
<th>Histotype</th>
<th>Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>56</td>
<td>IIb</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>65</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>64</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>32</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>35</td>
<td>IIIb</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>52</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>7</td>
<td>Yes</td>
<td>50</td>
<td>IIb</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>8</td>
<td>Yes</td>
<td>62</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>9</td>
<td>Yes</td>
<td>64</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>10</td>
<td>Yes</td>
<td>65</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>11</td>
<td>Yes</td>
<td>60</td>
<td>IIb</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>12</td>
<td>Yes</td>
<td>47</td>
<td>IIIa</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>13</td>
<td>Yes</td>
<td>60</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>14</td>
<td>Yes</td>
<td>41</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>15</td>
<td>No</td>
<td>65</td>
<td>IIIb</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>16</td>
<td>No</td>
<td>59</td>
<td>IIb</td>
<td>Squamous</td>
<td>G2</td>
</tr>
<tr>
<td>17</td>
<td>No</td>
<td>39</td>
<td>IIb</td>
<td>Adenocarcinoma</td>
<td>Not defined</td>
</tr>
<tr>
<td>18</td>
<td>No</td>
<td>42</td>
<td>IIa</td>
<td>Squamous</td>
<td>G3</td>
</tr>
<tr>
<td>19</td>
<td>No</td>
<td>65</td>
<td>IIb</td>
<td>Squamous</td>
<td>G3</td>
</tr>
</tbody>
</table>
higher than the percentage in the corresponding posttreatment samples (range, 27.7–83.8; median, 43.1; \(P = 0.0092\)).

MVD values in pre-celecoxib biopsies ranged from 28.0 to 55.6 (median, 38.5) and were significantly higher than the corresponding values in posttreatment samples (range, 16.0–49.5; median, 27.6; \(P = 0.012\)). In one case (7%) only, higher levels of MVD were found in posttreatment versus pretreatment tumor biopsy.

We also evaluated whether the difference in pretreatment versus posttreatment values of the markers examined could be related to the baseline COX-2 IDV levels in tumor cells.

As shown in Fig. 3, a direct correlation was found between the absolute basal levels of tumor COX-2 IDV and the difference in pretreatment versus posttreatment values of Ki67 (\(r = +0.77; P = 0.0032\)) and MVD (\(r = +0.55; P = 0.059\)).

We failed to find any modification of tumor and stroma COX-2 IDV, Ki67, and MVD expression in tumor tissue biopsies from patients not receiving the drug and used as control (Table 2).

**PGE\(_2\) Analysis.** Because of limited tissue size, analysis of PGE\(_2\) levels was performed in 10 cases of cervical tumor biopsies at baseline and at the end of celecoxib treatment (Fig. 4). At baseline, PGE\(_2\) levels ranged from 4.7 to 386.6 pg/mg wet tissue (median, 70.1) and showed a trend to be higher than PGE\(_2\) levels in posttreatment samples (range, 4.8–91.9 pg/mg wet tissue; median, 26.7; \(P = 0.092\)).

No correlation was found between celecoxib plasma levels at the end of treatment and the difference in PGE\(_2\) tissue levels before and after treatment (data not shown). Only in two cases (20%) was an increase in PGE\(_2\) levels found in posttreatment versus pretreatment biopsies. In these cases, celecoxib plasma levels at the end of treatment were 2.39 \(\mu\)M and 0.18 \(\mu\)M.

**Plasma Concentrations of Celecoxib.** Plasma concentrations of celecoxib showed a wide interindividual variation, with values ranging from 0.49 to 3.88 \(\mu\)M (median, 1.22 \(\mu\)M; mean \(\pm\) SD, 1.46 \(\pm\) 1.10 \(\mu\)M) at 24 h from the beginning of treatment, from 0.30 to 5.29 \(\mu\)M (median, 1.74 \(\mu\)M; mean \(\pm\) SD, 1.74 \(\pm\) 1.48 \(\mu\)M) at day 7, and from 0.77 to 3.80 \(\mu\)M (median, 2.03 \(\mu\)M; mean \(\pm\) SD, 1.81 \(\pm\) 0.93 \(\mu\)M) at the end of treatment.

There was no statistically significant difference among plasma concentrations of celecoxib at any interval time.

Finally, a trend to a direct correlation was found between the difference in pretreatment versus posttreatment values of COX-2 in tumor compartment and celecoxib plasma concentrations (\(r = +0.56; P = 0.05\)).

**SCC and Ultrasound Evaluation.** There were no treatment-related modifications of SCC levels in serum samples from pretreated cases (median, 3.8 ng/ml; range, 0.7–34.1 ng/ml) versus posttreated cases (median, 4.7 ng/ml; range, 0.4–30.3 ng/ml; \(P = 0.7\)).

The two-dimensional color Doppler evaluation showed no significant difference in peak systolic velocity in pretreated tumors (median, 23.0; range, 9–55) versus posttreated tumors (median, 15; range, 9–43; \(P = 0.4\)). Similar results were obtained when analyzing the time-averaged maximum velocity values (data not shown).

The three-dimensional-derived relative color was found not to differ in pretreated tumors (median, 25.8; range, 5.6–33.8)
versus posttreated tumors (median, 16.3; range, 5.2–41.5; \( P/H_11005 \) 0.3). As far as the other three-dimensional-derived indices of tumor vascularization are concerned, we failed to find any treatment-related modifications (data not shown).

Lymphocyte Mitogenic Response and Lymphocyte Subsets. The relative proportion of CD3, CD4, and CD8 cells were not significantly altered by celecoxib administration. Moreover, the proportion of a subset of CD4+ cells expressing the interleukin 2 receptor chain CD25 (T-regulatory cells) was also shown not to be modified by celecoxib administration (data not shown). Finally, there was no difference in the proliferative response to phytohemagglutinin in peripheral blood mononuclear cells collected before (PI, 4.08 ± 3.35) and after (PI, 6.2 ± 5.03) celecoxib treatment (\( P/H_11005 \) 0.15).

