Exclusive expression of the Gs-linked prostaglandin E_2 receptor subtype 4 mRNA in human mononuclear Jurkat and KM-3 cells and coexpression of subtype 4 and 2 mRNA in U-937 cells

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Abstract Prostaglandin E_2 (PGE_2) is regarded as a potent regulator of the immune system. It can regulate apoptosis in mononuclear cells and modulate the cytokine secretion pattern from T-helper cell subpopulations via an increase in cyclic AMP (cAMP). Of the 4 PGE_2 receptor subtypes (EP1–EP4) that are defined pharmacologically by their affinity to subtype-specific ligands and their coupling to G proteins, EP2 and EP4 receptors couple to Gs. It is as yet unknown which of these two receptor subtypes mediates the immunomodulatory effects. By quantitative RT-PCR, the mRNA for EP4 receptors was demonstrated and quantified in the human mononuclear cell lines Jurkat, KM-3 and U-937. However, EP2 receptor mRNA was only present in U-937 cells and was 100-fold less abundant than EP4 receptor mRNA. PGE_2 increased cAMP formation with an ED_{50} of 50–100 nM in all cell lines. cAMP formation was inhibited by the EP4R-specific antagonist AH23848. Since AH23848 inhibited PGE_2-induced cAMP formation in U-937 cells to a similar extent as in Jurkat and KM-3, EP2 receptors seem to play, if any, only a secondary role for the PGE_2-mediated cAMP formation in U-937 cells.

Key words: Prostaglandin E_2 receptor; Cyclic AMP; Immunomodulation; Quantitative reverse transcription polymerase chain reaction

1. Introduction

Prostaglandin receptors belong to the R7G class of receptors. They consist of an extracellular N-terminal domain, 7 transmembrane domains, 3 extracellular and 3 intracellular loops and the intracellular C-terminal domain. They are coupled to heterotrimeric G proteins, which mediate signalling by second messengers like cyclic AMP (cAMP) or $InsP_3/Ca^{2+}$. According to their affinity to various agonists and antagonists and their coupling to different G proteins, receptors for PGE_2 can by divided into four subtypes (EP1–EP4) [1]. Of these, the EP1 receptor utilizes $InsP_3/Ca^{2+}$ as a second messenger, while the others modulate intracellular cAMP. The EP3 receptors decrease cAMP by inhibiting adenylate cyclase through a Gi protein, the EP2 and EP4 receptors increase cAMP by activating adenylate cyclase through a Gs protein.

PGE_2 has been suggested to be a modulator of the immune system. It can modulate cytokine secretion from T-helper lymphocyte subsets, termed Th1 and Th2 [2]. Th1 cells secrete certain cytokines like IL-2 and IFN-γ, which stimulate cytotoxic T-lymphocytes, whereas Th2 cells secrete mainly IL-4 and IL-10 and thereby stimulate B-lymphocytes. The cytokines secreted by Th1 cells can inhibit Th2 cells and vice versa [3,4]. PGE_2 can suppress cytokine secretion of Th1 cells while cytokine secretion of Th2 cells remains unchanged or is even enhanced [5]. Therefore, PGE_2 can shift the balance of the immune system in favor of a humoral over a cell-mediated immune response. Furthermore, PGE_2 supports the class switch from IgM to IgE in B-lymphocytes [6,7]. Finally, it can modulate agonist-induced apoptosis in T- and B-lymphocytes [8–10]. It is as yet unknown by which type of PGE_2 receptor these effects are mediated. Some of the immunomodulatory effects could be mimicked by raising intracellular cAMP through cholera toxin, forskolin or dibutyryl cAMP [6,11,12]. It therefore seems likely that the immunomodulatory effects of PGE_2 are mediated by the Gs-coupled EP2 and/or EP4 receptors.

