Fig. 4 ISH of COX-2 and EP_{2-4} in gallbladder carcinoma tissue. A specimen of gallbladder carcinoma tissues from a case of pT_4 carcinoma was used for analysis. In *A*, COX-2 mRNA is expressed diffusely in the corresponding cancerous epithelia (original magnification: ×200). In *B*, COX-2 mRNA is expressed focally in the corresponding fibroblasts (original magnification: ×200). *C, D*, and *F*, EP_2, EP_3, and EP_4 mRNAs are diffusely expressed in the corresponding cancerous epithelia (original magnification: ×200). In *E*, EP_4 protein is expressed in the cancerous epithelia (original magnification: ×200).

in 11 specimens of pT_3 and pT_4 gallbladder carcinomas (257.9 ± 26.1 pg/mg × protein, $P < 0.01$), compared with the concentrations in 10 specimens of normal gallbladders (59.2 ± 7.9 pg/mg × protein; Table 2). Similarly, in terms of the depth of invasion, the concentration was significantly higher in the pT_3 and pT_4 carcinomas than in the pT_1 and pT_2 carcinomas ($P < 0.01$).

Effect of EP2-4 Agonists on Colony Formation and C-fos Expression in Gallbladder Carcinoma Cells. COX-2 protein and mRNA were expressed strongly in the Mz-ChA-1 cells but only slightly in the Mz-ChA-2 cells (Fig. 5*A*). The Mz-ChA-1 cells were observed to produce significant amounts of PGE_2 in response to treatment with arachidonate, whereas the Mz-ChA-2 cells were observed to produce only trace amounts (Fig. 5*A*). PGE_2 production in the cells appeared to be dependent on the expression level of COX-2, as reported for colorectal carcinoma cells (24, 55). The mRNAs of EP_2, EP_3, and EP_4 mRNAs were amplified in the Mz-ChA-2 cells (Fig. 5*B*), whereas EP_1 mRNA was not detected. In the ISH, EP_{2-4} mRNAs were diffusely and strongly expressed in the cells (Fig. 5*B*).

To elucidate whether the effect of PGE_2 via the PLA_2/COX-2 pathway on tumorigenicity in the Mz-ChA-2 cells is mediated by EPs, we evaluated the effect of EP_{2-4} agonists or PGE_2 treatment on colony formation in a monolayer culture (Fig. 5*C*). Because the number of tumor colonies can be affected by increased proliferation of carcinoma cells or a decreased rate of apoptosis, a colony formation assay was performed. The Mz-ChA-2 cells were used for the experiments because endogenous production of PGE_2 was observed to be very low in the cells, and, thus, the signaling pathway via the EP_{2-4} may be less activated. A dose-dependent increase in the colony number after treatment with 0.01, 0.1, 1, and 10 μM EP_4 agonist (ONO-AE1-329) for 14 days (a 1.4-fold increase at a concentration of 1 μM), as well as a 1.3-fold increase after 1 μM PGE_2 treatment, was observed in the cells. However, the colony number of Mz-ChA-2 cells after treatment with EP_2 agonist (ONO-AE1-259; Ref. 52) and the number with EP_3 agonist (ONO-AE-248; Ref. 52) were 109 ± 5% (at a concentration of 1 μM) and 103 ± 6% (at a concentration of 0.1 μM) of the nontreated cells, respectively. Treatment with an EP_2 or EP_3 agonist did not cause significant changes in the colony number of the cells. PGE_2 production via the PLA_2/COX-2 pathway and its related EP_4 activation could be important components in mediating colony growth of gallbladder carcinoma cells.

Furthermore, to investigate the signals mediating the biological functions of PGE_2 or the EP_4 agonist, *i.e.*, cell growth and proliferation, the expression of *c-fos*, a growth-related proto-oncogene, in the Mz-ChA-2 cells with or without treatment was determined. After (30 min) the addition of PGE_2 or the EP_4 agonist, total RNA was isolated from the cells and was subjected to RT-PCR analysis to observe the expression level of *c-fos* mRNA in the cells. The expression level of the *c-fos* product after being normalized to G3PDH was increased in a dose-dependent manner in the cells in response to treatment with the EP_4 agonist. *c-fos* mRNA was ~3-fold higher in the cells treated with 1 μM the EP_4 agonist than in the nontreated cells. The magnitude of increase in *c-fos* mRNA in the cells treated with 1 μM the EP_4 agonist was comparable with that in the cells treated with 1 μM PGE_2. The expression level of EP_4 mRNA did not change significantly in these cells. These data strongly suggest that PGE_2 up-regulates *c-fos* expression in gallbladder carcinoma cells, at least partly, through activation of the EP_4.

DISCUSSION

Overexpression of COX-2 has been reported in various types of gastrointestinal carcinomas (21–24, 56). However, the tissue localization of COX-2 in carcinoma tissues is not well understood. Localization of COX-2 is observed in tumor-derived epithelial cells of colonic adenocarcinomas (23), whereas the localization is found in stroma cells in tissues of colonic adenoma (57) and colorectal carcinoma (22, 23). In addition, the localization of COX-2 is found in interstitial cells of colonic adenomatous polyps formed in $Apc^{\Delta716}$ knockout mice (58), Apc^{Min} mice (59), and interleukin-10-deficient mice (60). These discrepant findings should be sorted out to determine the role of COX-2 in not only carcinogenesis but also

