Expression of messenger RNA for the prostaglandin D receptor in the leptomeninges of the mouse brain

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Abstract The localization of prostaglandin D receptor in the mouse brain was examined by in situ hybridization histochemistry. The autoradiography showed significant hybridization signals of mRNA for prostaglandin D receptor in the leptomeninges covering the surface of the brain, but not in neurons or glia in the brain parenchyma. This finding was confirmed by Northern blot analysis using mRNA prepared from either the whole brain with the leptomeninges, brain parenchyma without the leptomeninges or the leptomeninges alone. A weak signal corresponding to the major 3.5-kbp transcript was detected in the whole brain. This band was significantly enriched in the leptomeninges, but was not detected in the brain parenchyma. These results suggest that prostaglandin D receptor is most highly, if not exclusively, expressed in the leptomeninges of the mouse brain.

Key words: In situ hybridization; Northern blot analysis; Prostaglandin D_2; Prostaglandin D receptor; Leptomeninx; Mouse brain

1. Introduction

Prostanoids, including prostaglandins (PGs) and thromboxanes, exert a variety of effects which maintain local homeostasis in the body [1]. They are synthesized upon stimulation, released immediately after synthesis, and act on surface receptors of cells in the vicinity to elicit their actions [2]. Among them, PGD_2 is a prostanoid, the physiological functions of which are least known. The most characterized PGD_2 action is anti-aggregatory activity on human platelets. PGD_2 is produced during platelet aggregation and works as a negative feedback modulator [3]. The receptor for PGD_2 (DP receptor) mediating this activity has been characterized and several synthetic agonists and antagonists have been developed (for review see [4]). However, this activity is limited to a few species of mammals and is not seen in others including rodents [5]. PGD_2 is also synthesized and released during allergic challenge by activated mast cells [6]. This synthesis is catalyzed by mast cell-specific glutathione-requiring PGD synthase [7]. However, the target cells and the role of PGD_2 in allergic reactions remain unknown. PGD_2 is also one of the major PGs synthesized in the brain, and much attention has been paid to its possible physiological functions in the central nervous system [8]. Exogenous PGD_2 administered into the brain and the spinal cord exerts several actions such as sleep induction [9], hypothermia [10], modulation of olfactory function [11] and of LH-RH release [12], and analgesia [13]. However, the properties and localization of the receptor mediating these actions remain unknown. This knowledge may be essential to the elucidation of the physiological significance of PGD_2 and its actions in the brain. Recently, the DP receptor of mice [14] and humans [15] has been cloned. These receptors show pharmacological properties consistent with the DP receptor previously characterized in platelets and other systems [16]. Northern blot analysis of their mRNA expression, however, revealed very low expression in the brain. To clarify the apparent discrepancy between receptor expression and the reported actions of PGD_2 in the brain, we carried out an in situ hybridization study to examine localization of the DP receptor in the mouse brain.

2. Materials and methods

2.1. In situ hybridization

2.1.1. Synthesis of cRNA probes. An antisense cRNA probe for the mouse DP receptor was prepared as follows. A 1.2-kbp fragment, PGc9 [14], a functional cDNA encoding the mouse DP receptor, was subcloned into pBluescript SK(−) (Stratagene). After linearization with *Eco*RV, it was used as a template for riboprobe synthesis. An antisense riboprobe for in situ hybridization analysis was prepared by transcription with T3 RNA polymerase (Promega) in the presence of [α-^{35}S]CTP to a specific activity of 1.0×10^9 dpm/μg. The cold antisense riboprobe was synthesized by the same procedure using unlabeled nucleotides. After unincorporated nucleotides were removed, riboprobes were degraded to ~150 bases by alkaline hydrolysis.

2.1.2. Preparation of tissue sections and hybridization reaction. Adult male ddY mice were anesthetized with diethyl ether and decapitated. The brains were removed, immediately frozen in isopentane at −50°C, and then cut into coronal or sagittal sections 8 μm thick on a cryostat. These consecutive sections covered the whole brain. The sections were then thaw-mounted onto poly-L-lysine (Sigma) coated slides. They were briefly air-dried and kept at −80°C until use.

