Constitutive Expression and Involvement of Cyclooxygenase-2 in Human Megakaryocytopoiesis

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Objective—Cyclooxygenase-1 (COX-1), but not COX-2, is expressed in human platelets, and thromboxane A\(_2\) (TXA\(_2\)) produced via COX-1 induces platelet aggregation. The objectives of this study were to investigate the expression of COX-1 and COX-2 during platelet differentiation and to determine whether these enzymes are involved in the differentiation.

Methods and Results—CD34\(^+\) progenitor cells isolated from human cord blood were cultured with thrombopoietin and c-kit ligand. The cells differentiated into megakaryocytes (CD34\(^-\)/CD41\(^+\)) after 8 days of culture and into platelets (CD41\(^+\)/proidium iodide\(^-\)) after 14 days of culture. The CD34\(^+\) cells expressed a trace of COX-1 gene and no COX-2 gene. On day 5, COX-2 gene expression was observed and continued throughout the remainder of the culture. COX-1 gene expression increased after 8 days of culture. The treatment of this liquid culture with indomethacin, a dual inhibitor of COX-1 and COX-2, and NS-398, a COX-2–specific inhibitor, suppressed megakaryocyte differentiation. In contrast, at a dose of 10\(^{-7}\) M, mofezolac, which is a highly selective inhibitor of COX-1, did not affect differentiation. NS-398–induced suppression of megakaryocyte differentiation was partly abrogated by stable analogues of TXA\(_2\).

Conclusions—We report here that COX-2 and COX-1 are constitutively expressed in megakaryocytes, and TXA\(_2\) produced by COX-2 plays an important role in megakaryocytopoiesis. (Arterioscler Thromb Vasc Biol. 2004;24:607-612.)

Key Words: megakaryocytopoiesis ■ cyclooxygenase-1 ■ cyclooxygenase-2 ■ platelets ■ thromboxane A\(_2\)
megakaryocyte terminal differentiation from hematopoietic stem cells into proplatelet formation. We have also demonstrated that inhibition of COX-2 activity suppresses megakaryocytopoiesis and a TXA2 analogue partially abrogated this suppression.

**Methods**

**Purification and Culture of CD34+ Cells**

Umbilical cord blood (CB) samples from normal full-term newborn infants were obtained from Tokyo Metropolitan Bokutou Hospital after informed consents were obtained from the mothers. CB samples were diluted 2-fold with phosphate-buffered saline (PBS) and separated by centrifugation (800g, 20 minutes) on Ficoll-Paque (density=1.077 g/mL; Pharmacia Biotech AB, Uppsala, Sweden) to obtain mononuclear cell preparations. CD34+ progenitor cells were purified from these preparations using a Dynal CD34 Progenitor Cell Selection System (Dynal AS, Oslo, Norway). First, anti-CD34 antibody-conjugated beads bound to the progenitor cells to allow separation from other cells in the preparation. Then, the progenitor cells were detached from the antibody-coated beads. Flow cytometric analysis of purified cell preparations using a phycoerythrin-conjugated anti-CD34 monoclonal antibody (clone BirMA-K3; DAKO, Glostrup, Denmark) showed that >95% of the selected cells were positive for CD34. These cells were cultured in X-vivo 20 medium (BioWhittaker, Walkersville, Md) containing 50 ng/mL TPO (PeproTech EC, London, UK) and 40 ng/mL c-kit ligand (KL) (Biosource, Camarillo, Calif) at an initial density of approximate 1×10^6 cells/mL. Cultures were maintained at 37°C in humidified 5% CO2 atmosphere.

**RNA Preparation and Semi-quantification of mRNA**

Total RNA was extracted from cells using Trizol Reagent (GIBCO BRL, Gaithersburg, Md).^2^ cDNA was synthesized from 2 μg of total RNA using the SUPER SCRIPT First-Strand Synthesis System (GIBCO BRL) according to the manufacturer’s protocol, and reaction product was submitted to PCR amplification using a Gene Amp PCR System 9600 (Perkin Elmer, Emeryville, Calif). The primers (CLONTECH Laboratories) for detection of COX-1, COX-2, thromboxane receptor (TP) and hypoxanthine guanine phosphoribosyltransferase (HPRT) were as follows: COX-1: 5'-TGCC-CAGCTCTGGGCGCCGCTT-3' and 5'-GTGACATACAGAACATGCCGACATGCA-3'; COX-2: 5'-TTCAATAGGACTTTG-TGGGAAAATGTGCT-3' and 5'-AGATCATCTTGCTGATGT-ATCT-3'; TP: 5'-CTCCTCTCATCTTGGCGGT-3' and 5'-CAGGGTCAAGAGCATGCAA-3'; and HPRT: 5'-TCCTCGATTTGATGATGATGAC-3' and 5'-CTTGCGACC-TTGACATCTTGGTA-3'.