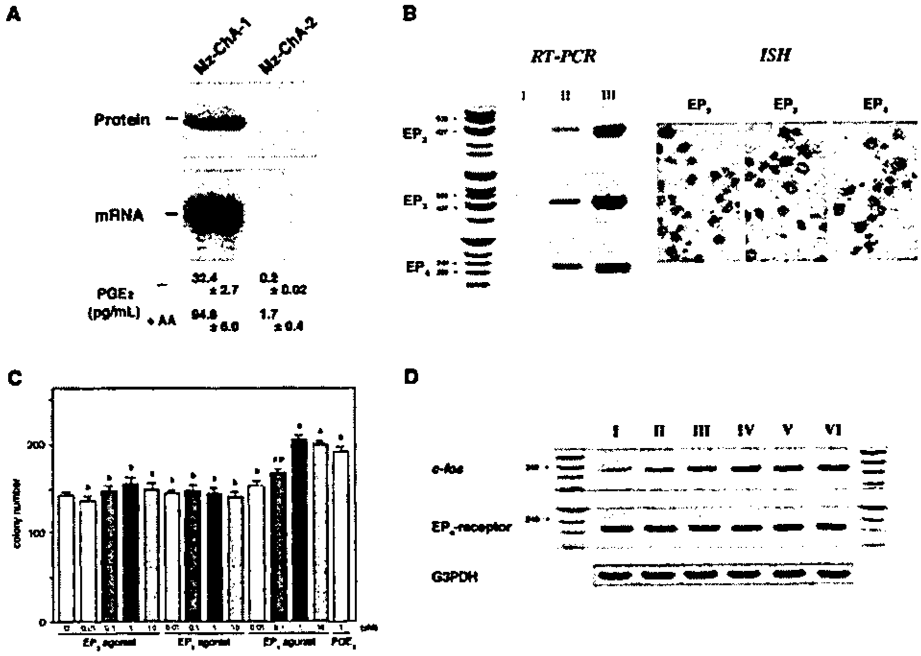

Fig. 5 Characterization of Mz-ChA-1 and Mz-ChA-2 cells and the effect of EP agonists on colony formation and *c-fos* expression in Mz-ChA-2 cells. *A*, expression levels of COX-2 in the Mz-ChA-1 and Mz-ChA-2 cells. COX-2 protein and mRNA were expressed strongly in the Mz-ChA-1 cells but were hardly detectable in the Mz-ChA-2 cells. The Mz-ChA-1 cells were observed to produce significant amounts of PGE_2 in response to treatment with 10 μM arachidonate, whereas the Mz-ChA-2 cells were observed to produce only trace amounts. The results are expressed as means ± SE, and the experiment was performed in triplicate. *B*, RT-PCR and ISH of EP_{2-4} mRNAs in the Mz-ChA-2 cells. In the RT-PCR, the mRNAs of EP_2, EP_3, and EP_4 are amplified in the cells. *Lane I*, reverse transcriptase-negative controls. *Lane II*, the PCR products of expected size from Mz-ChA-2 mRNA. *Lane III*, the PCR products from positive control cDNAs. In the ISH, EP_2, EP_3, and EP_4 mRNAs were diffusely and strongly expressed in the cells. *C*, effect of the EP_{2-4} agonist or PGE_2 treatment on Mz-ChA-2 colony number. The results are expressed as means (*bars*, SE), and the experiment was performed in triplicate. *a*, $P < 0.01$, significantly different from the nontreated cells; *b*, $P < 0.01$, significantly different from the cells treated with PGE_2. *D*, effect of the EP_4 agonist or PGE_2 treatment on *c-fos* expression. *Lane I*, nontreated; *Lane II*, 0.01 μM EP_4 agonist; *Lane III*, 0.1 μM EP_4 agonist; *Lane IV*, 1 μM EP_4 agonist; *Lane V*, 10 μM EP_4 agonist; *Lane VI*, 1 μM PGE_2. The PCR products were 236 bp in size for *c-fos* and 311 bp for G3PDH.

tumor growth and progression of human carcinomas in terms of epithelial-stromal interactions.

The important finding in the present study was that the expression levels of COX-2 in gallbladder carcinoma was increased in parallel to the depth of invasion; in pT_3 or pT_4 carcinoma of the gallbladder, a substantial increase in COX-2 mRNA and protein levels was observed compared with the levels in pT_1 or pT_2 gallbladder carcinoma or normal gallbladder tissue. In addition, ISH and immunohistochemistry revealed increased expression of COX-2 mRNA and protein in stroma cells adjacent to the cancerous epithelia of advanced carcinoma. Therefore, the main sources of COX-2 in the tissues of pT_3 or pT_4 gallbladder carcinoma may be not only the cancerous epithelia but also the adjacent stroma, and both the epithelia and stroma probably produce PGE_2, which regulates tumor biology in terms of epithelial-stromal interactions.

Besides COX-2, it is well known that $sPLA_2$-IIA is involved in the inflammatory response and can provide arachidonate for prostanoid production. Previous studies have shown that, like COX-2, PLA_2 activity (13) and arachidonate levels (61) are increased in human colorectal carcinoma. As overexpression of $sPLA_2$-IIA has been found in other carcinomas (26–28), the expression level of $sPLA_2$-IIA mRNA was significantly increased in pT_3 and pT_4 gallbladder carcinomas compared with the concentration in pT_1 and pT_2 carcinomas and normal gallbladder tissues. The high level of $sPLA_2$-IIA mRNA expression in advanced gallbladder carcinoma, in conjunction with the elevated expression of COX-2, could provide a substrate for COX-2 and lead to increased PG production. In another regard, $sPLA_2$ itself could be directly related to growth and differentiation in the human gastrointestinal tract, because the $sPLA_2$ receptor-mediated biological responses include stimulation of cellular proliferation (DNA synthesis; Ref. 62) and prostanoid production (63).

Interest should be focused on the biological effects of either COX-2 itself or PLA_2-/COX-2-derived PGE_2 on tumor growth and progression of gallbladder carcinoma, because the tissue concentration of PGE_2 was increased significantly in pT_3 and pT_4 gallbladder carcinomas in the present study. As indicated in several reports (50, 64–66). PGE_2 produced by COX-2-expressing carcinoma cells and stroma cells may play an important role in tumor growth and progression. This is because PGE_2 may stimulate carcinoma cell proliferation (51), inhibit apoptosis in carcinoma cells (51), promote immunosuppression in carcinoma tissues by preventing activation of inflammatory cells (67, 68), and induce growth factors important for the progression of carcinomas (66). Furthermore, COX-2-derived PGE_2 may play an important role in the formation and maintenance of the stroma and vessel structure in carcinoma tissues, because PGE_2 stimulates mitogenesis in fibroblasts (66) and induces angiogenesis (69, 70). A markedly increased production of hepatocyte growth factor in COX-2-expressing human fibroblasts via a PG-mediated pathway (71) is most interesting in epithelial-stromal interactions and may explain the crucial role of stromal cells adjacent to carcinoma cells in tumor growth and progression. Thus, "field-effect" alterations in stromal cell biology might contribute to the development of gallbladder carcinoma.

It is of particular interest to determine the effect of the PLA_2-/COX-2-derived PGE_2 on the biology of gallbladder carcinoma. In an experiment to determine the effect of PGE_2 treatment on the formation of colonies by plating gallbladder carcinoma cells (Mz-ChA-2) in a monolayer culture, we observed an increase in the number of Mz-ChA-2 cells in response to PGE_2 treatment through an up-regulation of c-fos expression. Supporting this, PGE_2 has been shown to potentiate a replication of gallbladder carcinoma cells (72). As found in several studies (73–75), the biological effect of PGE_2 in gastrointestinal tissues involves signaling via EP subtypes. Importantly, treatment with an EP_4 agonist was found to increase the number of Mz-ChA-2 cells to a similar degree through an up-regulation of *c-fos* expression. A key step by which PGE_2 potentiates growth of gallbladder carcinoma cells may be the activation of the EP_4 as observed recently in colorectal carcinoma cells (76). The activation of EP_4 in turn would mediate signals inside the nucleus to induce *c-fos* gene transcription, and the increased expression of *c-fos*, a growth-related proto-oncogene, may, at least in part, account for the increased number of colonies of the carcinoma cells as observed previously (54, 77). In contrast, treatment with an EP_2 or EP_3 agonist did not cause significant changes in the colony formation.

In summary, the results of the present study suggest that in cases of advanced pT_3 and pT_4 carcinoma of the gallbladder, the enhanced expression of COX-2 mRNA and protein is observed in the adjacent stroma rather than in the cancerous epithelia and that the stroma in these advanced gallbladder carcinomas is a potent source of PG synthesis. In epithelial-stromal interactions, the increased production of PLA_2-/COX-2-derived PGE_2 in the adjacent stroma and its biological effect via EP_4 on the carcinoma cells in a paracrine fashion may contribute to the development of gallbladder carcinoma.

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Prostaglandin Receptors: Advances in the Study of EP3 Receptor Signaling

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Received March 14, 2002; accepted April 1, 2002

Prostaglandin (PG) E_2 produces a broad range of physiological and pharmacological actions in diverse tissues through specific receptors on plasma membranes for maintenance of local homeostasis in the body. PGE receptors are divided into four subtypes, EP1, EP2, EP3, and EP4, which have been identified and cloned. These EP receptors are members of the G-protein coupled receptor family. Among these subtypes, the EP3 receptor is unique in its ability to couple to multiple G proteins. EP3 receptor signals are primarily involved in inhibition of adenylyl cyclase *via* G_i activation, and in Ca^{2+}-mobilization through Gβγ from G_i. Along with G_i activation, the EP3 receptor can stimulate cAMP production *via* G_s activation. Recent evidence indicates that the EP3 receptor can augment G_s-coupled receptor-stimulated adenylyl cyclase activity, and can also be coupled to the G_{13} protein, resulting in activation of the small G protein Rho followed by morphological changes in neuronal cells. This article focuses on recent studies on the novel pathways of EP3 receptor signaling.

