The cytosol fraction of human platelets did not convert prostaglandin (PG) H₂ to PGD₂. However, a homogenate of human megakaryoblastic CMK cells (precursor cells of platelets) produced PGD₂ from PGH₂. The PGD synthase activity was localized in the cytosol of CMK cells, and absolutely required glutathione. The catalytic properties and Western and Northern blottings indicated that the enzyme was PGD synthase of the hematopoietic type rather than the lipocalin type. When CMK cells were differentiated to megakaryocytes with phorbol ester along with induction of cyclooxygenase-1, the PGD synthase activity increased about 2-fold for 2 days and then decreased. In another human megakaryoblastic cell line, Dami, the PGD synthase increased about 10-fold by the addition of phorbol ester. Thus, the PGD synthase, which was undetectable in platelets, appeared during differentiation of megakaryoblasts to megakaryocytes.

Prostaglandin (PG)\(^1\) \(\Delta^2\) is an anti-aggregatory, vasodilating and bronchoconstrictive eicosanoid (1). Furthermore, the role of PGD₂ in sleep induction has been extensively investigated (2). The PGD₂ is formed from arachidonic acid by successive enzyme reactions: the oxygenation of arachidonic acid to PGH₂ via PGG₂ by PG endoperoxide synthase and the isomerization of PGH₂ to PGD₂ by PG synthase. There are two different types of PG synthase: the hematopoietic type and the lipocalin type (3). The former was purified from rat spleen (4, 5), identified as a \(\alpha\)-type glutathione S-transferase (6, 7), and immunohistochemically detected in mast cells of various organs (8) and antigen-presenting cells, histiocytes, dendritic cells, Kupffer cells, and Langerhans cells (9). The latter was purified from rat brain (10), and later found to be identical with \(\beta\)-trace, a major constituent in cerebrospinal fluid (11, 12).

A human megakaryoblastic cell line CMK was established from the peripheral blood of an acute megakaryoblastic leukemia patient with Down’s syndrome (13, 14). The cells differentiated to mature megakaryocyte-like cells by treatment with phorbol ester (TPA) or dimethyl sulfoxide (13, 14). Recently we showed that cyclooxygenase-1, but not cyclooxygenase-2, was induced in CMK cells during the differentiation (15). The cyclooxygenase-1 is well known as a constitutive enzyme present in platelets (16, 17), and is responsible for the production of proaggregatory thromboxane \(\Delta^2\) essentially as a sole cyclooxygenase metabolite. However, we found that the TPA-treated CMK cells produced PGD₂ as a major product in addition to thromboxane \(\Delta^2\) (15). Since PGD₂ was reported not to be produced in platelets (18), we were interested in the megakaryoblastic production of PGD₂ and attempted to characterize the responsible enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—\([1^{-14}C]\)Arachidonic acid (2.1 GBq/mmol) was purchased from Amersham International (Amersham, UK); arachidonic acid from Nu-Chek-Prep (Elysian, MN); lyophilized powder of sheep seminal vesicle microsomes from Novamed (Jerusalem, Israel); glutathione (reduced form), dithiothreitol, cysteine, and 1-chloro-2,4-dinitrobenzene from Wako Pure Chemical Industries (Osaka, Japan); 2-mercaptoethanol from Nakalai Tesque (Kyoto, Japan); precoated Silica Gel 60 F254 glass plates for TLC (20 cm × 20 cm, 0.25-mm thickness) from Merck (Darmstadt, Germany); fetal calf serum from Nippon Bio-Supplies Supplies Center (Tokyo, Japan); RPMI 1640 medium and Iscove’s modified Dulbecco’s medium from Life Technologies, Inc. (Gaithersburg, MD); TPA, esculetin, stromelysin, and penicillin G from Sigma; and glutathione-S-transferase from Pharmacia Biotech Inc. (Uppsala, Sweden). Other reagents used for Northern (15) and Western (19) blots were purchased as described previously. CMK cells were kindly provided by Dr. Eiji Shimizu of the Third Department of Internal Medicine, Tokushima University School of Medicine, with the consent of Dr. Takeyuki Sato of Chiba University, and Dami cells by Dr. Hiroshi Miyazaki of Kirin Brewery Company (Takasaki, Japan). Standard PGs and thromboxane \(\Delta^2\) were gifts from Ono Pharmaceutical Co. (Osaka, Japan). The anti-serum was raised in a rabbit against the recombinant human hematopoietic PGD synthase which was expressed in *Escherichia coli* and purified by glutathione-affinity chromatography (7). The rabbit antisera to human PGD synthase was prepared as described previously (20). CDNA for human hematopoietic PGD synthase was isolated by the 3-rapid amplification of cDNA end method (7).