Discussion
We first showed in human cancer that short-term treatment with celecoxib at therapeutic doses is associated with the reduction of COX-2, Ki67, and MVD, while being uneffective on COX-2 expression in cells from tumor surrounding stroma. These modifications are not related to flogistic or repair processes caused by biopsy, because no changes of the markers were observed in biopsies from patients not receiving the drug. Our data on the concomitant reduction of COX-2, Ki67, and PGE2 production after celecoxib treatment confirm previous data by Kaur et al. (34), who used rofecoxib in precancerous esophageal lesions, and suggest that COX-2 inhibitors might negatively affect tumor proliferative potential, as evaluated by the reduction of intermediate markers.

We failed to find any treatment-related modification of ultrasound-derived indices of tumor vascularization. In contrast, we first documented in humans that COX-2 inhibitors decrease CD31-positive tumor microvasculature. The antiangiogenic activity of COX-2 inhibitors has been documented in several experimental models and was proposed as an additional mechanism supporting the \textit{in vivo} antitumor effects of COX-2 inhibitors in COX-2 positive, and also in COX-2 negative, tumors (3, 9, 35, 36).

In contrast, differently from preclinical models in which the antiangiogenic activity of COX-2 inhibitors has been linked to induction of apoptosis (36), we failed to observe any relevant modulation of apoptotic effect. Indeed, M30 staining was almost undetectable in pretreatment biopsies, consistent with previous studies demonstrating low spontaneous apoptotic index in cervical cancer cells (27, 37).

A growing body of evidence based on \textit{in vitro} results has recently proposed that the antitumor activity of COX inhibitors might occur independently of COX-2-mediated mechanisms.
Celecoxib Modulates Biochemical Factors in Cervical Cancer

(21, 38) via the interference with the serine/threonine kinase Akt, or ERK2 (21, 38). However, great caution should be taken when extrapolating in vitro results to in vivo models, given the possible occurrence of several yet unknown mechanisms regulating the cross-talk, in terms of COX-2 activity, among tumor, stromal, and endothelial cells and extracellular matrix, as already emphasized (39, 40). Indeed, the concentrations of coxibs shown to induce apoptosis in vitro are several fold higher than the concentrations obtained with our schedule and are achievable in humans (20, 21, 26, 38). Moreover, the concomitant reduction of COX-2, Ki67, MVD, and PGE2 values in posttreatment biopsies suggests that all these effects may be COX-2 dependent, as also supported by the direct correlation between the basal tumor COX-2 IDV and the amount of Ki67 and MVD reduction in posttreatment biopsies. Finally, although we failed to find a significant correlation between the reduction in PGE2 levels and celecoxib concentrations, a trend to a correlation between the extent of tumor COX-2 reduction and celecoxib plasma concentrations was observed.

All these observations suggest that it is the concerted targeting of COX-2 both in tumor cells and in the microvasculature that supports the overall antitumor activity of COX-2 inhibitors, as proposed recently by Zweifel et al. (36).

Interestingly enough, we found that treatment with celecoxib failed to induce any significant modification of COX-2 staining in nonvascular cells from tumor surrounding stroma.

In our previous study (18), we showed that in cervical cancer the presence of high COX-2 expression in tumor cells is associated with scarce cellular infiltrate in the stroma and, notably, with a lower proportion of immunoregulatory cells (18). In contrast, low/absent COX-2 expression in cervical tumor cells is associated with stronger COX-2 positivity and a higher percentage of CD3+, CD4+, and CD25+ cells (18) in the stroma. It is noteworthy that although COX-2 in tumor cells identifies cervical cancer patients with unfavorable prognosis, COX-2 expression in stroma inflammatory compartment is associated with better clinical outcome, suggesting that COX-2-positive immunoregulatory stromal cells can play a role in reducing tumor cell aggressiveness (18).

In this context, the lack of activity of celecoxib on COX-2 expression in the nonvascular component of tumor surrounding stroma suggests that the potential favorable effects of COX-2-positive stromal cellular infiltrate in cervical cancer is not impaired by COX-2 inhibitors. Likewise, no modification of peripheral lymphocyte subsets and proliferative potential was observed after celecoxib treatment.

Additional studies are needed to provide a deeper characterization of the subcellular profile of tumor stroma to understand the contribution of each cell type to tumor/stroma cross-talk in terms of COX-2 activity (36).

In conclusion, we report that in cervical cancer, celecoxib treatment decreases tumor COX-2 expression and markers of proliferation and neoangiogenesis, thus suggesting that selective COX-2 inhibitors may be a promising strategy not only for chemopreventive approaches but also for therapeutic approaches in this neoplasia. In this context, considering also the documented chemosensitizing and radiosensitizing activity of these drugs in preclinical models (41, 42), the definition of selected clinical settings would be of clinical relevance to plan combined multimodal interventions.

Finally, attention has to be focused on defining the true impact of COX-2-dependent/independent mechanisms in anti-tumor effects of COX-2 inhibitors, especially in light of the growing availability of newly developed COX-2 inhibitors (38, 40, 43), the potential antitumor activity and safety profile of which is under investigation.

References


38. Song, X., Lin, H. P., Johnson, A. J., Tseng, P. H., Yang, Y. T., Kulp, S. K., and Chen, C. S. Cyclooxygenase-2, player or spectator in cyto-
Celecoxib Modulates the Expression of Cyclooxygenase-2, Ki67, Apoptosis-Related Marker, and Microvessel Density in Human Cervical Cancer: A Pilot Study