Key words: calcium mobilization, EP3 receptor, G_{13} protein, prostaglandin receptor.

Prostanoids comprising the prostaglandins (PGs) and thromboxanes (TXs) are potent eicosanoid lipid mediators generated by the cyclooxygenase (COX) isozymes. Prostanoids are quickly released from cells after synthesis and act as local hormones in the vicinity of their production site to maintain local homeostasis. The ability of each prostanoid to affect various biological responses is dependent on its binding to specific receptors on the plasma membrane. These prostanoid receptors are classified into five basic types, termed DP, EP, FP, IP, and TP receptors, on the basis of their sensitivities to the five primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, respectively. Furthermore, there are several receptor subtypes for PGD_2 and PGE_2. PGD_2 acts through two receptors, the DP receptor and the recently identified CRTH2 receptor (chemoattractant receptor homologous molecule expressed on Th2) (*1*). EP receptor is subdivided into four subtypes, EP1, EP2, EP3, and EP4, on the basis of their responses to various agonists and antagonists.

Prostanoid receptors are G-protein coupled, rhodopsin-type receptors with seven transmembrane domains. Knowledge accumulated from analyses on the structure and function of the prostanoid receptor molecules has been described elsewhere (*2*). The DP, EP2, EP4, IP receptors, and one isoform of the EP3 receptor can couple to G_s and thus increase intracellular cAMP concentration. The FP, IP, and TP receptors can couple to G_q, and activation of these receptors leads to an increase in intracellular calcium levels. Finally, the TP, CRTH2, and EP3 receptor can couple to G_i, causing a decrease in the cAMP levels while also mobilizing intracellular calcium. The EP1 receptor can also mobilize intracellular calcium, but activation of G proteins by the EP1 receptor has not been confirmed.

Of the prostanoid receptor molecules, the EP3 receptor has different C-terminal tail isoforms, which are generated by alternative splicing. It has been reported that the mouse EP3 receptor has three isoforms, EP3α, EP3β, and EP3γ (*3–5*), the bovine EP3 receptor has four isoforms (*6*), the rabbit EP3 receptor has five isoforms (*7, 8*), and the human EP3 receptor has seven isoforms (*9*). G_i activation mediated by the mouse EP3 receptor isoforms has been well investigated. The three mouse EP3 receptor isoforms couple to G_i with different IC_{50} values, of which EP3γ < EP3α < EP3β (*3, 4*). Regarding the agonist-dependency for G_i activation, the mouse EP3α and γ isoforms have partially constitutive G_i activity (EP3γ > EP3α), but the EP3β isoform has no constitutive G_i activity (*10, 11*). Moreover, the C-terminal tail-truncated mutant receptor, abbreviated as T-335, showed fully constitutive G_i activity (*11*). Along with G_i activity, the three isoforms and T-335 can cause agonist-dependent G_s activity (*4*). The order of potency is EP3γ > T-335 ≫ EP3α = EP3β = 0. This shows that the core of the EP3 receptor has the ability to associate with and activate G_i/G_s proteins, while the C-terminal tail of the EP3 receptor can suppress G protein activation.

Recently, novel actions of the EP3 receptor other than in G_i/G_s signaling have been identified using EP3-expressing cells and cultured neuronal cells. This review summarizes the current information regarding the EP3 receptor with a focus on its novel actions.

Ca^{2+} mobilization mediated by the EP3 receptor

Activation of the mouse EP3α, EP3β, and EP3γ receptors

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Abbreviations: PG, prostaglandin; TX, thromboxane; COX, cyclooxygenase; G protein, heterotrimeric GTP-binding protein; PT, pertussis toxin.

is known to lead to intracellular Ca^{2+} mobilization in a PT-sensitive manner in CHO cells (*12*). This Ca^{2+} mobilization mediated by the EP3 receptor is conducted by the Gβγ subunits from the $G_{i/o}$ protein, since the PLCβ isoform is activated by these subunits.

We recently reported that the mouse EP3β receptor and the T-335 receptor can significantly augment G_s-coupled EP2-induced adenylyl cyclase activity, and that this augmentation is mediated by a PT-insensitive Ca^{2+} pathway (*13*). G_i-coupled receptors such as α_2 adrenoceptor (*14*) and bradykinin B_2 receptor (*15*) are also known to lead to augmentation of G_s-stimulated adenylyl cyclase in COS-7 cells. This augmentation is suspected to be *via* an increase in adenylyl cyclase type II activity by direct interaction of the Gβγ subunits released from activated $G_{i/o}$ proteins with the receptors. However, the adenylyl cyclase augmentation induced by the EP3 receptor was not attenuated by either PT treatment or expression of the PH domain of rat βARK1, which serves as a scavenger of Gβγ subunits. This result suggests that adenylyl cyclase augmentation is mediated *via* a novel signaling pathway without the involvement of Gβγ subunits released from $G_{i/o}$ proteins. In fact, the adenylyl cyclase augmentation was almost completely attenuated by pretreatment with either 1,2-bis(*o*-aminophenoxymethyl)ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl)-ester, an intracellular Ca^{2+} chelator, or W-7, a calmodulin inhibitor. These findings suggest that the adenylyl cyclase augmentation induced by the EP3 receptor is achieved *via* a signaling pathway involving a Ca^{2+}/calmodulin reaction. Moreover, the T-335 receptor caused a similar augmentation in EP2-stimulated adenylyl cyclase activation, indicating that the C-terminal tail of the EP3β receptor is not essential for this reaction. This cross-talk between the EP3β and EP2 receptors was also reproduced by combination of the G_s-coupled luteinizing hormone (LH) receptor with the EP3β receptor in COS-7 cells. The putative EP1/EP3 agonist sulprostone significantly augmented the cAMP levels produced by LH stimulation in COS-7 cells coexpressing EP3 and LH receptors (Fig. 1). In preliminary experiments, we found that sulprostone augmented cAMP production stimulated by the EP4 agonist ONO-AE-1-329 in mouse mastocytoma P-815 cells, which mainly express the EP3 and EP4 receptors. Southhall and Vasko reported that the bovine EP3C and EP4 receptors mediate PGE_2-induced cAMP production and the sensitization of sensory neurons (*16*). Despite the extensive facts showing that G_i-coupled receptors can augment G_s-coupled receptor-stimulated adenylyl cyclase activity, it remains unknown why the G_i-coupled receptor does not preferentially interact with the G_i protein in COS-7 cells. Recent evidence suggests that many signaling molecules localize in microdomains in the plasma membrane, particularly in the caveolae. For example, the EP2 receptor does not activate adenylyl cyclase type VI, although the β-adrenergic receptor activates this adenylyl cyclase (*17*). Hence, the selective interaction of the EP3 receptor with G_s-coupled EP2-stimulated adenylyl cyclase, even in the presence of an excess of G_i protein in the plasma membrane, may be crucial for agonist-dependent augmentation of cAMP synthesis.