The frozen sections were warmed to room temperature, fixed with 4% formaldehyde in phosphate-buffered saline for 10 min, rinsed in phosphate-buffered saline, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl for 10 min at room temperature. After dehydration in an ascending ethanol series, the sections were air-dried and stored at −80°C until use. Hybridization was carried out in 10 mM Tris-HCl, pH 7.5 containing 50% formamide, 2×SSC, 1×Denhardt's solution, 10% dextran sulfate, 0.2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 500 μg/ml sheared single-stranded salmon sperm DNA (ssDNA) and 250 μg/ml yeast transfer RNA (tRNA). The riboprobe, preheated at 80°C for 10 min in 1 M DTT, was added to the hybridization buffer at 7×10^4 dpm/μl. After the hybridization solution was applied, the sections were covered with a coverslip and sealed with rubber cement. After incubation at 57°C for 6 h, the slides were immersed at room temperature in 2×SSC to remove the coverslips, and then washed for 1 h in 2×SSC containing 10 mM β-mercaptoethanol. They were then warmed from room temperature to 60°C and cooled back to room temperature. The sections were then treated with 20 μg/ml ribonu-

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clease A in 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM ethylenediamine tetraacetic acid for 30 min at 37°C, followed by an additional wash in 0.1×SSC at 60°C for 1 h. After dehydration in an ascending ethanol series, the slides were air-dried and exposed to β-Max film (Amersham) for 7 days at room temperature, or were dipped in NTB 2 (Kodak), a nuclear track emulsion which was diluted 1:1 with distilled water. After exposure for 30 days at 4°C, the dipped slides were developed in D-19 developer (Kodak), fixed, and counterstained with 1% cresyl violet.

2.2. Northern blot analysis

DDY mice were killed by cervical dislocation and the brains were carefully removed so that the leptomeninges remained intact as much as possible. The leptomeninges, composed of the pia mater and the arachnoid membrane, were then peeled off with forceps under microscopy, and pooled. The remainder of the brain was used as brain parenchyma. Total RNA was isolated from the whole brain, brain parenchyma and the leptomeninges using the AGPC method, and poly(A)$^+$ RNA was prepared using Oligotex dT30 (Takara, Japan). A cRNA probe was synthesized as described in Section 2.1.1 using [α-^{32}P]UTP instead of [α-^{32}P]CTP. Hybridization was carried out at 70°C in 5×SSC, 50% formamide, 5×Denhardt's solution, 0.2% SDS, 250 μg/ml heat-denatured ssDNA, 200 μg/ml yeast tRNA and 5 ng/ml of cRNA probe. The filter was washed in 0.1×SSC, 0.1% SDS at 70°C for 20 min, treated with 1 μg/ml RNase A in 2×SSC for 10 min at room temperature, and washed again in 0.1×SSC, 0.1% SDS at 70°C for 20 min. The filter was exposed to X-ray film (Konica, Japan) for 12 h.

3. Results and discussion

We previously performed in situ hybridization to study expression of PGE receptor EP_3 subtype mRNA and revealed that this receptor is expressed in a variety of nuclei in the mouse brain [17]. We employed a similar procedure to examine the regional distribution and cellular localization of DP receptor mRNA in the brain with particular attention to those areas such as the preoptic area, hypothalamus and olfactory bulb where PGD_2 is thought to exert the reported actions. However, we could not observe specific signals in any nuclei in these and other areas of the mouse brain. Instead, in the film autoradiography, we detected weak but significant signals discontinuously covering the surface of the brain in both sagittal and coronal sections (Fig. 1a,b). This labeling was more evident in the coronal sections of the olfactory bulb, where intense signals were found in the inter-hemispheric region (Fig. 1b,d). This signal appeared specific, because it was absent in a neighboring section incubated in the presence of an excess of unlabeled probe (Fig. 1c). In the emulsion-dipped, counterstained sections, it was confirmed that the hybridization signals were localized in the leptomeninges covering the brain (Fig. 2), and that no specific signals were distributed on neurons or glia in the brain parenchyma. Localization of signals in the leptomeninges could explain the discontinuity of the signal on the brain surface and its enrichment in the inter-hemispheric region, because the leptomeninges tend to detach from the brain during sample preparation.

