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A small amount of cyclooxygenase 2 (COX2) is constitutively expressed in platelets

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Abstract

Cyclooxygenase (COX) is the rate-limiting enzyme in conversion of arachidonic acid to prostanoids, and has two isoforms, COX1 and COX2, which share ~65% amino acid homology. COX1 is universally expressed in many cell types including platelets; however, expression of COX2 is known to be more limited. We examined expression of COX2 mRNA and protein in platelets and platelet-derived microparticles; using quantitative RT-PCR, immunostaining, and Western-blotting. We have detected a significant amount of COX2 in platelets, both at mRNA and protein levels. We found that COX1/COX2 mRNA and protein ratios in platelets were 3–7:1, and 17:1, respectively. Expression level of COX2 in platelets was less than COX1, but comparable to the expression of COX2 in malignant epithelial cells. Considering the important role of COX2 in tumorigenesis and thrombosis, and the large number of circulating platelets, we propose that platelet COX2 may play an important role in physiologic and pathologic conditions.

Introduction

Cyclooxygenases (COX) are the rate-limiting enzymes in the biosynthesis of prostanoids. It has two primary isoforms, COX1 and COX2 each responsible for production of preferential prostanoids. COX1 is constitutively expressed in many tissues but expression of COX2 is mostly inducible by proinflammatory stimuli, growth factor, cytokines. [1;2] Overexpression of COX2 has been detected in malignant epithelial cells, such as in colorectal, breast,

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Declaration of Interest

The authors report no declarations of interest.

pancreatic, and lung cancers. Upregulation of COX2 is involved in tumor initiation, growth, and metastasis via production of PGE2 and possibly other COX2-generated prostanoids. [3] The anti-cancer activity of COX2-specific inhibitors such as celecoxib and rofecoxib has been shown by a number of studies. [1] However, the promising use of COX2 inhibitors in treatment of cancer patients was overshadowed by the detection of a higher risk of myocardial infarction and thrombotic events associated with these drugs. [4]

COX1 is expressed in platelets and is an important therapeutic target for aspirin. However, expression of COX-2 in platelet is controversial and is not commonly accepted. Weber et al. reported the presence of COX2 mRNA and protein in platelets [5] and speculated that COX2 in platelets is responsible for aspirin resistance. In another study, COX2 was detected during later stages of maturation of murine megakaryocytes [6]. Platelets isolated from COX2-deficient mice showed an increased aggregatory response to collagen and thrombin. [7] In that study, less than 10% of murine platelets expressed COX2 as detected by immunohistochemistry.

In the current manuscript, we compared the expression of COX2 to that of COX1 in platelets, and detected a significant amount of COX2 in platelets and platelet-derived microparticles. Considering the large number of platelets, this might have a significant impact on the amount of prostanoids available in plasma and in the tumor microenvironment, and ultimately on the risk of arterial thrombosis and on the behavior of malignant tumors.

Materials and methods

Studies were approved by the Institutional Review Board of The University of Texas M. D. Anderson Cancer Center and in accordance with an assurance approved by the US Department of Health and Human Services.

Preparation of platelets and microparticles

Platelets were isolated from human blood following a previously described gel-filtration method. [8] Platelet-derived MPs were prepared by activating the washed platelets with a combination of thrombin (0.1 unit/ml) and collagen (50 µg/ml), or calcium ionophore A23187 (4 µM) at 37°C for 30 min, as described before. [9;10]

Western blot analysis and immunofluorescence microscopy

Protein concentrations were determined by a BCA Protein Reagent Kit (Pierce Biotech.), and 30 µg (if not otherwise indicated) of proteins were subjected to gel electrophoresis on 10% SDS-PAGE gels. Standard recombinant proteins COX2 and COX1 were purchased from ThermoFisher Scientific and United States Biological, respectively. Antibodies used were against COX2 (Abcam, ab15191, at 1:500 dilution; ab52237, at 1:200 dilution), COX1 (Abcam, ab109025, 1:1000), and β-ACTIN (Sigma-Aldrich, A5316, 1:5000).