The study of PGE_2 receptors on mononuclear cells by binding and functional studies has been hampered by the fact that binding characteristics of the PGE_2 receptor subtypes are very similar. The subtype specificity of 'selective' agonists and antagonists is limited and usually more than one type of prostanoid receptor or even PGE_2 receptor is present on the same cell. It is essentially impossible to differentiate between the multiple binding sites in a population. Especially low abundance sites might be missed. Only a few copies of PGE_2 receptors are present per cell [13–16]. The corresponding mRNAs are of low abundance and therefore hard to quantify by Northern blot techniques. Instead, a competitive reverse transcription polymerase chain reaction (RT-PCR) was used here as a highly sensitive method to demonstrate and quantify subtype specific receptor mRNAs in established human mononuclear cell lines as models for monocytes as well as B- and T-lymphocytes.

2. Materials and methods

2.1. Cell lines and culture

The established human cell lines Jurkat, KM-3 and U-937 were continuously cultured in suspension at a density of $0.5–2\times10^6$ cells/ml in medium containing 80% RPMI-1640 (Gibco, Eggenstein, Germany) and 20% M199 (Gibco). To this mixture 10% heat-inactivated fetal calf serum was added.

2.2. Stimulation of cAMP generation by PGE_2 and its inhibition by AH23848 in membrane preparations

Cells were lysed in 5 mM Tris-HCl (pH 7.4)/2 mM EDTA at 5×10^6 cells/ml with a rotor/stator homogenizer four times 5 s on ice. The homogenate was centrifuged for 2 min at $800\times g$ at 4°C and the supernatant centrifuged at $100\,000\times g$ for 1 h at 4°C. The crude membrane pellet was resuspended in 75 mM Tris-HCl (pH 7.4), 25

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Abbreviations: EP2R, prostaglandin E_2 receptor subtype 2; EP4R, prostaglandin E_2 receptor subtype 4; PGE_2, prostaglandin E_2; RT, reverse transcriptase

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mM $MgCl_2$, 2 mM EDTA and aliquots frozen at −70°C until further use.

An aliquot representing 25 μg of crude membrane protein was preincubated with AH23848 (Glaxo, UK, final concentration 25 μM) in 6% ethanol or plain 6% ethanol as control on ice for 10 min. PGE_2 was added at final concentrations from 10 nM to 10 μM and the incubation was continued for 10 min at 37°C. Samples were then incubated at 95°C for 5 min and stored at −20°C until detection of cAMP by radioimmunoassay (Amersham, Braunschweig, Germany).

2.3. Quantification of cell proliferation

On day 1, 1×10^4 cells were seeded in 100 μl culture medium in triplicate into a 96-well tissue culture plate (Sarstedt, Nümbrecht, Germany). PGE_2 was added every 8 h at final concentrations of 1 nM to 100 μM. On day 3, cell proliferation was determined by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the DNA of proliferating cells by ELISA (Boehringer Mannheim, Mannheim, Germany). Tests were performed according to the instructions of the manufacturer.

2.4. RNA isolation and cDNA synthesis by reverse transcription

Total RNA was extracted from approximately 20×10^6 cells by homogenization in guanidinium isothiocyanate, followed by cesium chloride gradient ultracentrifugation [17]. Total RNA concentration was determined spectrophotometrically. mRNA was isolated from roughly 50 μg total RNA by oligo-dT affinity chromatography (Qiagen, Hilden, Germany). For cDNA synthesis, the mRNA eluate was preincubated with oligo-$d(T)_{12-18}$ and transcribed with reverse transcriptase (RT) (Superscript II, Gibco) into cDNA in 30 μl buffer. The reaction was terminated by heating to 80°C for 5 min; then 30 μl of deionized water was added. Of this solution, 1 μl (representing about 16 ng cDNA) was taken for PCR.

To monitor the efficiency of cDNA synthesis, 1 μCi of [^{35}S]dATP (1000 Ci/mmol, ICN Pharmaceuticals, Eschwege, Germany) was included in the RT mix. The cDNA was precipitated with ammonium acetate/ethanol, washed 3 times with 70% ethanol and resuspended in 30 μl deionized water. The radioactivity in the final precipitate was proportional to the initial amount of mRNA and therefore represents the amount of newly synthesized cDNA. Equal amounts of radioactivity from the different RT reactions were taken for quantitative PCR.