PCR reaction for HPRT and COX-1 was repeated for 36 cycles, and each cycle included denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and primer extension at 72°C for 1 minute. PCR reaction for COX-2 and TP was repeated for 36 cycles, and each cycle included denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, and primer extension at 72°C for 1 minute. The PCR products were electrophoresed through a 1.2% agarose gel and visualized by staining the gel with ethidium bromide.

**COX Enzyme Assay**

Cells from day 14 of culture (3×10^6 cells/0.5 mL) were incubated with 10 μmol/L [1-14C]arachidonic acid for 10 minutes at 37°C. The reaction mixture was then acidified (pH 3.0) and extracted with 2 mL of ethyl acetate. The resulting organic phase was evaporated to dryness and the residue was applied to thin-layer chromatographic plates. The plates were developed with a solvent system of isocane/ethyl acetate/water/acetic acid (50:110:100:20, by volume). Distribution of radioactivity on the plate was detected by BAS 2000 imaging analyzer (Fuji X, Tokyo, Japan).

**Immunocytochemistry of COX-1 and COX-2**

Cells in X-vivo 20 medium were seeded on 48-well plates at a density of 1×10^6 cells/mL and incubated for 14 days at 37°C. The medium was removed, and the cells were fixed in PBS–2% formaldehyde for 30 minutes at room temperature. After two washings with PBS, the cells were permeabilized in PBS buffer containing 1% fetal bovine serum (FBS) and 0.5% saponin for 15 minutes. The cells were subsequently incubated with the primary antibodies (anti-COX-1 and anti-COX-2; Oxford Biomedical Research, Oxford, Mich) diluted 1:20 in PBS with 1% FBS for 60 minutes at room temperature. Samples were washed with PBS containing 1% FBS, then incubated for 60 minutes at room temperature with the secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG) diluted 1:40 in PBS with 1% FBS. The samples were washed with PBS containing 1% FBS and then rinsed with PBS. For negative control staining, the same procedure as described was performed, but without the primary antibody. Fluorescence confocal microscopy (FV300/FLUOVIEW, Olympus, Japan) was used with an argon laser as the excitation source. A 40× objective and laser-power setting of 30 milliwatts were used for detection of subcellular COX-1 and COX-2 after immunocytochemical staining.

**Determination of Megakaryocyte and Platelet Number in Culture by Flow Cytometry**

On day 14, cultured cells were collected and rinsed with PBS, then centrifuged at 1000 g for 10 minutes and fixed with 2% formaldehyde for 60 minutes. After the cells were washed twice with 0.1% BSA-PBS, they were incubated with a 1:100 dilution of FITC-conjugated mAb against human CD41 antibody (clone SB12; DAKO, Glostrup, Denmark) and 15 μg/mL propidium iodide (PI) (Calbiochem, San Diego, Calif). Cell-associated immunofluorescence was analyzed, and cells were sorted by FACScan and a Vantage flow cytometer using Cell Quest software (Becton Dickinson, San Jose, Calif). Cells that were doubly positive for CD41 and PI (CD41+/PI+) on FACScan were counted as megakaryocytes, and the number of CD41-positive and PI-negative (CD41+/PI-) cells (excluding small debris or microparticles) were counted as proplatelets. The identification of megakaryocytes and platelets was performed by electron microscopy using forward scatter as an indicator of cell size and the expression of CD42b proteins.^1^ The identification of proplatelets was also performed by electron microscopy using as a reference the same distribution of forward scatter as platelets isolated from human peripheral blood and aggregated by thrombin.

**Statistics**

Data are expressed as mean±SEM. The statistical significance of differences of the means was determined by one-way analysis of variance (P<0.05).

**Results**

**Expression of COX-1 and COX-2 in Human Megakaryocytes and Platelets Differentiated From CD34+ Cells**

CD41 was substantially expressed at day 8 after induction by TPO and KL. The percent of CD41+ cells obtained was 3.7% (background level), 74.1%, 82.6%, and 88.9% on day 3, 8, 13, and 18, respectively. In this system, we first examined the profile of PG production in megakaryocytes. As shown in Figure 1, TXB2 was mainly converted from arachidonic acid (Figure 1). Next, we determined the expression levels of COX-1 and COX-2 mRNA during megakaryocyte differentiation (Figure 1). As expected, the expression of COX-1 mRNA was slightly detected on days 3 and 5, and it gradually increased with culture age. The expression of COX-2 mRNA
of megakaryocytes (Figure 2, inset). Revealed that COX-2 proteins were localized in the cytoplasm but not in platelets on day 15. Microscopic observation whereas COX-2 proteins were still detected in megakaryocytes that displayed large size with multinuclei, platelets that displayed small size without nuclei, but not in!

