FEBS Letters 409 (1997) 242–246 FEBS 18664

Inverse gene expression of prostacyclin and thromboxane synthases in resident and activated peritoneal macrophages

Shinsuke Kuwamoto[a,b], Hiroyasu Inoue[a], Yoshinori Tone[a], Yoshikazu Izumi[b], Tadashi Tanabe[a,]*

[a] *Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan*
[b] *Department of Biotechnology, Faculty of Engineering, University of Tottori, Tottori 680, Japan*

Received 19 March 1997; revised version received 21 April 1997

Abstract **Prostacyclin and thromboxane A_2 produced from prostaglandin H_2 are known to be important modulators with opposite biological activities. To examine possible roles of these prostanoids in immune responses, we have studied the gene expression of prostacyclin synthase (PGIS) and thromboxane synthase (TXS) in murine resident macrophages or in macrophages elicited with casein or *bacillus Calmette-Guérin* (BCG). Northern blot analyses showed that the PGIS mRNA was expressed in a decreasing order in the resident, and casein- and BCG-elicited macrophages. In contrast, the TXS mRNA was expressed in an increasing order in the resident, and casein- and BCG-elicited macrophages. On the other hand, the mRNA for cyclooxygenase-2, which produces PGH_2 and participates in the production of prostanoids in inflammation, was expressed in both the resident and BCG-elicited macrophages but barely in the casein-elicited cells. In situ hybridization analysis showed that the expression of mRNAs for PGIS and TXS was ascribable not only to the alteration of the expression levels of both mRNAs in the each macrophage but also to the changes in subpopulations of the cells expressing these mRNAs. These observations suggested that the inverse gene expression of PGIS and TXS in macrophages contributes to immune responses by modulating the relative levels of prostacyclin and thromboxane A_2.**
© 1997 Federation of European Biochemical Societies.

Key words: Prostacyclin synthase; Thromboxane synthase; Cyclooxygenase; Prostaglandin; Peritoneal macrophage

1. Introduction

Prostacyclin (prostaglandin I_2: PGI_2) and thromboxane (TX) A_2 are produced from the same substrate PGH_2 by their specific synthases, prostacyclin synthase (PGIS) and thromboxane synthase (TXS), respectively. Since PGI_2 and TXA_2 are opposing modulators in the vascular construction and platelet aggregation, the balance between production of PGI_2 by vessel walls and the production of TXA_2 by platelets is postulated to be important in the maintenance of vascular integrity [1]. PGI_2 and TXA_2 have been also considered to be important modulators in the immune system. PGI_2 has been suggested to be an inhibitor of macrophage activation and its analog OP-41483 is reported to be effective at suppressing the lymphokine-stimulated Ia antigen expression [2]. On the other hand, a TXA_2 agonist is reported to induce apoptosis of immature thymocytes highly expressing TXA_2 receptor [3]. However, the physiological significance of the balance between the production of PGI_2 and TXA_2 in the immune system still remains to be elucidated.

We and other investigators have isolated the cDNAs encoding human, porcine, rat and murine TXS [4–7], and bovine, human and rat PGIS [8–12]. Interestingly, rat TXS and PGIS cDNAs have been isolated from the identical cDNA library derived from peritoneal macrophages [11], indicating a possible coexistence of the both enzymes in the same preparation of the cells. It has been reported that the production of PGs including PGI_2 in elicited macrophages was less than that in resident macrophages, whereas the production of TXA_2 was selectively conserved when stimulated with agonists such as zymosan and arachidonic acid [13,14]. Furthermore, the profile of prostanoids produced by macrophages elicited with an acute bacterial infection of *bacillus Calmette-Guérin* (BCG) or *Listeria monocytogenes* was different from that by resident peritoneal macrophages or macrophages elicited with non-bacterial inflammatory reagents such as casein [15]. Recent studies have also shown that the inducible isoform of cyclooxygenase (COX), COX-2 predominantly regulates the production of PGs and TXA_2 in activated macrophages [16,17]. Therefore, the balance of the production of PGI_2 and TXA_2 appears to be important in macrophages as cellular regulators of immune responses. However, there has been no report describing the balance of the expression of TXS and PGIS in macrophages.