2.5. PCR detection of EP2 receptor and EP4 receptor cDNA

PCR was carried out in a 50 μl reaction mix consisting of 0.75 μM of each forward and reverse oligonucleotide primer (Table 1), 1.5 mM $MgCl_2$, 0.2 mM of each of the four deoxynucleotide triphosphates (dNTPs), 6% DMSO (only for EP4R), 1 μl of cDNA preparation and 0.25 U of thermostable DNA polymerase (Goldstar Red, Eurogentec, Seraing, Belgium). The cDNA was denatured for 3 min at 95°C and then subjected to 35 cycles of 1 min at 95°C, 1 min at 60°C for EP2R or 63°C for EP4R and 2 min at 72°C. A final elongation step was carried out for 10 min at 72°C. EP2R and EP4R PCR products were cloned into the plasmid vector pBlueScript (pBS) and sequenced for identification using dyedeoxy terminator NTPs (Perkin Elmer, Weiterstadt, Germany).

2.6. Generation of internal PCR standards for the EP2 and EP4 receptors

A 930 bp RT-PCR fragment of the EP4 receptor [18] (primers EP4-3F and -2R) and a 1149 bp fragment of the EP2 receptor [19] (primers EP2-3F and -2R) were cloned into the *Sma*I restriction site of pBlueScript. From the construct containing the EP4R insert, a 171 bp fragment was removed by restriction enzyme digestion with *Bss*HII (at positions 1037 and 1208, GenBank accession number L25124), followed by gel electrophoresis, extraction (Genomed, Bad Oeynhausen, Germany) and religation of the new construct. Similarly, a 186 bp fragment was deleted from the EP2R construct by *Sma*I digestion (positions 703 and 889, GenBank accession number U19487). The shortened inserts (termed internal standards) were isolated by restriction enzyme digestion of the flanking polylinker region with *Eco*RI and *Bam*HI and purified by agarose gel electrophoresis and extraction. The concentrations of the extracted standards were determined and 10-fold dilution series were prepared, ranging from 500 amol/μl to 0.0005 amol/μl in deionized water containing 50 ng/ml glycogen.

2.7. Quantitative PCR for EP2R and EP4R cDNA

For quantitative PCR, 2 μl of the dilution series of the internal standards was coamplified with a constant amount of test sample cDNA. Aliquots representing equal numbers of counts and therefore equal amounts of test sample cDNA from different preparations were used to ensure an equal cDNA load in the PCR reaction. After amplification, PCR products were separated and visualized on a 1% agarose gel.

3. Results and discussion

3.1. RT-PCR detection of EP2 and EP4 receptor mRNA in human mononuclear cell lines

PGE_2 receptors are expressed on human lymphocytes at low copy number; some cells express more than one type of PGE_2 receptor, rendering analysis of cellular receptor equipment by binding studies with synthetic ligands and [^{3}H]PGE_2 difficult [13–16]. With the cloning of the human EP2 and EP4 receptors, molecular analysis with highly sensitive methods like RT-PCR has become possible. Therefore, this method was used to study receptor expression in the human cell lines Jurkat, KM-3 and U-937 as models for human T- and B-lymphocytes and monocytes, respectively.

After RT-PCR amplification, cDNA for β-actin was observed in all three cell lines, indicating successful cDNA synthesis (Fig. 1). In a control setup, no transcripts were obtained with mock transcribed mRNA, indicating that PCR products

Table 1
Oligonucleotide primers used for RT-PCR

Name	Sequence (5′→3′)	Position	GenBank accession number
EP2-1F	ctc tga gtc tcg gaa cgc tcc agc tc	64–89	U19487
EP2-2R	cat ttc tcc agg gaa caa ttt c	1305–1284	
EP2-3F	atg ggc aat gcc tcc aat gac tcc cag	157–183	
EP2-4R	tgg cga aag cga agt agg tgc acg cgc	502–476	
EP4-1F	atg aag ggc caa tgg ccc ggg ggc cag	826–852	L25124
EP4-2R	ctc ggc gcc cgc gat gcg gcg gaa gc	1386–1361	
EP4-3F	gtg aaa gca ggt tgg agg cgg gtc cag	457–483	
EP4-4R	cag gat ttt ata agg gtc cag aaa cag	2102–2076	
β-actin F	gtg ggc cgc tct agg cac ca	25–44	M12481
β-actin R	cgg ttg gcc tta ggg ttc agg ggg g	270–245	

Primers were designed for the human EP2 and EP4 receptors. Given are the primer positions in the published sequences. Mouse β-actin primers, which also serve to amplify a cDNA fragment of human β-actin (positions 144–163 and 364–388 in GenBank J00074), were purchased from Stratagene (La Jolla, CA, USA).