As shown in Figure 2, COX-1 proteins were detected in electron microscope, the cells were characterized as platelets. As previously mentioned,18 morphological identification of these cells was performed on day 15. Some cells had abundant cytoplasm and multinucleated cells. Using an electron microscope, the cells were characterized as mature megakaryocytes. The other cells displayed very small size without nuclei, and using propidium iodide staining and electron microscope, the cells were characterized as platelets. As shown in Figure 2, COX-1 proteins were detected in platelets that displayed small size without nuclei, but not in megakaryocytes that displayed large size with multinuclei, whereas COX-2 proteins were still detected in megakaryocytes but not in platelets on day 15. Microscopic observation revealed that COX-2 proteins were localized in the cytoplasm of megakaryocytes (Figure 2, inset).

Effects of COX Inhibitors on Megakaryocytopoiesis
Because COX-2 is constitutively expressed in megakaryocytes, we next examined whether COX-2-induced PG synthesis was involved in megakaryocyte differentiation and platelet formation. The treatment of this culture with indomethacin or NS-398 (a specific inhibitor of COX-2)26 caused a decrease in the number of CD41+/PI+ cells on day 14 (Figure 3). The number of platelets (CD41+/PI-) also decreased, but treatment in the late stage (days 10 to 14) failed to suppress platelet formation (control: 14.10±0.46×10^5 cells; NS-398: 14.91±0.10×10^5 cells; no significant difference). In contrast, mofezolac, a specific inhibitor of COX-1,27,28 did not affect the megakaryocyte differentiation and platelet formation (Figure 4). These data indicate that COX-2 is involved in megakaryocyte formation.

Involvement of TXA2 in Megakaryocytopoiesis
It is known that megakaryocytes and platelets produce TXA2 and PGD2,29,30 and the main product in megakaryocytes that differentiated in our system was TXA2 (Figure 1). Therefore, we next investigated whether PGs produced by COX-2 affect megakaryocytopoiesis. The inhibition of megakaryocytopoiesis by NS-398 was specifically abrogated by the simultaneous addition of TXA2-receptor agonists, U44619 (1 mmol/L), and I-BOP (10 μmol/L) (Figure 5a).

However, the simultaneous addition of PGD2 did not abrogate the suppression (data not shown). To assess the involvement of TXA2 on megakaryocytopoiesis, we examined the gene expressions of thromboxane synthase (TXS) and TXA2 receptors during megakaryocyte differentiation. TXS was detected during megakaryocytopoiesis (Figure 6). During our cultures, we detected gene expressions of TPα and TPβ (Figure 6). Finally, the treatment with U51605, an inhibitor of TXS, caused suppression of megakaryocytopoiesis and thrombocytopoiesis, as shown in
Figure 4. Dose-dependent effect of mofezolac on megakaryocytopoiesis and thrombocytopoiesis. CD34+ cells were purified from human umbilical cord blood and cultured for 14 days with 50 ng/mL TPO and 40 ng/mL c-kit ligand. NS-398 (10^{-7}M) added throughout the 14 days of culture. Mofezolac at the indicated doses was added throughout the 14 days of culture. Columns and vertical bars represent mean±SEM of triplicate samples. **P<0.01 versus control.

Figure 5. Involvement of TXA2 in megakaryocytopoiesis. a, CD34+ cells were purified from human umbilical cord blood and cultured for 14 days with 50 ng/mL TPO and 40 ng/mL c-kit ligand. NS-398 (10^{-7}M), U44069 (3 μM), U44619 (1 μmol/L), and I-BOP (10 μmol/L) were added throughout the 14 days of culture. Columns and vertical bars represent mean±SEM of triplicate samples. **P<0.01 versus control. #P<0.01 versus NS-398 alone. b, CD34+ cells were purified from human umbilical cord blood and cultured for 14 days with 50 ng/mL TPO and 40 ng/mL c-kit ligand, then U51605 (10 μmol/L) was added throughout the 14 days of culture. Columns and vertical bars represent mean±SEM of triplicate samples. **P<0.01 versus control.