Here, we have studied the murine gene expression of PGIS and TXS as well as COX-2 in resident macrophages or in macrophages elicited with casein or *bacillus Calmette-Guérin* (BCG) in vivo, indicating a possibility that the inverse gene expression of PGIS and TXS contribute to immune responses by modulating the relative levels of PGI_2 and TXA_2.

2. Materials and methods

2.1. Molecular cloning and characterization of mouse PGIS and TXS cDNAs

Murine PGIS cDNA clone (λMPGIS5) was obtained from a mouse macrophage cDNA (λgt11) library (Clontech) by a plaque hybridization method using the bovine PGIS cDNA [8] radiolabeled with [α-^{32}P]dCTP (Amersham) by the random-priming method [18]. Hybridization was carried out overnight at 60°C and the membranes were washed at 60°C for 1 h in 3×SSC (0.45 M NaCl and 45 mM

*Corresponding author. Fax: (81) 6-872-8092

Abbreviations: PG, prostaglandin; PGI_2, prostacyclin; TX, thromboxane; PGIS, prostacyclin synthase; TXS, thromboxane synthase; BCG, *bacillus Calmette-Guérin*; PES, prostaglandin endoperoxide synthase; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

The nucleotide sequence reported in this paper has been submitted to the GenBank/DDBJ/EMBL Data Bank under the accession number AB001607.

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(97)00516-4

sodium citrate, pH 7.0) containing 0.1% SDS. The positive clone λMPGIS5 was isolated and the cDNA insert excised by *Eco*RI digestion was ligated into pBluescript II SK(−) for sequence analysis. A murine TXS cDNA clone was obtained by polymerase chain reaction (PCR) using DNA from the macrophage cDNA library as a template. Primers, 5′-AATGGAAGTGTTGGGGCTTC-3′, nucleotide residues −1 to 19 and 5′-AACTCTCCATCTCCACACGA-3′, complementary to nucleotide residues 1671 to 1692, were synthesized according to the nucleotide sequence of murine TXS cDNA according to [7]. The conditions of PCR amplification were as follows: denaturation at 94°C for 1 min, annealing at 53°C for 2 min and extension at 72°C for 4 min for a total of 30 cycles after incubation at 95°C for 5 min. The recombinant plasmid pMTX1 was obtained by ligation with the PCR product into pCR^{TM}II by the aid of TA cloning kit (Stratagene) for further analysis. The nucleotide sequence analysis was performed on a model 373A DNA sequencer (Applied Biosystems) and some sequences were further confirmed using [α-^{32}P]dCTP and BcaBEST dideoxy sequencing kit (Takara Shuzo, Kyoto). Computer analysis was performed with the aid of the GENETYX-MAC program supplied by Software Development (Tokyo).

2.2. Preparation of murine peritoneal macrophages

Male C57BL/6 mice at 8–12 weeks of age were purchased from Japan SLC (Shizuoka). Peritoneal macrophages were harvested from 34 mice with no treatment as resident macrophages, 21 mice stimulated with casein and 17 mice stimulated with BCG. For in vivo stimulation, mice were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) containing 5% casein or 1 mg dry weight of BCG before 4 days of the harvest. Peritoneal macrophages were obtained by lavaging of the peritoneal cavity with 5 ml of PBS on ice. Harvested peritoneal cells were centrifuged at $400\times g$ for 10 min at 4°C, resuspended in Dulbecco's modified Eagle medium (Life Technologies) containing 10% heat-inactivated fetal calf serum and 4×10^6 cells were seeded in each 90-mm tissue culture dish. After incubation for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air, the nonadherent cells were removed by washing with PBS 3 times. Using this method, 4.6×10^7, 5.8×10^7 and 9.0×10^7 cells were obtained for RNA preparations from mice with no treatment and mice elicited with casein and BCG, respectively. Over 90% of the adherent cells prepared were identified as macrophages by Wright's staining.

2.3. Northern blot analysis

Total RNA was isolated from the adherent macrophages according to the acid guanidium thiocyanate procedure [19]. Twenty micrograms of total RNA was electrophoresed on a 1.0% agarose gel containing 1.5% formaldehyde, and transferred to a nylon membrane. The membrane was hybridized overnight with ^{32}P-labeled cDNA for murine PGIS (nucleotide −35 to 1127) and was washed at 65°C for 1 h in $0.1\times$SSC containing 0.1% SDS. The same membrane was also hybridized with labeled cDNAs amplified by PCR for mouse TXS (nucleotides 102 to 1221) [7], rat COX-1 (nucleotides 299 to 1733) [20], COX-2 (nucleotides 202 to 1685) [21], murine α chain of Ia antigen and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (nucleotides 83 to 1130), respectively. The cDNA probe for the murine α chain of Ia antigen was prepared by the reverse transcription-PCR method with total RNA from the murine BCG-elicited macrophages as described [22].