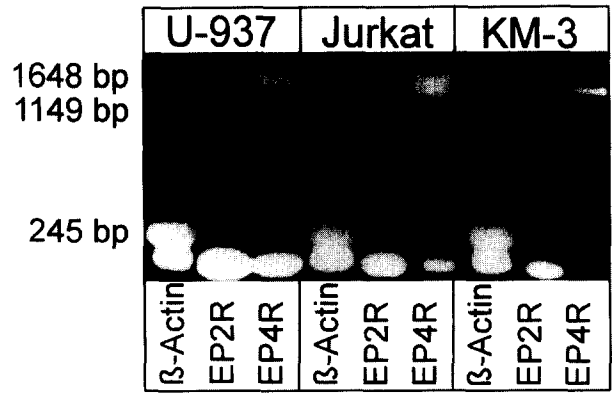

Fig. 1. Detection of mRNA for the EP2 and EP4 receptors in Jurkat, KM-3 and U-937 cells by RT-PCR. cDNA pepared from mRNA by reverse transcription was subjected to 35 cycles of PCR in the presence of sequence specific primers for the EP2 (primers EP2-3F/EP2-2R, 1149 bp) and EP4 receptor (primers EP4-3F/EP4-4R, 1648 bp) and β-actin (245 bp).

truly reflect cDNA transcripts and are not due to genomic DNA contamination (data not shown). All PCR reactions were prepared as master mixes before the addition of cDNA and oligonucleotide primers, and the same cDNA was used for the detection of EP2 and EP4 receptor mRNA. While the mRNA of the EP4 receptor was found in all cell lines, the mRNA of the EP2 receptor could only be detected in U-937 cells (Fig. 1). To exclude that EP2 receptor mRNA was missed because of ineffective reverse transcription or PCR, cDNA prepared from mRNA of human placenta, from which the EP2 receptor was originally cloned, served as a positive control (data not shown).

Receptors of the EP1, 3 and 4 subtypes but not of the EP2 subtype have recently been described on murine B-lymphocytic cell lines by molecular analysis [20]. Although EP2 receptors were not demonstrated directly, their presence was postulated on immature, but not on mature B-cell lines from functional studies with various prostaglandin analogues. In the present study, mRNA of the cloned human EP2 subtype receptor could not be demonstrated in the immature, pre-B-lymphoblastic cell line KM-3 by RT-PCR (Fig. 1), although this method is far more sensitive. These findings are further supported by the detection of mRNA of the EP4, but not of the EP2 receptor in various other T- and B-lymphocytic cell lines (CEM, HUT78, Raji, Daudi, Wi-L2 and H2LCL, own data not shown). It is therefore possible that human and murine B-cell lines differ with respect to the expression of EP2 receptors.

3.2. *Validation of quantitative competitive RT-PCR and the quantitation of mRNA for the EP2 and EP4 receptor in human mononuclear cell lines*

RT-PCR is not only a sensitive tool for the qualitative detection of mRNA, it can also be used for mRNA quantification when certain important prerequisites are met [21,22]. Due to the exponential nature of the amplification process, small differences in either the amount of initial cDNA or the efficiency of the amplification process can lead to significant differences in product quantity. Therefore, for the comparison of mRNA levels in different samples, equal amounts of initial cDNA, i.e. identical amounts of radioactivity of the reverse transcription in the presence of [^{35}S]dATP, were used and possible differences in the amplification efficiency were accounted for by the use of a second cDNA template coamplified in the same reaction. Since amplification efficiency crucially depends on the oligonucleotide primers but only to a negligible extent on the amplified sequence [21], an internal cDNA standard that was slightly smaller than the target cDNA and that was amplified with the same primer pair as efficiently as the target cDNA (own data not shown) was used [22].