**Cell Culture**—CMK cells (1 × 10⁷ cells/dish) and Dami cells (0.5 × 10⁷ cells/dish) were plated in a 150-mm plastic dish with 30 ml of RPMI 1640 medium containing 10% fetal calf serum, 23.8 mM sodium bicarbonate, and 34.3 \(\mu\)M streptomycin sulfate or with 30 ml of Iscove’s modified Dulbecco’s medium containing 10% horse serum, 35.7 mM sodium bicarbonate, 69 \(\mu\)M streptomycin sulfate, and 0.29 mM penicillin G. The Petri dishes were placed in a humidified 7% CO₂, 93% air incubator at 37 °C. The cells were subcultured every 3 days. For cell differentiation, TPA was dissolved in 30 \(\mu\)l of ethanol, and added to the cells in 30 ml of the medium. After incubation for various periods, the cells were scraped from the dishes, washed with phosphate-buffered
saline at pH 7.4, and resuspended in 0.5 ml of phosphate-buffered saline per 1 x 10^7 cells. Platelets were prepared from whole blood as described previously (21).

Enzyme Preparation—The cells were sonicated three times for 3 s at 30 kHz with an interval of 1 min after each sonication. The homogenate was centrifuged at 187,000 x g at 4 °C for 20 min, and the resultant supernatant was used as the cytosol. Protein concentration was determined by the method of Bradford (22) using bovine serum albumin as standard. The cytosol (2 ml containing 10 mg of protein) of CMK cells was loaded onto a glutathione-Sepharose 4B column (a bed volume of 10 ml) equilibrated with phosphate-buffered saline at pH 7.4 including 1 mM glutathione. The enzyme was highly stabilized by adding γ-globulin at a final concentration of 1 mg/ml.

Enzyme Assay—[1-14C]PGH_2 as the radioactive substrate was prepared by incubation of 100 μM [1-14C]arachidonic acid (2,500 cpm/nmol) with sheep seminal vesicle microsomes (5 mg) at 24 °C for 2 min in a 2-ml reaction mixture containing 0.1 M Tris-HCl (pH 8.0), 2 μM hematin, and 5 μM tryptophan. The radioactive PGH_2 was extracted with diethyl ether, and purified by TLC in a solvent system consisting of diethyl ether/petroleum ether/acetetic acid (85:15:0.1, v/v/v) at –20 °C. The PGD synthase assay was performed using 40 μM [1-14C]PGH_2 (10,000 cpm/5 μl of ethanol) for 1 min at 24 °C. The 0.1-μl reaction mixture contained 100 μM Tris-HCl (pH 8.0) and 1 mM glutathione. The reaction was terminated by addition of 0.3 ml of diethyl ether/methanol, 1 M citric acid (30:4:1, v/v/v). The etheral extracts were then separated by TLC with a solvent system of diethyl ether/methanol/acetic acid (90:2:0.1, v/v/v) at –20 °C. Distribution of radioactivity on the plate was detected by a BAS2000 imaging analyzer (Fujix, Tokyo, Japan). The cyclooxygenase assay was performed as described previously (15).

Western Blot—The cytosol (15 μg of protein) was subjected to 12% polyacrylamide gel electrophoresis in the presence of 0.1% SDS. Immunostaining was performed as described for cyclooxygenase (15) by the use of rabbit antiserum against recombinant human hematopoietic PGD synthase (1:2000 dilution). Immunostained bands were visualized by the enhanced chemiluminescence method.

Northern Blot—Total RNA was extracted with an ISOGEN solution (Nippon Gene, Tokyo, Japan) (23). Electrophoresis and transfer of RNA onto the membrane were carried out as described for cyclooxygenase (15). The blots were hybridized with 32P-labeled cDNA probes for human hematopoietic PGD synthase or glyceraldehyde-3-phosphate dehydrogenase in Rapid-hyb buffer (Amersham International). Distribution of the radioactivity on the membrane was detected by a BAS2000 imaging analyzer.

RESULTS

When the homogenate of human megakaryoblastic CMK cells was incubated with [14C]PGH_2, the major product was PGD_2 (lane 2 of Fig. 1A). Under this assay condition only a small amount of unstable PGH_2 was spontaneously converted to PGD_2 and PGE_2 in a protein-free buffer used as a control (lane 1). The activity to generate PGD_2 in the homogenate was mostly recovered in the cytosol fraction (lane 4), but was hardly detectable in the particulate fraction (lane 3). The enzyme activity required glutathione and the PGD_2 production was very low in the absence of glutathione (lanes 5 and 6 of Fig. 1B). Furthermore, when pretreated at 90 °C for 5 min, the cytosol totally lost the activity (lane 7). These results showed the presence of a cytosolic glutathione-dependent PGD synthase in CMK cells.

It should be noted that the cytosol fraction of human platelets did not significantly convert PGH_2 to PGD_2 either in the presence (Fig. 1, lane 8) or absence (lane 9) of glutathione. In addition, the cytosol fractions of porcine and rat platelets did not show any significant activity to produce PGD_2 (data not shown).

The PGD synthase activity was dependent on the concentration of PGH_2 (Fig. 2A), and a high K_m value for PGH_2 (about 200 μM) was obtained by Lineweaver-Burk plots. The specific enzyme activity was about 150 nmol/min/mg with a subsaturating concentration (40 μM) of PGH_2.