It has also been reported that the rabbit EP3 receptor can couple to the activation of cAMP response element (CRE)–mediated gene transcription, which is a PT-insensitive Ca^{2+} pathway in HEK293tsA201 cells (*8*). The rabbit EP3 receptor was able to elicit this activation in an agonist-dependent manner, although their EC_{50} values were 15-fold higher than that for G_i activity. This CRE activation is mediated by a Ca^{2+}-dependent kinase pathway, since activation was partially inhibited by the selective PKC inhibitor, bisindolylmaleimide I, and completely inhibited by staurosporine, a strong inhibitor of PKC, PKA, and other serine/threonine kinases. These two signals mediated by either the mouse EP3 receptor or the rabbit EP3 receptor elicited an increase in Ca^{2+} levels in a G_i-independent manner. Furthermore, the C-terminal tail–deleted receptors, T-335 being the mouse derivative and NT being the rabbit EP3 derivative, activated these PT-insensitive Ca^{2+} related pathways in an agonist-dependent manner. Since T-335 results in agonist-independent constitutive G_i activity (*11*), the C-terminal tails of the EP3 receptors have different functions in PT-sensitive G_i activity and PT-insensitive Ca^{2+} signaling. These results indicate that the conformation of the EP3 receptor may be quite different in these different signaling pathways.

It has recently been reported that EP3 receptor–mediated signals may promote a novel form of neutrophil cell death, which differs from typical apoptosis or necrosis (*18*). Incubation of neutrophils with staurosporine or H-7, which are inhibitors of PKC, prevented this EP3 receptor agonist–induced neutrophil cell death, though it remained unclear whether this neutrophil death occurs by a PT-sensitive or insensitive pathway. This study showed that the EP3 receptor promoted neutrophil cell death through the activation of PKC, indicating that Ca^{2+} signaling mediated by the EP3 receptor may play a role in various diseases.

G_{13} activity mediated by the EP3 receptor

The bovine EP3 isoform receptors (EP3A, EP3B, EP3C, EP3D) are known to couple to various G proteins. EP3A receptor can couple to G_i, EP3B and EP3C receptors to G_s and G_o, EP3D receptor to G_i, G_s, and G_q. Along with these G proteins, the bovine EP3 receptor was found to lead to the activation of G_{13} in PC12 cells (*19, 20*). The bovine EP3B receptor was able to induce neurite retraction in differenti-

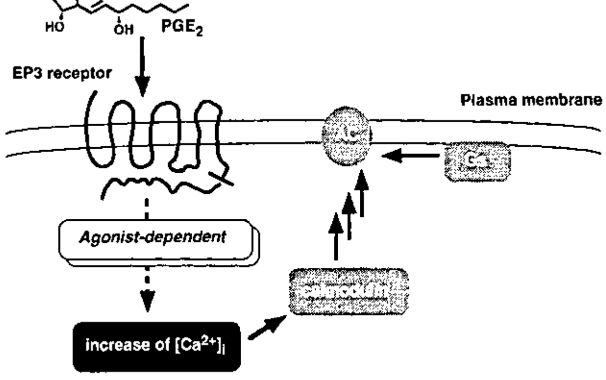

Fig. 1. **Schematic illustration of the mechanism of EP3 receptor–induced Ca^{2+}-dependent augmentation of cAMP synthesis.** The mouse EP3 receptors stimulate an increase in intracellular Ca^{2+} levels, and promote G_s-activated adenylyl cyclase (AC) through the Ca^{2+}-calmodulin pathway in an agonist-dependent and PT-insensitive manner.

ated PC12 cells in a PT-insensitive and agonist-dependent manner. *Clostridium botulinum* C3 exozyme completely inhibited EP3 receptor-induced neurite retraction when microinjected into the PC12 cells, indicating that the morphological effect of the EP3B receptor is dependent on Rho activity. Small GTPases of the Rho family, Rac, CDC42, and Rho, are involved in morphological changes in various cells. In neuronal cells, Rac or CDC42 appears to be required for the outgrowth of neurites, while Rho is required for neurite retraction (*21*). It has been reported that G_{12}, G_{13}, and G_q induce Rho-dependent neurite retraction in nerve growth factor (NGF)-differentiated PC12 cells (*22*). The bovine EP3B receptor–induced neurite retraction was blocked by tyrphostin A25, which inhibits the G_{13} and G_q-mediated morphological changes *via* Rho. Moreover, EP3 receptor activation did not increase the intracellular Ca^{2+} concentration in PC12 cells, and the neuronal morphological changes induced by the EP3 receptor were not blocked by the inhibition of protein kinase C activity. These results indicate that the bovine EP3B receptor induces neurite retraction *via* a G_{13}-small GTPase Rho pathway in PC12 cells.

The mouse EP3 receptor isoforms induced the formation of stress fibers in MDCK cells (*23*). This receptor-mediated stress-fiber formation was completely inhibited by *Clostridium botulinum* C3 exozyme, indicating the involvement of Rho in the formation of stress fibers in MDCK cells. However, since the EP3 receptor–mediated stress-fiber formation was not inhibited by PT treatment, it may be mediated *via* a G_{13}-Rho pathway, as in the case of receptor-mediated neurite retraction in PC12 cells. The EP3α and EP3β receptors differed in their agonist-dependencies for stress-fiber formation: the EP3α isoform acted agonist-independently, while the EP3β isoform acted agonist-dependently. These observations indicate that the mouse EP3 isoforms differ in agonist-independent constitutive G_{13} activity, and that the carboxyl-terminal tail of the EP3 receptor can suppress G_{13} protein activation mediated by the core region of the EP3 receptor (Fig. 2).

PGE_2 is one of the major PGs synthesized in the nervous system (*24*). PGE_2 has several important functions in the nervous system, such as the generation of fever, regulation of LH-releasing hormone secretion, pain modulation, and regulation of neurotransmitter release. Furthermore, the EP3 receptor is involved in pyrogen-induced fever generation (*25*). Among the EP subtypes, the EP3 receptor is the most abundant in the brain and is specifically localized to neurons (*26*). When the brain is injured, newly synthesized PGE_2 may cause retraction of neurites of EP3 receptor-expressing neurons and mediate reorganization of damaged neuronal connections. In addition, the levels of PGE_2 are also increased in the brain upon synaptic activity or during development (*27*). PGE_2 may therefore also be involved in the refining and remodeling of initial neuronal connections through the EP3 receptors.

Conclusion

Among the PGE receptor subtypes, the EP3 receptor has been shown to mediate various physiological and pathophysiological functions. These functions are mediated through the different actions of the EP receptor subtypes, which are coupled to different G proteins, leading to the stimulation of multiple signal transduction pathways. EP3 receptor signals have been extensively studied using cells

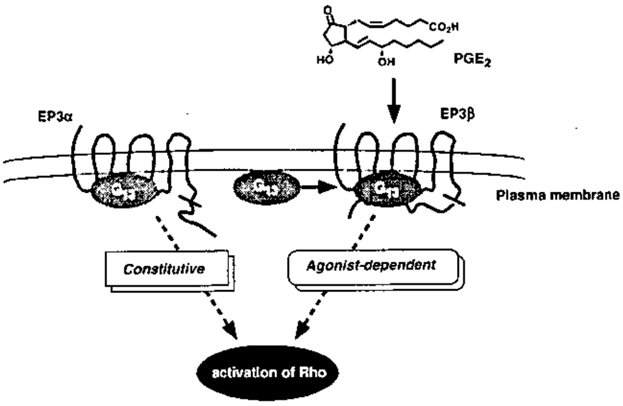

Fig. 2. **Schematic illustration of the mechanism of Rho activation induced by EP3 receptor isoform–G_{13} coupling.** The mouse EP3 receptor isoforms EP3α and EP3β constitutively and agonist-dependently activate the G_{13} protein respectively, leading to the activation of the small GTPase Rho.

expressing a single receptor subtype. However, regular cells probably express multiple EP receptor subtypes or different hormone receptors on their plasma membranes. Hence, defining the cross-talk of multiple signaling pathways induced by the different EP receptor subtypes or hormone receptors is crucial for the biochemical and molecular biological understanding of hormone actions. Such analyses are essential for the evaluation of receptor-induced signaling pathways and receptor-induced physiological responses. In addition, these advanced studies will promote the development of the specific agonists and antagonists for clinical use against various hormone-related diseases.