2.4. In situ hybridization

The adherent macrophages described as above were removed from the plates by gently scraping after the incubation of PBS supplemented with 10 mM EDTA in 10 min. The collected macrophages were cytospun onto glass microscope slides. The *Eco*RI-digested fragment (nucleotides −35 to 1127) of murine PGIS cDNA from λMPGIS5 or the fragment (nucleotides 102 to 1221) of murine TXS cDNA from pMTXS1 were subcloned into the *Eco*RI site of pBluescript, respectively. In situ hybridization was carried out according to the method described previously [10]. Each antisense RNA was synthesized in vitro by T7 RNA polymerase in the presence of [^{35}S]CTP (NEN, 37 TBq/mmol). The resulting RNAs were hydrolyzed in 0.1 M sodium bicarbonate buffer (pH 10.6) to give fragments with sizes of approximately 150 nucleotides. Cell preparations were fixed with 4% paraformaldehyde, treated with 0.25% acetic anhydride, dehydrated in ethanol, and incubated with the ^{35}S-labeled antisense RNA probe for PGIS or TXS in the hybridization solution (50% formamide, $2\times$SSC, 10 mM Tris-HCl (pH 8.0), $1\times$Denhardt's, 10% dextran sulfate, 0.2% SDS, salmon sperm DNA (500 μg/ml), tRNA (250 μg/ml) and 100 mM dithiothreitol) at 42°C for 16 h. The preparations were washed with $2\times$SSC containing 10 mM 2-mercaptoethanol at 60°C for 1 h, treated with RNase-A (20 μg/ml) at 37°C for 30 min and then washed with $0.1\times$SSC containing 10 mM 2-mercaptoethanol at 60°C for 1 h. After dehydration in ethanol, the cell preparations were dipped into emulsion NTB-2 (Kodak) diluted with an equal volume of distilled water, developed with Freshline-L (Chugai Photo, Tokyo) after 3 weeks of exposure, and counterstained with hematoxylin-eosin. Inasmuch as the limits of cytoplasms were slightly defined with our stain, we counted the grains over the cell or those that were around the nucleus. A minimum of 100 cells was counted. The in situ hybridization of macrophages in this study was repeated 3 times with the separately prepared cells.

3. Results and discussion

3.1. Cloning and sequencing of murine PGIS and TXS cDNAs

Screening a murine macrophage cDNA library with a bovine PGIS cDNA as a probe [8] yielded a PGIS cDNA clone λMPGIS5 with a 1665-bp insert. The nucleotide sequence analysis showed that λMPGIS5 contained the 35-bp 5′-untranslated region, the 1503-bp open reading frame encoding a 501-amino-acid protein which has a calculated molecular mass of 57 046 Da, and the 127 bp of the 3′-untranslated region (data not shown). The predicted amino acid sequence of the murine PGIS exhibited 84.6%, 85.8% and 94.6% similarities with those of the bovine [8,9], human [10] and rat [11] enzymes, respectively. On the other hand, murine TXS cDNA (1713 bp) was obtained by PCR method using the primers prepared according to Zang et al. [7] and DNA from the murine macrophage cDNA library as a template. The nucleotide sequence of the cDNA was agreed with the sequence in [7] except four nucleotide differences, one nucleotide insertion and one nucleotide deletion in the nucleotide sequence from 229 to 262 (corresponding to the amino acid residues 77–87). The predicted amino acid sequence of the residues 77–87 from our sequence was almost identical to those of human [4], porcine [5] and rat [6] TXS, but was different from that of murine TXS [7].