While the above findings suggest the expression of DP receptor in the leptomeningeal cells, it is also known that radioactive probes tend to accumulate along the cutting edges of tissue samples. In order to exclude the possibility of this so-called edging effect, we examined DP receptor expression in the isolated leptomeninges by Northern blot analysis, and compared it with the expression levels in whole brain with the leptomeninges and in the brain parenchyma (Fig. 3). Consistent with the previous findings [14], only a very faint hybridization signal was detected in the whole brain. This 3.5-kbp signal corresponding to the previously detected transcript in peripheral tissues [14] was very much enriched in the leptomeninges. In addition, minor hybridization bands were detected at 0.5, 2.0 and 6.0 kbp in this tissue. The expression level in the leptomeninges was much higher than that found in any other tissues reported in the previous study [14] (data not shown). In contrast, no hybridization signals were found in the brain parenchymal fraction in the present analysis.

The present study has thus revealed that the DP receptor is most highly expressed in the leptomeninges of the mouse brain. We cannot, of course, exclude the possibility that the DP receptor is also expressed by neurons and glia at levels

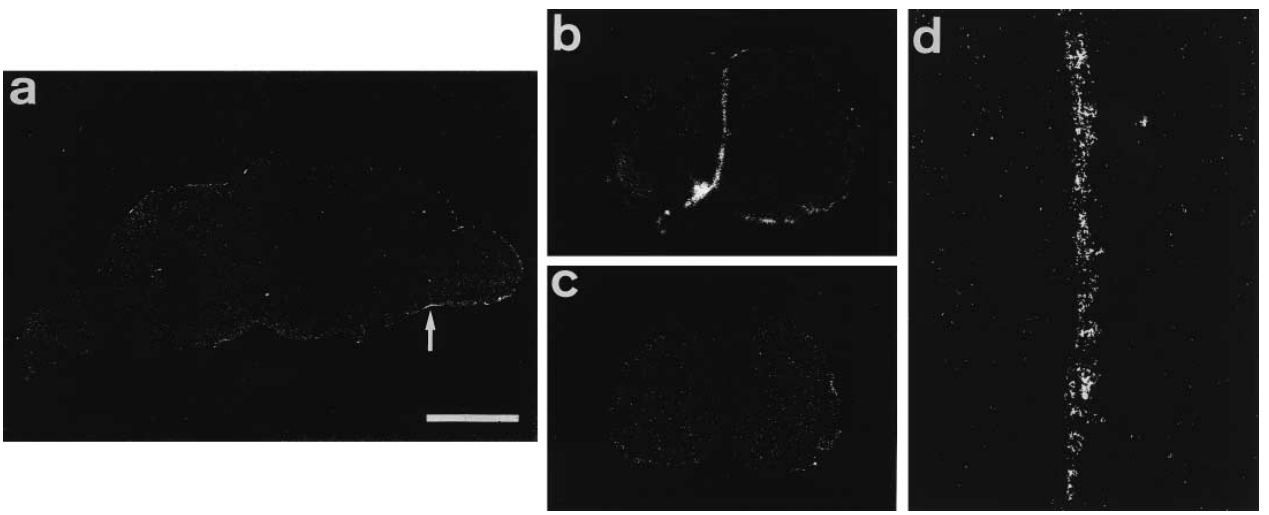

Fig. 1. Negative film images of in situ hybridization analysis of the mouse brain. Sections were hybridized with ^{35}S-labeled antisense riboprobe in the absence (a, b and d) or presence (c) of a 100-fold excess of the unlabeled probe. Panel a is shown in a sagittal plane approximately 0.3 mm lateral to the midline. Panel b is a transverse section of the olfactory bulb, which is located about 3.3 mm anterior to bregma indicated by an arrow in a. Panel c is the section adjacent to b, and panel d shows a higher magnification of the inter-hemispheric region shown in b. Bar, 3 mm for a, 1.7 mm for b and c, and 650 μm for d.