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Inflamm. res. 51, *Supplement 1* (2002) S17–S18
1023-3830/02/01S17-02 $ 1.50+0.20/0

Inflammation Research

Histamine synthesis in mouse polymorphonuclear neutrophils

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Introduction

Histamine is involved in both the early and late phase of inflammatory and allergic responses. Late phase responses are characterized by infiltration of neutrophils, eosinophils and macrophages into tissues. Although mast cells and basophils are recognized as the main storage sites for histamine in the tissues, it is not yet clear which cell type produces histamine in the late phase of the inflammatory response. In in vitro studies, mouse macrophages activated by lipopolysaccharide were found to express histidine decarboxylase (HDC) and to produce histamine [1, 2]. It was reported that a significant amount of histamine was produced by infiltrated leukocytes in an air pouch-type allergic inflammation model in rats [3]. In the current study, we have investigated histamine synthesis by peritoneal cells in an experimental peritonitis model in order to identify the responsible cell type.

Materials and methods

Anti-GST-fusion HDC antiserum was prepared as described previously [4]. The following materials were purchased from the sources indicated: anti-matrix metalloproteinase (MMP)-9 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), fluorescein isothiocyanate (FITC)-conjugated anti-CD11b antibody from Pharmingen (San Diego, CA), Alexa594-conjugated anti-rabbit IgG antibody from Molecular Probes, Inc. (Eugene, OR), Percoll from AmershamPharmacia (Uppsala, Sweden), and [^{35}S]methionine (1,000 Ci/mmol) from DuPont-New England Nuclear (Boston, MA). All other chemicals were commercial products of reagent grade.

Preparation of polymorphonuclear neutrophils (PMNs)

Female Balb/c mice (7–8 weeks of age) were used for all the experiments. Casein in saline (5%, w/v) was injected intraperitoneally. Classification of the peritoneal cell type was determined by microscopic observation after May-Grünwald-Giemsa staining. Five hours after the injection, the cells in the peritoneal cavity were harvested by lavage of the cavities with 3 ml of sterile phosphate-buffered saline. Other time points were also studied (0, 2, 8, 12, 24 h), however the maximal HDC activity was obtained after 5 h thus this timepoint was chosen for further study. PMNs from the peritoneal cavity and from peripheral blood were purified by centrifugation on discontinuous Percoll gradients [5].

Culture of purified PMNs

Purified PMNs were cultured in RPMI-1640 medium containing 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 % heat-inactivated fetal calf serum for the indicated times. Cell viability was greater than 98%, as confirmed by the trypan blue exclusion test.

Immunoprecipitation

Immunoprecipitation using anti-GST-fusion HDC antiserum was performed as previously described [6].

Immunofluorescence study

PMNs were centrifuged onto round cover glasses and the immunofluorescence study was performed as described previously [6]. An anti-HDC antibody (1:500) and an anti-MMP-9 antibody (1:500) were used as a first antibody. Alexa594-conjugated anti-rabbit IgG antibody (1:1000) and an FITC-conjugated anti-CD11b antibody (1:1000) were used for HDC/CD11b double staining.

Results and discussion

In a casein-induced mouse peritonitis model, a biphasic infiltration of leukocytes was observed in the peritoneal cavity; the early phase (~8 h) was characterized by PMNs and the late phase (~24 h) by macrophages. HDC activity in the peritoneal cells was markedly and transiently induced 5 h after the initial stimulation, when more than 80% of peritoneal cells could be identified as PMNs by a May-Grünwald-Giemsa staining. Immunofluorescence study using an anti-CD11b (a marker of neutrophils) and an anti-HDC antibody revealed that about 90% of the peritoneal cells were CD11b+/HDC+. On the other hand, peripheral blood leukocytes were found to be negative to an anti-HDC antibody. These observations indicate that HDC may be induced in the peritoneal cavity. Although which kind of factors could induce HDC remains unknown, tumor necrosis factor-α

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(TNF-α) may be one candidate, since systemic injection of TNF-α was reported to induce histamine synthesis in mouse bone marrow and spleen cells [7].

We purified PMNs from the peritoneal cells 5 h after the initial stimulation by a Percoll density gradient method. Determination of the cell population by May-Grünwald-Giemsa staining indicated that more than 98% of the cells obtained were neutrophils and the rest were mononuclear cells. Specific HDC activity of purified PMNs was comparable to that of mucosal-type mast cells whereas much less histamine content was obtained; HDC, 99.3 ± 21.3 pmol/min/mg protein (n = 6), histamine, 239 ± 36 pmol/10E7 cells (n = 6). The HDC activity was decreased under a standard culture condition, indicating again that HDC may be induced by some humoral factors in the peritoneal cavity. Immunoblot analysis with an anti-HDC antibody demonstrated the expression of a 53-kDa mature form of HDC in the PMNs. Furthermore, the post-translational processing of HDC, which was metabolically labelled with [^{35}S]methionine, was found to be very rapid in a pulse-chase study; a 74-kDa form of HDC was converted to its 53-kDa form within 30 min. Immunofluorescence study with an anti-HDC and anti-matrix metalloproteinase-9 (MMP-9) antibody demonstrated that HDC was localized in the granules of PMNs. These observations are consistent with our previous results in a rat mast cell line that a 53-kDa form of HDC was localized in the granular fractions of the mast cells [6].

The function of histamine produced by PMNs remains to be fully clarified. Recently, we have demonstrated in an experimental syngenic tumor model that a large number of PMNs infiltrated the tumor tissues and the growth of the tumor could be suppressed by a daily treatment with cimetidine (0.12 mg/kg) [8]. In situ hybridization demonstrated that the PMNs infiltrating the tumor tissue expressed HDC mRNA. A daily cimetidine treatment augmented the intratumoral expression of some cytokines, such as TNF-α and IFN-γ, which have been reported to have antitumoral effects [8]. These results suggest that histamine may have a suppressive effect on the tumor immunity acting on H2 receptors of the infiltrated immune cells. Histamine has also been reported to have suppressive effects on the function of PMNs, such as degranulation, superoxide production and chemotaxis. It is possible that histamine may modulate the function of PMNs in an autocrine fashion.

In summary, we have revealed that infiltrated PMNs are possible sources of histamine in a late phase of inflammatory responses in mice.

Acknowledgements. This study was supported by grants-in-aid for Scientific Research form the Ministry of Education, Science, Sports and Culture, Japan.

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International Congress Series 1233 (2002) 397–404

Prostaglandin E_2 and $F_{2\alpha}$ in mouse reproduction

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Abstract

The prostaglandins (PGs) are involved in various mammalian female reproductive processes including ovulation, fertilization, luteolysis, and uterine contraction. To determine which specific PG and receptor subtype are crucial for each of the reproductive processes, we generated PG receptor-deficient mice. Among the eight types of PG receptors, only the $PGF_{2\alpha}$ receptor FP-deficient and PGE_2 receptor subtype EP_2-deficient mice exhibited a failure of parturition and a decrease in litter size, respectively. FP-deficient mice failed to show both the up-regulation of the oxytocin receptor in uterine tissues at term and the prepartum decline in serum progesterone levels, indicating that $PGF_{2\alpha}$ is essential for the induction of parturition via its luteolytic activity. Furthermore, expression analyses of the cyclooxygenases (COXs) suggested that the COX-2 isozyme in the myometrium at term is responsible for producing uterotonic PGs. On the other hand, EP_2-deficient female mice consistently delivered fewer pups than their wild-type counterparts. They showed phenotypes of slightly impaired ovulation and a dramatic reduction in fertilization due to impaired expansion of the cumulus cells, indicating that PGE_2 plays a role in ovulation and fertilization by inducing cumulus expansion via the EP_2 receptor. These results show that PGE_2 and $PGF_{2\alpha}$ play important roles in the mouse physiological reproduction processes.