3.2. Expression of PGIS and TXS mRNAs in murine peritoneal macrophages

To evaluate the gene expression levels of PGIS and TXS in macrophages, total RNAs have been isolated from resident macrophages or macrophages elicited with casein or BCG. Expression of the mRNA for α chain of Ia antigen in BCG-elicited macrophages was higher than that in resident or casein-elicited macrophages (Fig. 1B, lanes 1–3). This result agrees with the previous finding that Ia antigen is induced during activation of macrophages [23]. The murine mRNA for COX-1, a house-keeping isozyme of COX, was constitutively expressed (Fig. 1A, lanes 10–12) and the intensity of ethidium bromide-stained rRNA was also not changed in each macrophages (Fig. 1B, lanes 7–9), although the expression of GAPDH mRNA, which was usually used as an internal control of RNA loaded, was altered by activation of macrophages (Fig. 1B, lanes 4–6). From these results, expression of COX-1 not GAPDH mRNA was regarded as the internal control in this study.

The murine PGIS mRNA as a single species of 1.9 kb was expressed in a decreasing order in the resident, and casein- and BCG-elicited macrophages (Fig. 1A, lanes 1–3). In con-

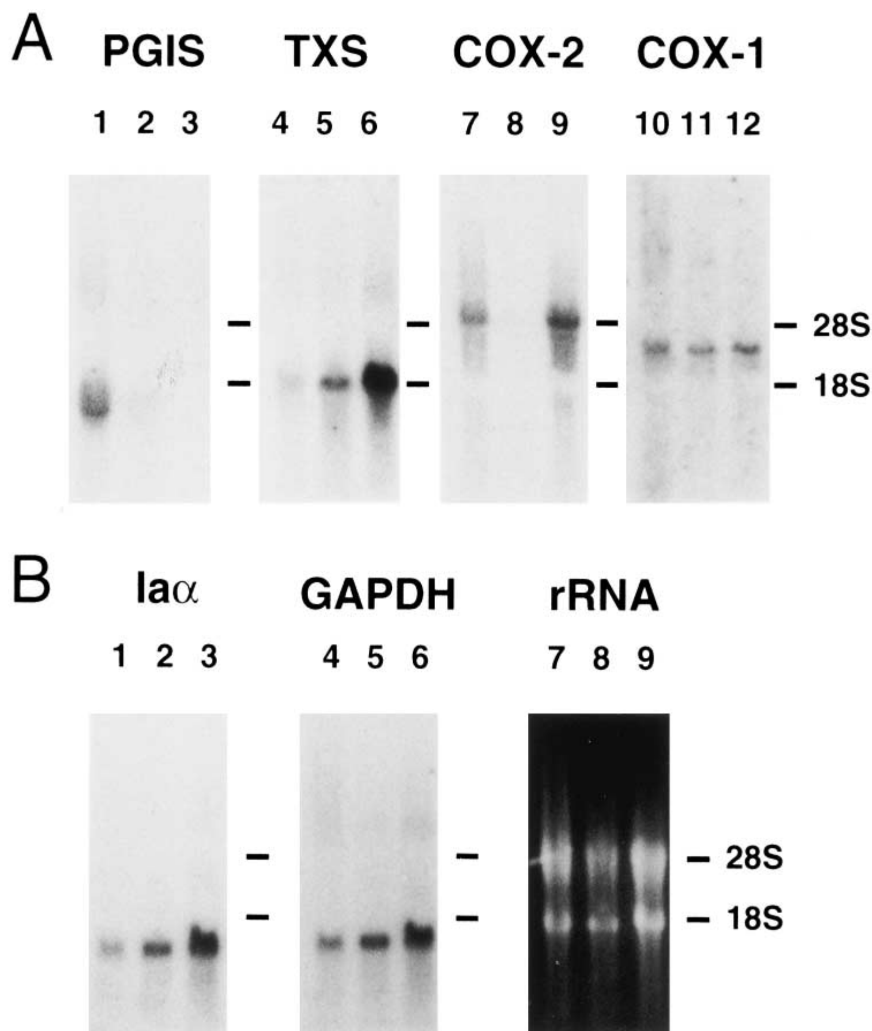

Fig. 1. Expressions of PGIS, TXS, COX-1 and COX-2 (A) or α chain of Ia antigen and GAPDH (B) mRNAs in murine peritoneal macrophages. Total RNA was isolated from resident (lanes 1, 4, 7 and 10), casein-induced (lanes 2, 5, 8 and 11) and BCG-elicited (lanes 3, 6, 9 and 12) macrophages, and 20 μg was fractionated through formaldehyde-containing agarose gels. The fractionated RNAs were transferred to a nylon membrane and hybridized with a ^{32}P-labeled murine PGIS probe. After analysis of the hybridization signals, the blot was stripped in boiling 0.1% SDS and rehybridized with a ^{32}P-labeled murine TXS probe and then, with a ^{32}P-labeled COX-2, COX-1, α chain of Ia antigen and GAPDH probes after stripping, successively. The positions of 28S and 18S ribosomal RNAs with ethidium bromide-staining patterns on the nylon membrane (B, lanes 7–9), are indicated on each right.