Double and indirect immunofluorescence (IF) staining was performed on 1.5 million platelets plated onto the coverslips. Platelets were washed twice with PBS, fixed with -20°C methanol for 10 min, and permeabilized with -20°C acetone for 90 sec. After blocking with

1% BSA in PBS for 30 min at room temperature, antibodies against COX2 (Abcam, ab15191, at 1:200 dilution) and CD42b (Santa Cruz, sc-7070, at 1:100 dilution) were incubated with platelets for 90 min. Platelets were washed with PBS 3 times, each wash for 5 min, and incubated with appropriate fluorescence-labeled secondary antibodies (ThermoFisher Scientific) for 1 h in dark at room temperature. After 3 washes with PBS, A ProLong antifade reagent (ThermoFisher Scientific) was used to complete the mounting of the coverslip. Images were then acquired with a Leica DMR epifluorescent microscope and the Metamorph software program (Universal Imaging).

RT-PCR and absolute quantification of mRNA

Total RNA was isolated from platelets or ovarian cancer cell lines (OVCAR5, HeyA8, and A2780) using mirVana miRNA Isolation Kit (ThermoFisher Scientific) and gene expression quantified by SYBR Green assays (40 ng cDNA per assay with triplicate) using the ViiTM 7 Rea-Time PCR System (Applied Biosystem of ThermoFisher Scientific) with an Absolute Quantification approach. [11;12] For RNA samples, first strand cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Threshold cycle number (Ct) was recorded for data analysis. The Ct value for the negative control was beyond the 40 cycle limit (Not detectable). The following RT-PCR primers were used. COX2, 5'-TGAGCATCTACGGTTTGCTG-3' (forward), and 5'-TGCTTGTCTGGAACAACTGC-3' (reverse). COX1, 5'-GAGTACTGGAAGCCGAGCAC-3' (forward), and 5'-GCACTCTGGAATGACAAGCA-3' (reverse).

For quantification of mRNA copies, cDNAs of both COX2 (PTGS2) and COX1 (PTGS1) were obtained from a commercial source (Transomic), linearized, cleaned up by QIAquick Spin Kits (Qiagen), quantified by a spectrophotometer, and used for generating standard curves in qRT-PCR. mRNA copy numbers for COX2 and COX1 in platelets and ovarian cancer cell lines were determined by using the Ct value of each PCR interpolated against the linear regression of the standard curve and subsequently performing an exponential transformation. The calculated data were finally normalized to the total RNA amount and expressed as copy number per ng of total RNA.

Results and Discussion

Platelets have COX2 mRNA

We quantified COX2 and COX1 mRNAs in platelets and in malignant ovarian epithelial cells (OVCAR5, HeyA8, and A2780) by quantitative RT-PCR. COX2 and COX1 encoding cDNAs were used as control templates. The PCR amplification efficiency is determined by the slope of the plot, which was very close for COX2 and COX1 (-5.6698 vs -5.3883, Figure 1A-B). [13] The linear regression of Ct vs log input of cDNA copy number generated models with a high correlation coefficient of 0.99 for both COX2 and COX1 (Figure 1A-B). COX2 mRNA copy number in platelets (145 ± 13 per ng of total RNA) was comparable to those in ovarian cancer cells (in average 948 ± 30 per ng of total RNA) (Fig. 1C). On the other hand, platelet expressed much more COX1 mRNA than ovarian cancer cells ($56,238 \pm 1369$ and 4602 ± 207 , respectively). As a result, COX1/COX2 mRNA ratio

in platelets was larger than ovarian cancer cells (370 vs 3–7 folds, respectively), while platelets express a significant amount of remnant COX2 mRNA from megakaryocytes.

Platelets have COX2 protein

We used Western-blotting on platelet lysates and immunofluorescence microscopy on intact platelets to investigate the presence of COX2 in platelets. COX2 was easily detectable in platelets lysate using 2 different anti COX-2 antibodies (Fig 2.A). We used COX2 and COX1 recombinant proteins as controls to confirm that the detection of COX2 by Western-blotting is not due to a cross reactivity of COX2 antibody detecting both COXs. COX2 antibodies were COX2-specific and did not bind to recombinant COX1 (Figure 2A). We further quantified COX1 and COX2 using Western-blotting and multiple concentrations of recombinant COX1 and COX2 standards. About 105 ng COX1 and 6.0 ng COX2 were detected in 10 µg of platelet lysate, which was equivalent to the protein content of ~20 million platelets (Figure 1B and 1C). The ratio of COX1 to COX2 in platelets was ~ 17:1. We also investigated the presence of COX2 in platelet-derived microparticles. We used calcium ionophore or collagen/thrombin to generate platelet microparticles as is described in the Methods. We found that COX2 and COX1 were both present in platelet-derived microparticles (Fig. 2D), and had similar expression pattern.