Keywords: Prostanoid; Gene-targeting; Labor; Uterus; Ovary

1. Introduction

Prostaglandins (PGs) are arachidonate metabolites, which are synthesized via the cyclooxygenase (COX) pathway [1]. PGs exert a wide variety of physiological and pathophysiological actions in the whole body [2]. The actions of the PGs are mediated by specific receptors on the cell surface, which are classified into five types and four subtypes

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PII: S0531-5131(02)00160-7

of receptors; DP, FP, IP, TP, and EP (subtypes: EP_1, EP_2, EP_3, and EP_4) receptors for PGD_2, $PGF_{2\alpha}$, PGI_2, TXA_2, and PGE_2, respectively [3]. The involvement of PGs in biological actions has been suspected by the actions of aspirin-like drugs which inhibit the enzyme activity of COX. Indeed, aspirin-like drugs are known to cause delayed parturition in many species [4]. Furthermore, COX-2-deficient mice have recently been shown to induce multiple failures in female reproduction, including impaired ovulation, fertilization, implantation, and decidualization [5]. Indeed, a large amount of PGs, especially PGE_2 and $PGF_{2\alpha}$, is produced and released in uterine tissues during parturition [6]. Both PGE_2 and $PGF_{2\alpha}$ have been considered to be important mediators and/or modulators of several processes in female reproduction [7]. However, it has been obscure as to which type of PG and PG receptor mediate each of the processes of female reproduction. In addition, it has not yet been clarified as to what extent the PGs play physiologically significant roles. To address these issues, we generated mice deficient in each of the eight PG receptors by gene-targeting, and analyzed their phenotypes in female reproduction. Here, we review our recent studies on the significance of the FP and EP_2 receptors in the female reproductive systems. These studies indicate that $PGF_{2\alpha}$ plays a crucial role in the induction of parturition during late pregnancy and that PGE_2 plays an important role in ovulation and fertilization via the EP_2 receptor during early pregnancy.

2. Role of $PGF_{2\alpha}$ and the FP receptor in luteolysis and parturition

In the ovary, the granulosa cells differentiate into luteal cells after ovulation, forming the corpus luteum. The corpus luteum secretes progesterone (P_4), which is essential for the maintenance of pregnancy. Although the life span of the corpus luteum depends on the animal species, $PGF_{2\alpha}$ has been suggested to induce luteolysis among a widespread number of species [8,9]. In support of this luteolytic function of $PGF_{2\alpha}$, the expression levels of FP transcripts in the ovary, especially at the corpus luteum, are the highest in the mouse [10]. To examine the physiological significance of $PGF_{2\alpha}$ on luteolysis, we generated and analyzed FP-deficient mice [11]. Unexpectedly, there was no change in the length of normal estrous cycle between the wild-type and homozygous mice. This result shows that $PGF_{2\alpha}$ is not essential for luteolysis in the normal estrous cycles at least in the mouse. Furthermore, FP-deficient mice became pregnant and the numbers of their corpora lutea and implants were normal, indicating that $PGF_{2\alpha}$ is dispensable for the processes of ovulation, fertilization, and implantation in the mouse. Since genetic inactivation of COX-2 in the mouse leads to the impairment of these three processes [5], other PG may be crucial for these processes.

In contrast to luteolysis in the normal estrous cycle, we found that FP-deficient mice did not show a prepartum decline in serum P_4 levels, indicating that endogenous $PGF_{2\alpha}$ is essential for luteolysis during late pregnancy [11]. As the prepartum withdrawal of the P_4 initiates the parturition process in rodents, parturition does not occur in these FP-deficient mice. Concomitant with the persistently high serum P_4 levels in the FP-deficient mice, both the uterine sensitivity to oxytocin and the up-regulation of the uterine oxytocin receptor gene were impaired in these mice. When we removed the ovaries from these FP-deficient mice at the expected term, both parturition and up-regulation of the oxytocin receptor gene were restored at 20 h after the treatment. Thus, $PGF_{2\alpha}$ is essential for luteolysis and parturition during late pregnancy, acting upstream of the oxytocin system (Fig. 1).

It has been reported that mice deficient in cytosolic phospholipase A_2 ($cPLA_2$) or COX-1 also exhibit impaired luteolysis and delayed parturition [12–14], suggesting that these isozymes may synthesize luteolytic $PGF_{2\alpha}$ during late pregnancy. Considering that the luteolytic $PGF_{2\alpha}$ originates from the uterus in many species [8], uterine COX-1 may be responsible for producing luteolytic $PGF_{2\alpha}$. On the other hand, an abundant expression of COX-2 has been detected in the periparturient uterus [15]. Therefore, it is likely that COX-1 and COX-2 have distinct roles in the onset of parturition. We then analyzed the uterine expression of COX-1 and COX-2 in FP-deficient mice at term, compared with those of wild-type mice [16]. In the wild-type mice, uterine COX-1 mRNA, which was localized dominantly in endometrial epithelial cells, gradually increased during the latter half of pregnancy, and decreased on the day of parturition. This result supports the role of COX-1 in luteolysis by producing luteolytic $PGF_{2\alpha}$. In contrast, FP-deficient mice persistently expressed COX-1 at high levels at the expected term. This suggests that a fall in serum P_4 levels may down-regulate uterine COX-1 in a negative feedback system. The P_4 withdrawal by ovariectomy of the FP-deficient mice at term indeed led to a decrease in uterine COX-1 gene expression. On the other hand, strong expression of COX-2 was induced in the myometrium during parturition in wild-type mice (Fig. 2A and B). This expression was not observed at term in FP-deficient mice which lacked parturition, whereas COX-2 expression was restored when parturition was induced by ovariectomy in the FP-deficient

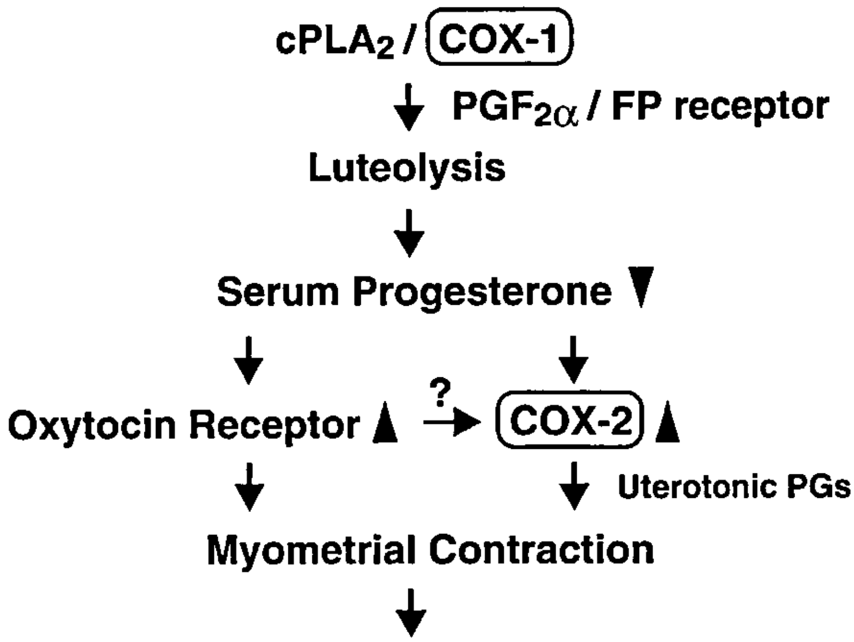

Fig. 1. Proposed role of $PGF_{2\alpha}$ and the FP receptor in murine parturition.