trast, the single species of murine TXS mRNA with a size of 2.1 kb was expressed in an increasing order in the resident, and casein- and BCG-elicited macrophages (Fig. 1A, lanes 4–6). On the other hand, mRNA for COX-2, which possibly participates in the production of prostanoids in inflammation, was expressed in both the resident and BCG-elicited but barely in casein-elicited macrophages (Fig. 1A, lanes 7–9). From these results, not only COX-2 but also PGIS and TXS, which are terminal enzymes in synthesis of bioactive prostanoids, are regulated during immune responses in macrophages. Concerning the COX-2 expression, our data may also provide a novel insight on regulation of COX-2 gene expression, that is, not only in BCG-elicited macrophages under an inflammatory condition but also in resident macrophages under a physiological condition.

3.3. *In situ hybridization analysis of PGIS and TXS mRNAs*

A population of resident or activated macrophages is suggested to exist as a mixture of several phenotypes which exhibit different patterns of eicosanoid synthesis [14]. Thus, the altered expression of PGIS and TXS by activation in macrophages may reflect the change of the subpopulations of macrophages expressing these enzymes. To examine the possibility, the expression levels of PGIS and TXS mRNAs in each cell (Fig. 2) was measured by in situ hybridization analysis. According to the hybridization signals, the macrophages were roughly classified into two types of cells with and without expression of PGIS mRNA (Fig. 2A–C) or TXS mRNA (Fig. 2D–F), respectively. To estimate the population of cells expressing PGIS or TXS mRNA, the grains over a cell were counted for a minimum of 100 cells in each macrophage preparation. As a control experiment, over 99% of total cells gave less than nine grains corresponding to PGIS or TXS mRNA in the presence of a 100-fold excess of each unlabeled probe. Therefore, cells with more than 10 grains were defined as cells expressing PGIS or TXS mRNA in this study. As shown in Fig. 3, 37%, 24% or 10% of cells expressing PGIS mRNA and 16%, 25% or 58% of cells expressing TXS mRNA were ob-