When wild-type cDNA was amplified by PCR in the presence of increasing amounts of internal standard cDNA (0.001–1000 amol), both competed for the oligonucleotide primers according to their molar ratio in the reaction mix (Fig. 2). At a high molar excess of the internal standard cDNA, essentially only the standard was amplified by PCR, while only the wild-type cDNA was amplified when present in high molar excess. When both cDNAs were present in equimolar amounts, both products were formed in equal intensity. Thus, the initial amount of wild-type cDNA could be quantitated by determining the amount of internal standard that resulted in the same amount of PCR product as the wild-type cDNA.

EP4 receptor mRNA was present in all cell lines in similar amounts of about 5 amol in the PCR reaction mix (Fig. 2). In U-937 cells, mRNA for the EP2 receptor amounted to about 0.05 amol; it was 100-fold less abundant than the EP4 receptor mRNA (Fig. 2). Given the final specific activity of [^{35}S]dATP of 1 μCi/20 nmol dATP in the RT reaction and an estimated average length of mRNA of 2500 nt, 1 mol of cDNA equalled roughly 7×10^{16} decays per minute (DPM). From the RT reaction aliquots of 1000 DPM representing 1.4×10^{-14} mol cDNA were used in the PCR reaction. Since 5 amol of EP4R specific cDNA was present, it can be concluded that about 1 in every 2900 mRNA molecules coded for the EP4 receptor in Jurkat, KM-3 and U-937 and hence in U-

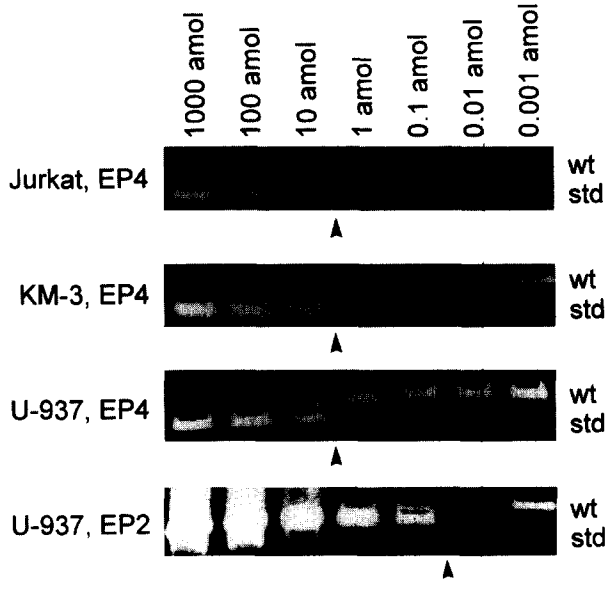

Fig. 2. Quantitation of cDNA for the EP2 and EP4 receptors in Jurkat, KM-3 and U-937 cells by competitive PCR. Equal amounts of cDNA were coamplified with a 10-fold dilution series (1000–0.001 amol) of the internal standards for the EP2 and EP4 receptor. The arrows denote the point of equimolarity between wild-type cDNA (wt) and internal standard (std). Data shown are representative of 3 experiments.

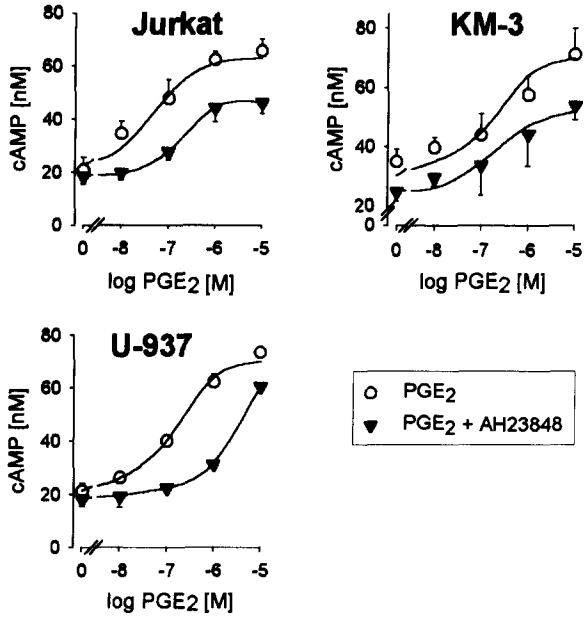

Fig. 3. Increase in cyclic AMP by PGE_2 and its inhibition by the EP4R specific antagonist AH23848 in plasma membranes of Jurkat, KM-3 and U-937 cells. Cell membranes were prepared by ultracentrifugation. Aliquots representing 25 μg of membrane protein were incubated with the indicated concentrations of PGE_2 alone or in the presence of 25 μM of AH23848. Values are means ± S.E. of 4 independent experiments.