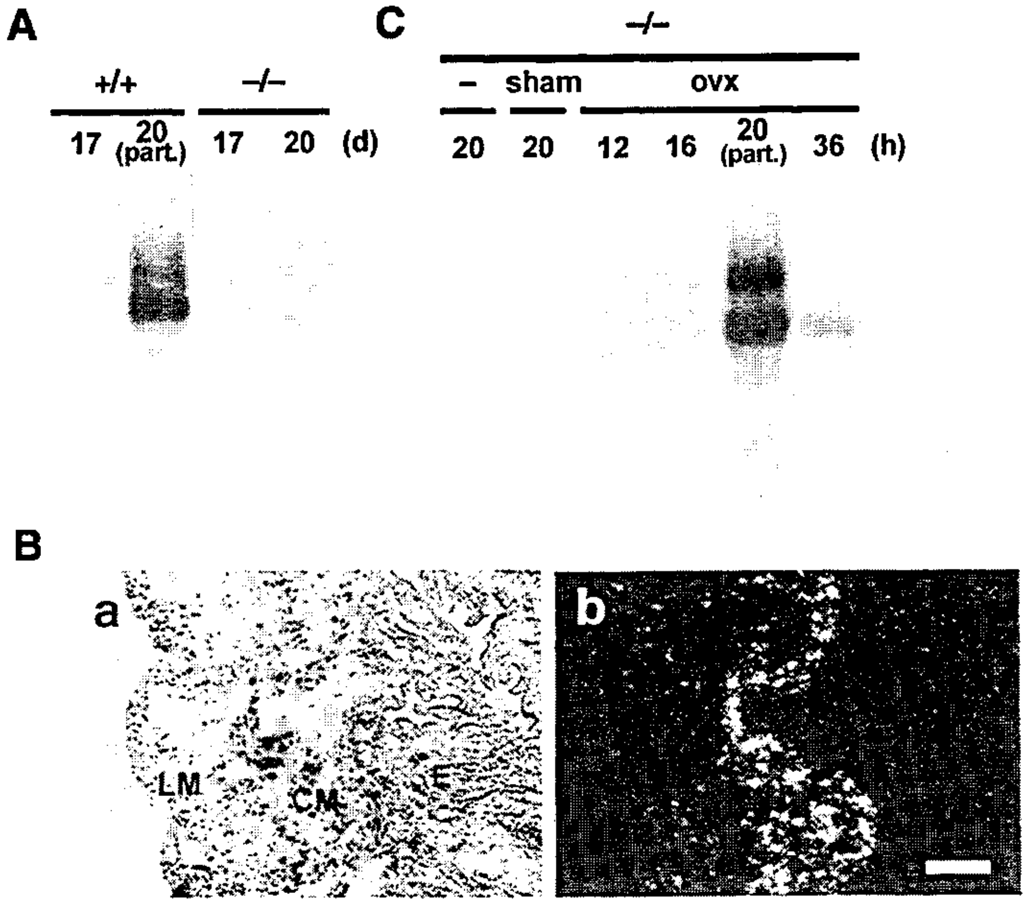

Fig. 2. (A) Uterine expression of COX-2 mRNA in late pregnancy of wild-type (+/+) and FP-deficient (−/−) mice. Uterine horns were collected from wild-type and FP-deficient mice on day 17 (17d), from wild-type mice undergoing parturition (20d part.), or from FP − / − mice on day 20 of pregnancy (20d), and they were subjected to Northern blot analyses. (B) Myometrial expression of COX-2 during parturition. Uterine sections of wild-type mice undergoing natural parturition were subjected to in situ hybridization analyses. Bright- (a) and dark-field (b) photomicrographs are shown. Longitudinal smooth muscle layer, LM; circular smooth muscle layer, CM; endometrial epithelium, E. Bar, 150 μm. (C) Effect of ovariectomy on uterine expression of COX-2 mRNA in FP-deficient mice. FP-deficient mice were ovariectomized bilaterally (ovx), sham-operated (sham), or left untreated (−) on day 19 of pregnancy, their uterine horns collected at the indicated hours after treatment and their RNA subjected to Northern blot analyses.

mice (Fig. 2C). Such a close association of COX-2 expression with the occurrence of parturition suggests that COX-2-derived PGs may be responsible for the final steps in the parturition process. Considering that PGE_2 and $PGF_{2\alpha}$ are the major products of uterine COX and that both are known to have potent uterotonic activities [17], COX-2-derived PGs may be responsible for myometrial contraction (Fig. 1). Parturition could be restored after ovariectomy even in the FP-deficient mice, and the mice deficient in any of the other PG receptors including the PGE receptor subtypes EP_1–EP_4, did not exhibit a phenotype of impaired parturition. Thus, a single deletion of either of the eight PG receptors had no effect on uterine contraction itself during parturition, suggesting that they may compensate each other.

In humans, COX-2 is reported to be dominantly expressed in the chorion and amnion, but COX-2 expression levels in these tissues are higher upon spontaneous parturition than that upon cesarean section, indicating that uterotonic PGs are produced via COX-2 [18,19]. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been found to be effective in delaying delivery in clinical trials, but the adverse side effects on the fetal ductus arteriosus have limited the use of such treatment in preterm labor [20]. Since the severe effects on the fetal ductus arteriosus appear only after genetic inactivation of both COX-1 and COX-2 in mice [21], a COX-2 specific inhibitor has the potential for the inhibition of parturition with less side effects than conventional NSAIDs [22]. Indeed, a COX-2 inhibitor has been reported to delay parturition in a murine LPS-induced preterm parturition model [23]. However, it has also been reported that a COX-2 inhibitor was able to delay murine spontaneous parturition at high doses [24]. Further investigations are necessary to clarify the role of COX-2 during parturition.

3. Role of PGE_2 and the EP_2 receptor in ovulation and fertilization

Ovulation and fertilization are the key processes in mammalian reproduction, and these processes are highly regulated by pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Ovulation is triggered when mature antral follicles are stimulated with LH, and the process of LH-induced ovulation is inhibited by NSAIDs. This inhibition is recovered by the administration of PGE_2 [25]. Indeed, an LH surge leads to a high expression of COX-2 in granulosa cells and a stimulation of PGE_2 synthesis in the antral follicle [26–28]. COX-2-deficient mice exhibit infertility due to impaired ovulation, fertilization, implantation, and decidualization [5]. This result suggests that PGs may play a role in one of a series of preovulatory processes that are required for both ovulation and fertilization. One candidate is the cumulus expansion step which is triggered by the endogenous preovulatory surge of gonadotropins [29]. Cumulus cells existing around the ovum have important roles in ovulation, transition to the oviducts and maturation of the ovum, and fertilization [30]. The cumulus cells secrete extracellular matrix components in response to gonadotropin, which induces cumulus expansion during the period of ovulation and fertilization. PGE_2 has been shown to mimic the action of gonadotropin, inducing cumulus expansion in vitro [29]. However, whether endogenous PGE_2 actually contributes to cumulus expansion, or which receptor subtype mediates this step has not been clarified.