Figure 6. Time-dependent expression of TXS, TPα, and TPβ mRNA during human megakaryocytopoiesis. At several stages of megakaryocytopoiesis, mRNAs were purified and reverse-transcribed in the presence of oligo dT. The total cDNAs were then amplified using a set of oligonucleotides specific for TXS, TPα, and TPβ. Ethidium bromide staining of PCR products yielded 2 bands. The larger and the smaller band in each reaction correspond to TPα and TPβ mRNA, respectively.

Figure 5b. By treatment with U51605, TXB2 secretion was completely suppressed but PGE2 secretion was increased (day-5 control culture: TXB2=280 pg/mL, PGE2=not detectable; the treatment of NS-398, TXB2, and PGE2=not detectable; and the treatment with US51605: TXB2=not detectable, PGE2=270 pg/mL, respectively) However, the treatment with PGE2 in this system did not affect the megakaryocyte formation (data not shown).

Discussion

It is widely recognized that TXA2 produced by COX-1 in platelets stimulates aggregation. However, Weber and Zimmermann\(^3\) have reported that COX-2 mRNA and proteins were expressed in human platelets, and the extent of COX-2 expression might be an important factor in aspirin resistance. These findings suggest the possibility that megakaryocytes express COX-2 and COX-1. In a related report, when CMK and MEG-01 leukemia cell lines were differentiated to megakaryocytes by phorbol-12-myristate acetate, COX-2 was expressed. However, in that system, COX-2 mRNA significantly increased with only 3 hours of phorbol-12-myristate acetate treatment, and the level decreased rapidly,\(^29,30\) so it was not clearly demonstrated whether COX-2 was expressed constitutively in megakaryocytes. Here, we have reported that megakaryocytes differentiated from human cord blood expressed COX-2 as well as COX-1. The expression of COX-2 mRNA during megakaryocytopoiesis was shown by RT-PCR, and COX-2 protein was expressed in megakaryocytes, as demonstrated by immunostaining. These results were supported by the study in which megakaryocytes from bone marrow biopsies and those derived from thrombopoietin-treated CD34(+) hemopoietic progenitor cells in culture were positive for COX-1 and COX-2.\(^2\) In our culture system, the extraction of RNA and immunostaining were performed on the indicated day before medium change; however, in those experiments after medium change, the expression of COX-2 did not vary (data not shown), indicating that COX-2 may be expressed constitutively. This is not surprising because constitutive expression of COX-2 has been observed in several tissues and cells such
as rat brain,32 maculadensa of the kidney,33 tracheal epithelium,34 pancreatic islets,35 and hepatic stellate cells.36 Thus, expression of COX-2 in many specialized cell types appears to be differentially sensitive to stimuli that regulate the unique physiological activities of each tissue.

During megakaryocytopoiesis, TXS mRNA, TPα mRNA, and TPβ mRNA were also detected by RT-PCR. A single gene encodes the human TP, of which there are 2 splice variants, TPα and TPβ. The mRNA for both splice variants have been demonstrated in platelets, but the protein of TPβ was not detected in human platelets.25

In our experiments, mRNA for TPα and TPβ were expressed during megakaryocytopoiesis. Although we did not determine the protein expression, TX agonist or TXS inhibitor affected megakaryocytopoiesis in our system, indicating that TPs are functionally active.

Lorenz et al47 have demonstrated that COX-2–deficient mice had markedly reduced numbers of erythroid and myeloid colony-forming cells in the recovery phase after treatment with 5-fluorouracil. They showed that the platelet number on day 8 after 5-fluorouracil treatment was also clearly reduced in COX-2–deficient mouse (COX-2−/− = 7.5 × 10⁹/µL versus COX-2+/− = 2.1 × 10⁹/µL).

However, the number of platelets did not change in COX-2–deficient mouse cells without 5-fluorouracil treatment. These results suggest that COX-2 is involved in megakaryocytopoiesis, but other factors may compensate for COX-2 deficiencies in development. In our experiments, the treatment of the late phase of culture with NS-398 did not affect the number of platelets. These data were supported by a previous report in which inhibition of TXS by aspirin after 10 days of culture has no effect on platelet production.18

Despite the inhibition of platelet aggregation by classic NSAIDs (inhibition activity: COX-1 > COX-2), a COX-1–specific inhibitor, mofezolac, failed to suppress megakaryocytopoiesis (Figure 4). These data are supported by clinical data that classic NSAID therapy does not cause thrombocytopoiesis. The results of large clinical trials treated with COX-2 specific inhibitors have recently raised some concerns regarding the cardiovascular safety.45,46 This problem is still disputed, but the data in the present study will support that COX-2 selective inhibitors were not associated with an increased risk of cardiovascular thrombotic events.

References


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