937 cells only 1 in every 290 000 mRNA molecules coded for the EP2 receptor.

Although the correlation between the number of mRNA molecules and the corresponding receptor is not necessarily linear due to possible differences in translational efficiencies and posttranslational modifications, fewer EP2 than EP4 receptors can be expected on U-937 cells. Since the Gs-linked EP2 receptor has a lower affinity for PGE_2 than the Gs-linked EP4 receptor, this would implicate a high number of the high affinity EP4 receptor (K_d 1–2 nM [18,23]) and a small number of the low affinity EP2 receptor (K_d 100 nM [19]). This contrasts with findings of a highly abundant low affinity (K_d 137 nM) and a rare high affinity (K_d 3.1 nM) PGE_2 receptor on U-937 cells [24]. Another study reported that the main PGE_2 receptor (> 90%) on U-937 cells is a high affinity (K_d 2.9 nM) Gi-linked EP3 subtype receptor [16]. In Scatchard plots, this group could not detect the other binding site of the Gs-linked EP4 subtype receptor, which had been detected by functional studies and Western blot, and thereby confirm previous reports for monocytes [13]. Since multiple PGE_2 receptor subtypes seem to be present on the same cell type, the differentiation by binding studies appears to be impossible.

3.3. Functional PGE_2 receptors of the EP4 subtype on human mononuclear cell lines

The presence of EP4 receptor mRNA in a human B-, T-lymphocytic and a monocytic cell line is in accordance with functional studies. In all three cell lines, PGE_2 increased cAMP (Fig. 3). This increase in cAMP was inhibited by the EP4 specific antagonist AH23848. This antagonist was initially described as a competitive antagonist of PGE_2 at EP4 receptors with low affinity (pA_2 5.36) but high selectivity [25]. In Jurkat, KM-3 and U-937 cell membranes, however, it did not behave as a true competitive antagonist. This is in line with previous findings in Jurkat cells [26] that led to the postulation of a new EP4 receptor related subtype with a different pharmacological profile. The reason for this discrepancy is as yet unclear, but the present study confirms at least the presence of mRNA for the published EP4 receptor in Jurkat, KM-3, U-937 and other human B- and T-lymphocytic cell lines such as CEM, HUT78, Raji, Daudi, Wi-L2 and H2LCL (own data not shown, see above). Certainly, a neutralizing antibody against the cloned EP4 receptor could shed more light on this matter.

PGE_2 can induce apoptosis in mononuclear cells in vitro as well as in vivo under various conditions [8,9,27,28], although it has also been reported to suppress activation-induced apoptosis [10]. In murine B-cell lines, a dose-dependent growth inhibition of immature cells was reported, whereas mature cell lines were less susceptible [20]. However, in the present study, immature KM-3 cells as well as Jurkat and U-937 cells were only inhibited by PGE_2 at doses as high as 10^{-4} M (data not shown). Inhibition was most pronounced in U-937 cells (87%) and less in KM-3 (73%) and Jurkat (50%) cells.

3.4. Conclusions

This study demonstrated the presence of the mRNA for the EP4 subtype prostaglandin E_2 receptor in Jurkat, KM-3 and U-937 cells. mRNA for the EP2 receptor was present only in U-937 cells and 100-fold less abundant than EP4 receptor mRNA. All cell lines responded to stimulation with PGE_2 with a similar increase in cAMP formation, which was inhibited by AH23848. Therefore, the EP2 receptor appeared to play, if any, only a minor role in mediating the PGE_2-stimulated cAMP increase in human mononuclear cells. In contrast to murine mononuclear cell lines, PGE_2 did not inhibit proliferation in Jurkat, KM-3 or U-937 cells.

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