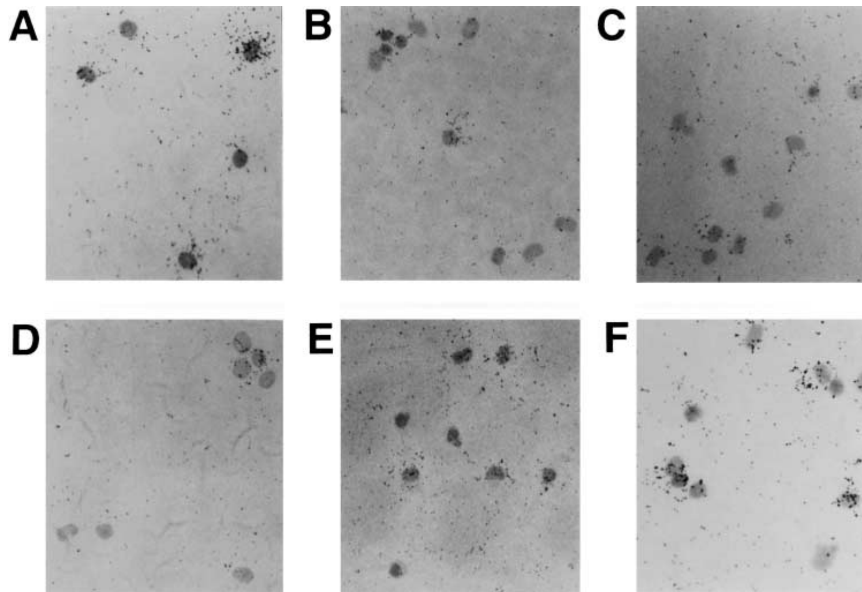

Fig. 2. In situ hybridization of murine peritoneal macrophages with a cRNA probe for PGIS or TXS. Macrophages (10^5 cells) were cytospun and fixed onto glass slides. After prehybridization, a ^{35}S-labeled cRNA for PGIS or TXS was allowed to hybridize to permeabilized cells overnight. Hybridized cRNAs were detected by overlaying photographic emulsion over the slides and were visualized by the development of grains within the emulsion, then counterstained with hematoxylin–eosin. Representative 400× amplified fields for resident (A,D), and casein- (B,E) and BCG-elicited (C,F) macrophages hybridized with a ^{35}S-labeled cRNA probe for PGIS (A–C) or TXS (D–F) are shown.

served in resident, casein- or BCG-treated macrophages, respectively. The decrease in the macrophage subpopulations expressing PGIS mRNA was accompanied by the increase in the subpopulations expressing TXS mRNA. This finding is, to some extent, explainable by monocyte migration during immune responses as previously reported [24]. In our experiments, however, about 1.4×10^6, 2.8×10^6 and 5.3×10^6 cells/mouse were obtained as resident, and casein- and BCG-elicited macrophages, respectively. Hence, only a 3.8-fold increase of the cell number by monocyte migration can not totally explain the significantly different levels of PGIS and TXS mRNAs between resident and BCG-elicited macrophages as shown in Fig. 1. From these results, the inverse expression of mRNAs for PGIS and TXS in resident and activated macrophages were ascribable not only to the alteration of the expression levels of both mRNAs in each cell but also to the changes in subpopulations of the cells expressing different levels of these mRNAs.

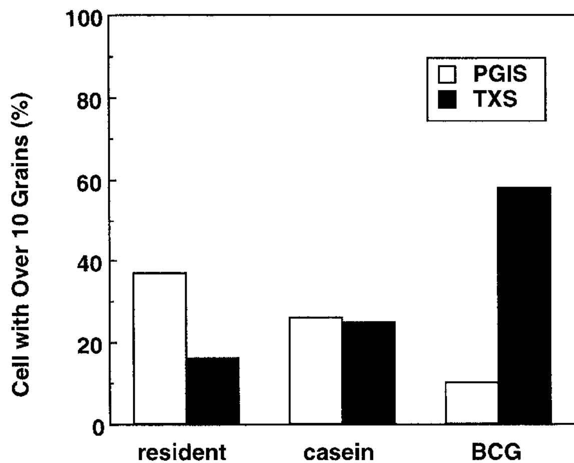

Fig. 3. Population of cells expressing PGIS or TXS mRNA in murine peritoneal macrophages. According to the results of in situ hybridization as shown in Fig. 2, the grains over the cell expressing PGIS or TXS mRNA were counted for a minimum of 100 cells. Percentages of cells with over 10 grains of resident, and casein- and BCG-elicited macrophages were indicated. Average grain numbers of PGIS mRNA in cells giving over 10 grains were 16 ± 5, 17 ± 6 and 15 ± 5 in resident, and casein- and BCG-elicited macrophages, respectively. Average grain numbers of TXS mRNA in cells yielding over 10 grains were 14 ± 5, 17 ± 6 and 20 ± 7 in resident, and casein- and BCG-elicited macrophages, respectively.

In this communication, we have shown that the expression of PGIS and TXS mRNAs is inversely regulated during activation in macrophages. It is clear that there exist a population of macrophages expressing neither PGIS nor TXS mRNA because the sums of percentages of cells expressing PGIS and TXS mRNAs are 53, 49 and 68% in resident, and casein- and BCG-treated macrophages, respectively (Fig. 3). At present, however, we were unable to show the presence of a population of cells expressing both PGIS and TXS mRNAs since we have not been successful in differential display of PGIS and TXS mRNAs on a cell. Remarkably, the expression pattern of TXS mRNA was similar to that of mRNA for α chain of Ia antigen known as an activation marker of macrophages (Fig. 2). On the other hand, COX-2 mRNA was barely expressed in casein-treated macrophages which are reported to be in an intermediate stage of the activation (Fig. 1) [25]. These results suggest a possibility that macrophages are classified in terms of the expression of PGIS, TXS and COX-2 as activation markers of the macrophage.