EP_2-deficient mice showed a decrease in litter size [31–33]. The ovulation number and the fertilization rate of the EP_2-deficient mice were 80% and 20%, respectively, compared to that of the wild-type mice. Thus, PGE_2 may contribute to the process of ovulation and fertilization, at least in part, via the EP_2 receptor. In vitro cumulus expansion experiments revealed that both FSH and PGE_2 elicited expansion in wild-type mice, whereas only FSH could elicit expansion in EP_2-deficient mice, indicating that the defects of fertilization in EP_2-deficient mice could be attributed to impaired cumulus expansion. Indeed, EP_2 gene expression was found in cumulus cells, as determined by in situ hybridization, and the cumulus cells of the cumuli oophori complexes isolated from oviducts of EP_2-deficient

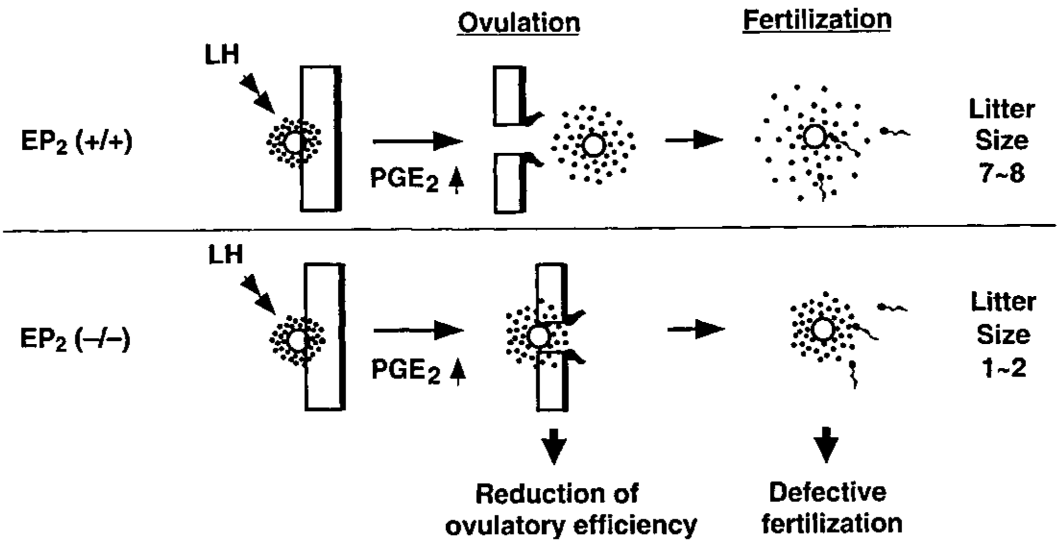

Fig. 3. Proposed role of PGE_2 and the EP_2 receptor in ovulation and fertilization.

mice just before fertilization were less expanded compared with wild-type cumulus cells. Furthermore, no difference in the fertilization rate between wild-type and EP_2-deficient mice was found in in vitro cumulus expansion experiments using cumulus-free oocytes. Collectively, the PGE_2/EP_2 system contributes to the ordered process of cumulus expansion required for successful fertilization (Fig. 3).

COX-2-deficient mice have been reported to show impaired implantation of wild-type blastocysts [5], suggesting that PGs may modulate the implantation process. Since the EP_2 gene was found to be transiently up-regulated in luminal epithelial cells during the peri-implantation period, the EP_2 receptor has been suggested to be involved in this process [34,35]. However, no defects have been found in the implantation of wild-type blastocysts in EP_2-deficient mice [31]. This discrepancy may be accounted for by compensation by the EP_4 receptor in the EP_2-deficient mice, since both EP_2 and EP_4 are expressed in the luminal epithelial cells during implantation and are coupled to the stimulation of cAMP synthesis. Alternatively, COX products may exert its effect on implantation through peroxisome proliferator-activated receptor-δ (PPARδ) since the defects of implantation in COX-2-deficient mice can be recovered by the administration of a PGI_2 analogue or a PPARδ agonist [36]. The analyses of PPARδ-deficient mice and double-knockouts of the EP_2 and EP_4 genes may help to address this issue.

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Placenta (2002), 23, 215–223
doi:10.1053/plac.2001.0759, available online at http://www.idealibrary.com on IDEAL®

Expression of Apoptosis in Placentae from Mice Lacking the Prostaglandin F Receptor

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Paper accepted 26 October 2001

This study aimed to investigate the changes in apoptosis in the placenta and decidua of pregnant mice lacking the prostaglandin F receptor. Mouse placentae were removed from fetuses on days 10–23 of pregnancy. Apoptotic cells were examined by a DNA fragmentation assay and the terminal deoxynucleotidyl transferase-mediated dUDP nick end-labelling (TUNEL) technique. The placenta and decidual weight increased before day 18 and 14 of pregnancy, and then decreased with gestational day. After day 19, the fetuses gradually died in the uterus. All fetuses died in the uterus on day 23 of pregnancy. The number of apoptosis was not significantly different between wild type and FP-deficient mice before day 18 of pregnancy by DNA fragmentation and TUNEL staining. The DNA fragmentation was always more pronounced in decidual tissue on each day of pregnancy. DNA laddering on placentae was more extensive on day 22 than day 18. In placenta, most TUNEL-positive cells were detected in trophoblast and stromal cells. A higher intensity of apoptotic cells was in the decidual basalis. The main area was the centre of the decidual basalis, and was in decrease toward to margin of placenta. The index of TUNEL positive cells increased as gestation progressed toward termination. Especially, it was prominent in the placentae on day 22 compared with that day 18 of pregnancy. The increased TUNEL-positive staining in syncytiotrophoblast surface was found in placenta at post-term, compared with those at term. Apoptosis may provide insights into both normal placental development and placental dysfunction during an abnormal pregnancy from post-term pregnancy.

Placenta (2002), 23, 215–223

INTRODUCTION

Apoptosis is a form of programmed cell death that is controlled at the gene level, and it plays important roles in embryonic development, the maintenance of tissue homeostasis, and the elimination of cells that have suffered serious DNA damage (Hale et al., 1996; Kerr et al., 1994). In general, apoptosis is characterized by activated specific endonuclease activities, as well as by chromatin condensation and margination, in sharply delineated masses at the nuclear envelope. Due to shrinkage of cell volume, cytoplasmic condensation and rigidification, budding of nuclear and plasma membranes, membrane-bound apoptotic bodies are then formed, which consequently cleave chromosomal DNA into oligomers of 180–200 bp nucleosomal units (Tilly and Hsueh, 1993; Vaux and Weissman, 1993). These DNA fragments can be detected by a 'DNA ladder' on agarose gel electrophoresis (Chen et al., 2001; Forsberg et al., 1998). Identification of apoptotic cells in histological sections was made possible by in terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL). This method can relate apoptosis to either histological localization or cell differentiation (Qiao et al., 1998).

In recent years, it has become clear that apoptosis is important in many aspects of reproduction. Apoptosis has recently been implicated in regulating various reproductive tissues, including those of the uterus (Watanabe et al., 1997), ovary (Hsueh et al., 1994), placenta (Smith et al., 1997b) and fetal membranes (Runic et al., 1998). In humans, apoptosis, a form of programmed cell death, has been described in placentae of normal pregnancies and increases in pregnancies complicated by fetal growth restriction (Axt et al., 1999a; Smith et al., 1997a). An increased incidence of apoptosis has been demonstrated in syncytiotrophoblasts in failing first trimester pregnancies (Kokawa et al., 1998a; Qiao et al., 1998). As far as we know, however, apoptosis in the placenta in the mouse throughout pregnancy, including post-term pregnancy, has not been reported.

Prostaglandins involved in various mammalian reproductive processes. Exogenous prostaglandins have been shown to

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