undetectable by the current methods, and that the receptor in the brain may be upregulated by certain stimuli. However, the DP receptor, if present in the brain, is at a level much lower than that found for the EP_3 receptor. Interestingly, Urade et al. [18] recently examined the localization of brain-specific PGD synthase by both immunohistochemistry and in situ hybridization and found that it is expressed abundantly in the leptomeninges and the choroid plexus. Thus, the enzyme responsible for PGD_2 synthesis and the receptor for this PG are both expressed in the leptomeninges, although no expression of the receptor mRNA was detected in the choroid plexus. This may indicate that the enzyme and the receptor are expressed by different types of cells in the leptomeninges. In any event, these findings taken together suggest that PGD_2 produced by cells in the leptomeninges acts in an autocrine or paracrine way on cells in the leptomeninges. It is not known what central action of PGD_2 is mediated by the DP receptor in the leptomeninges. Interestingly, Matumura et al. examined the site of the sleep inducing action of PGD_2 by microinjecting the PG into various parts of the brain and found that it induces sleep more effectively when injected into the subarachnoid space than when injected into the brain parenchyma. From these studies, they concluded that PGD_2 acts on the ventral surface of the rostral basal forebrain [19]. However, no expression of DP receptor mRNA was detected in the parenchyma of the brain in this area. On the other hand, the leptomeninges are markedly thick in this area with high levels of signals for DP receptor mRNA. It is possible, therefore, that PGD_2 injected into the subarachnoid space of this region works on the DP receptor in the leptomeninges to induce sleep. If this is the case, these observations suggest a new mode of regulation of brain function in which humoral factor released by the leptomeninges is delivered to the brain and affects its function. The leptomeninges are located in a strategic position between the peripheral circulation and the brain and may mediate the transmission of blood-borne signals such as cytokines to the brain by this mode of regulation. We have recently succeeded in preparing DP-deficient knockout mice. The above hypothesis and the possible involvement of the DP receptor studied here in sleep induction will be tested by the analysis of these DP-deficient mutant mice.

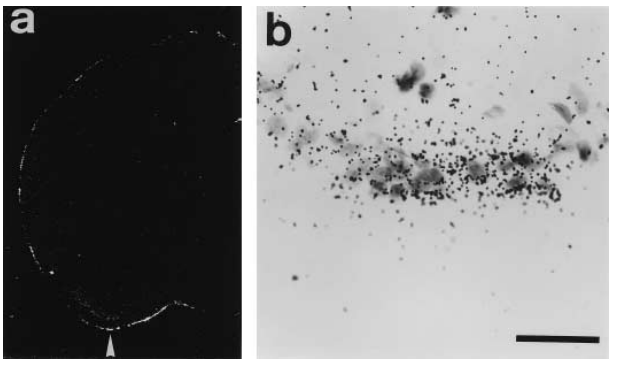

Fig. 2. Localization of DP receptor mRNA in the leptomeninges. A dark-field photomicrograph of the coronal section 1.2 mm posterior to bregma is shown in panel a, and a higher magnification bright-field photomicrograph of a ventral area (indicated by an arrow in a) of the adjacent section is shown in b. Bar, 1.7 mm for a and 100 μm for b.

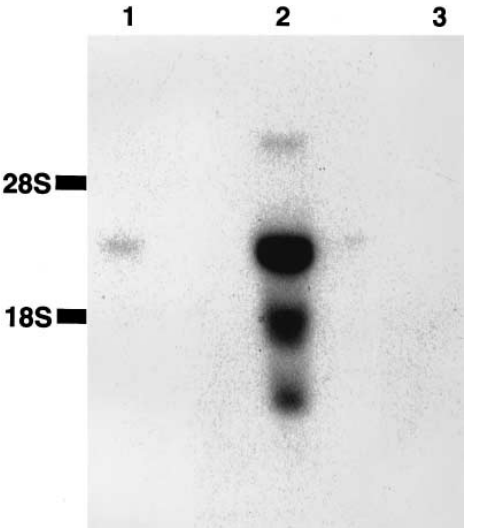

Fig. 3. Northern blot analysis of DP receptor mRNA in the mouse brain. Poly(A)$^+$ RNAs were isolated from the brain with or without the leptomeninges and from the leptomeninges themselves, and 5 μg of each RNA was applied to gel electrophoresis. After transfer to a membrane, hybridization was carried out using a ^{32}P-labeled antisense cRNA probe as described in Section 2. Lane 1, brain with the leptomeninges. Lane 2, leptomeninges. Lane 3, brain without the leptomeninges.

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