Acknowledgements: We thank Dr. Ryotaro Yoshida (Osaka Bioscience Institute), Drs. Chieko Yokoyama, Shuntaro Hara, Toshihisa Hatae and Tomoko Yabuki (National Cardiovascular Center) for helpful discussions and also Tamiko Sugimoto, Setsuko Bandoh and Fumie Kishigami for technical assistance. This work was supported in part by grants from the Ministry of Health, and Welfare and the Ministry of Education, Science, Culture and Sports of Japan and also by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan.

References

[1] S. Moncada, J.R. Vane, N. Engl. J. Med. 300 (1979) 1142–1147.
[2] C.S. Tripp, A. Wyche, E.R. Unanue, P. Needleman, J. Immnol. 137 (1986) 3915–3920.
[3] F. Ushikubi, Y. Aiba, K. Nakamura, T. Namba, M. Hirata, O. Mazda, Y. Katsura, S. Narumiya, J. Exp. Med. 178 (1993) 1825–1830.
[4] C. Yokoyama, A. Miyata, H. Ihara, V. Ullrich, T. Tanabe, Biochem. Biophys. Res. Commun. 178 (1991) 1479–1484.
[5] R.-F. Shen, L. Zhang, S.J. Baek, H.-H. Tai, K.-D. Lee, Gene 140 (1994) 261–265.
[6] Y. Tone, A. Miyata, S. Hara, S. Yukawa, T. Tanabe, FEBS Lett. 340 (1994) 241–244.
[7] L. Zhang, M.B. Chase, R.-F. Shen, Biochem. Biophys. Res. Commun. 194 (1993) 741–748.
[8] S. Hara, A. Miyata, C. Yokoyama, H. Inoue, R. Brugger, F. Lottspeich, V. Ullrich, T. Tanabe, J. Biol. Chem. 269 (1994) 19897–19903.
[9] B. Pereira, K.K. Wu, L. Wang, Biochem. Biophys. Res. Commun. 203 (1994) 59–66.
[10] A. Miyata, S. Hara, C. Yokoyama, H. Inoue, V. Ullrich, T. Tanabe, Biochem. Biophys. Res. Commun. 200 (1994) 1728–1734.
[11] Y. Tone, H. Inoue, S. Hara, C. Yokoyama, T. Hatae, H. Oida, S. Narumiya, R. Shigemoto, S. Yukawa, T. Tanabe, Eur. J. Cell Biol. 72 (1997) 268–277.
[12] T. Tanabe, V. Ullrich, J. Lipid Mediators Cell Signal. 12 (1995) 243–256.
[13] W.A. Scott, N.A. Pawlowski, M. Andreach, Z.A. Cohn, J. Exp. Med. 155 (1982) 535–547.
[14] C.S. Tripp, K.M. Leahy, P. Needleman, J. Clin. Invest. 76 (1985) 898–901.
[15] S.E. Wenzel, J.B. Trudeau, D.W.H. Riches, J.Y. Westcott, P.M. Henson, Inflammation 7 (1993) 743–756.
[16] S.T. Reddy, H.R. Herschman, J. Biol. Chem. 269 (1994) 15473–15480.
[17] T. Nanayama, S. Hara, H. Inoue, C. Yokoyama, T. Tanabe, Prostaglandins 49 (1995) 371–1995.
[18] A.P. Feinberg, B. Vogelstein, Anal. Biochem. 132 (1983) 6–13.
[19] P. Chomczynski, N. Sacchi, Anal. Biochem. 162 (1987) 156–159.
[20] L. Feng, W. Sun, Y. Xia, W.W. Tang, P. Chanmugam, E. Soyoola, C.B. Wilson, D. Hwang, Arch. Biochem. Biophys. 307 (1993) 361–368.
[21] J. Sirois, L.O. Levy, D.L. Simmons, J.S. Richards, J. Biol. Chem. 268 (1993) 12199–12206.
[22] K. Hayakawa, D. Tarlinton, R.R. Hardy, J. Immunol. 152 (1994) 4801–4807.
[23] D.I. Beller, J.M. Kiely, E.R. Unanue, J. Immunol. 124 (1980) 1426–1432.
[24] C.S. Tripp, E.R. Unanue, P. Neeedleman, Proc. Natl. Acad. Sci. USA 83 (1986) 9655–9659.
[25] T.A. Hamilton, J.E. Weiel, D.O. Adams, J. Immunol. 132 (1984) 2285–2290.