In view of the glutathione requirement, the cytosol of CMK cells was applied to glutathione-affinity chromatography (Fig. 3A). A bulk of proteins passed through the column, and the PGD synthase activity was eluted with a significant delay in the absence of glutathione, suggesting a weak interaction between the enzyme and glutathione. The enzyme was highly
purified by the glutathione-affinity chromatography, and the specific activity of three active fractions pooled was about 300 μmol/min/mg protein. Active fractions showed a 26-kDa protein band stained with silver nitrate (Fig. 3B). This protein band was immunostained with rabbit antisera against human hematopoietic PGD synthase (Fig. 3C), but not with antiserum against human lipocalin type PGD synthase activity.

The PGD synthase activity was followed during differentiation of CMK cells. When the cells were cultured in the presence of 0.1 μM TPA for different days, the cyclooxygenase activity in the cell homogenate was almost undetectable at first, but increased markedly in a time-dependent manner (Fig. 4). The maximum PGD synthase activity (10-fold at day 3) was observed at day 2, and thereafter the enzyme activity decreased. As examined by Western blotting, the cytosol fraction of CMK cells (15 μg of protein) was applied to Western blot analysis using anti-human hematopoietic PGD synthase antibody. C, total RNA (10 μg) was isolated from CMK cells, and applied to Northern blot analyses using cDNAs of human hematopoietic PGD synthase (hPGDS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 18S indicates the position of 18S rRNA.

DISCUSSION

CMK and Dami cells were established from the peripheral blood of patients with megakaryoblastic leukemia. The cells exhibit various biochemical and morphological characteristics
Prostaglandin D Synthase in Human Megakaryoblastic Cells

of megakaryoblasts or megakaryocytes of bone marrow, and are useful tools for biochemical studies on megakaryoblast differentiation (13, 24).

In the present study we revealed potent PGD₂ production by the cytosol fraction of CMK cells with a specific activity as high as about 150 nmol/min/mg protein. This value is comparable to the PGD synthase activity in the cytosol of rat peritoneal mast cells (105 nmol/min/mg protein under the same assay conditions) which was reported to be the highest among various rat tissues (8). Serum albumin was previously shown to convert PGH₂ to PGD₂ in a glutathione-independent manner (18), but the PGD synthase in CMK cells was absolutely dependent on glutathione. Therefore, it is unlikely that the PGD₂ formation which we observed is attributed to albumin derived from fetal calf serum in the cell culture medium. Several lines of evidence including catalytic properties and Western and Northern blot analyses demonstrated that the enzyme activity was due to the hematopoietic type of PGD synthase rather than the lipocalin type. Dami cells also contained the hematopoietic type of the enzyme. The hematopoietic PGD synthase has so far been found in mast cells of various tissues (8) and antigen-presenting cells (9). To the best of our knowledge, this is the first report for the presence of PGD synthase in megakaryoblastic cell lines, precursor cells of platelets which have no PGD synthase.

The cyclooxygenase-1 activity was almost undetectable in untreated CMK cells, but increased for 5 days up to 10 nmol/2 min/mg protein by the addition of TPA. In contrast, the PGD synthase activity was detected in the untreated cells with a specific activity of about 40 nmol/min/mg protein of the homogenate, and increased only by 2-fold in the presence of TPA. These observations indicate that the cyclooxygenase reaction rather than the PGD synthase reaction is a rate-limiting step in the biosynthetic pathway of PGD₂ starting with arachidonic acid released from phospholipid. Interestingly, the original level of PGD synthase activity in Dami cells was low, but the enzyme activity was increased markedly by the addition of TPA. The induced cyclooxygenase was also lower than the increased PGD synthase in Dami cells.

Previously it was a debatable subject whether or not platelets generated PGD₂. Later, it was clearly demonstrated that the capacity to generate PGD₂ in platelet-rich plasma was attributable to serum albumin and that neither washed platelets nor platelet lysate formed PGD₂ enzymatically (18). Indeed we could not detect the PGD synthase activity in the cytosol of peripheral platelets from different animal species. Unlike cyclooxygenase-1 and thromboxane A synthase, the induced PGD synthase is not transferred to platelets which are derived from megakaryocytes. Since CMK and Dami cells are leukemia cells, a high expression of PGD synthase may not be a physiological event. Therefore, it is important to examine its expression level in native megakaryoblasts and megakaryocytes, especially by immunohistochemical study, which may lead to a finding of unknown physiological function of PGD₂. Furthermore, PGD₂ may be further transformed to PGJ₂ and its derivatives which are known to have anti-proliferative activity and may regulate cell proliferation (25). Recently, 15-deoxy-D₁₂,14-PGJ₂, a further metabolite of PGJ₂, was reported to be a ligand for peroxisome proliferator-activated receptor γ which determined differentiation of fibroblasts to adipocytes (26).

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