Next we used immunofluorescence staining to investigate the expression of COX2 in platelets. Washed platelets detected by the expression of CD42b were examined for the presence of COX2. Double immunofluorescence staining showed colocalization of COX2 with CD42b (Figure 1E) in more than 60% of platelets.

We examined three human ovarian cancer cell lines for COX1 and COX2 expression and compared those to platelets and platelet-derived MPs. The cell lines expressed varying amounts of COX2 but all had much lower levels than that of platelets and MPs. COX1 was abundant in platelets but could not be detected in the cell lines with Western-blotting, probably due to low sensitivity of the detection method, consistent with the results of a previous report. [14]

COX2 is reported to be expressed in later stages of megakaryocyte maturation and in a small percentage of platelets [6]. In this study, we showed the expression of COX2 mRNA and protein in platelets. We used COX1 as an internal control for our studies, and used COX2 and COX1 cDNAs to accurately quantify the respective mRNA copy numbers and used recombinant COX2 and COX1 proteins and two different COX2 antibodies in Western-blotting to overcome antibody specificity as a potential problem in measuring the relative amount of COX proteins. COX2 was easily detectable by Western-blotting, and present in more than 60% of platelets in immunofluorescence microscopy, which is much higher than the percentage of reticulated platelets in the peripheral blood. We compared the expression of COX2 to that of COX1 in platelets and platelet-derived microparticles. The copy number of COX1 mRNA was much more than COX2 in platelets (370:1) but this difference was less when the amount of COX1 and COX2 proteins was compared (17:1). This might be due to a relative instability of COX2 mRNA as compared to COX1 mRNA. [15] We identified a significant amount of COX2 protein in platelet-derived microparticles, and this was regardless of the method used for generation of microparticles from platelets (both calcium

ionophore- and collagen/thrombin-induced platelet microparticles). Considering the large number of circulating platelets, COX2 originated from platelets can contribute to prostaglandin metabolism inside blood vessels and, via platelet-derived microparticles, in the tumor microenvironment and potentially in tumor cells. Platelets are anucleated cells and lack the same robust transcription machinery as epithelial cells; as a result COX2 detected in platelet are the result of a constitutive expression of this protein, contrary to the COX2's inducible expression in many other cell types. COX2 expressed by platelets might have some functional effects as shown by the increased aggregation of COX2-deficient platelets in response to platelet agonists [7] and reduction of Thromboxane B₂ generated by washed human platelets in the presence of a selective COX2 inhibitor, although to a smaller magnitude than a selective COX1 inhibitor [16].

COX2 from platelets and platelet-derived microparticles may participate in the progrowth effect of platelets on cancer cells [17] and in thrombocytosis-induced chemoresistance [18]. As a result the anti-cancer benefit of COX2 inhibitors can be due to inhibition of COX2 in platelets. The possible benefit of platelet COX2 in preventing thrombotic vascular event and its potential risk in promoting cancer have significant clinical implications, and need to be further examined in clinical studies.

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Figure 1

Figure 1. COX2 mRNA is detected in human platelets

(A–B) Establishment of linear regressional equations for output variable Ct of RT-PCR and input variable logged cDNA copy number of COX2 and COX1, respectively. (C) Estimate of the COX2 mRNA level relative to that of COX1 in platelets and ovarian cell lines by RT-PCR. Total RNA samples from platelets and ovarian cell lines were assayed by RT-PCR to obtain corresponding Ct values. COX2 and COX1 mRNA copy numbers were calculated by using the above mentioned linear regressional equations and subsequent exponential transformation.

Figure 2. A significant amount of COX2 protein is present in human platelets

(A) Both COX2 and COX1 proteins were detected in platelets by Western blot analysis on platelet lysates along with recombinant COX2 and COX1 proteins (rCOX2, rCOX1). (B) Estimate of COX1 protein level in 10 μ g of platelet lysate by Western-blotting using a series of rCOX1 standards. (C) Estimate of COX2 protein level in 10 μ g of platelet lysate by Western-blotting using a series of rCOX2 standards. (D) Comparison (by Western blot) of COX2 and COX1 protein levels in ovarian cancer cells and platelets, platelet microparticles (MP). MP(I): MP prepared by using calcium ionophore as the agonist. MO(Col): MP prepared by using collagen and thrombin as the agonists. (E) Colocalization of COX2 and platelet marker CD42b as demonstrated by double immunofluorescence staining.