

Maternal Glucocorticoids Make the Fetal Membrane Thinner: Involvement of Amniotic Macrophages

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Glucocorticoid use during pregnancy is known to increase the risk of preterm birth and preterm premature rupture of membranes (pPROM). Here, we investigated the mechanism of how glucocorticoids weaken the fetal membranes. The amnion mesenchymal layer was significantly thinner in pregnant women treated with prednisolone and in corticosterone-injected mice than in control groups. Matrix metalloproteinase (MMP)-9 mRNA and its activity, COX2 mRNA levels, and prostaglandin E₂ synthesis were increased, whereas type 1 collagen (COL1A1) mRNA levels were decreased in the fetal membranes of corticosterone-injected mice. Unexpectedly, the proliferation and migration of macrophages were observed around the corticosterone-injected amnion, and IL-1 β was released from these macrophages. In human amnion mesenchymal cells, cortisol did not change MMP mRNA expression, whereas IL-1 β treatment robustly increased MMP and COX2 mRNA expression. COL1A1 mRNA level was decreased by both cortisol and IL-1 β . These data suggest that the recruitment of amniotic macrophages by glucocorticoids plays a pivotal role in weakening of the fetal membranes, leading to the pathogenesis of pPROM. (*Endocrinology* 160: 925–937, 2019)

Preterm labor is the leading cause of perinatal morbidity and mortality (1). Preterm premature rupture of membranes (pPROM), defined as membrane rupture occurring before 37 weeks' gestation, is associated with 30% to 40% of preterm deliveries and occurs in approximately 1% to 3% of all pregnancies. Although the leading cause of pPROM is infection, many causes are unrelated to infection, such as low socioeconomic status, low body mass index, previous preterm delivery, obesity, cigarette smoking, maternal poor nutrition, maternal stresses, young or advanced age, periodontal disease, and genital bleeding in the first and second trimesters (1).

Glucocorticoids are necessary to treat patients with autoimmune diseases such as systematic lupus erythematosus and ulcerative colitis and for maintenance treatment after organ transplantation. However, glucocorticoid use during pregnancy is known to increase the risk of preterm birth and pPROM (2). Treatment with

>10 mg/d of prednisone during pregnancy was associated with preterm birth irrespective of disease activity (3), and corticosteroid use was associated with increased risk of preterm birth (4, 5). The mechanism by which glucocorticoids increase the risk of preterm birth, however, is unclear.

The primary load-bearing structure of the fetal membranes is the amnion, which is composed of a single layer of epithelial cells and an underlying collagen-rich layer of mesenchymal cells (6). Mesenchymal cells are the primary source of collagen and matrix support for the amnion. Interstitial collagen (types I and III) maintains the mechanical integrity of the amnion, and collagen degradation is mediated primarily by matrix metalloproteinases (MMPs). Cleavage of the triple helix of fibrillar collagen is mediated primarily by the interstitial collagenase MMP1. Collagen fragments are then further degraded by the gelatinases MMP2 and MMP9.

Prostaglandins (PGs) play pivotal roles in human parturition by stimulating cervical ripening and myometrial contraction (7). In human pregnancy, the principal source of PG is the amnion (8). PG is also involved in membrane rupture, upregulating MMP9 (9).

In this study, we hypothesized that glucocorticoids weaken the fetal membranes and increase the risk of pPROM. First, we retrospectively compared the thickness of the fetal membranes between women treated with glucocorticoids and those who received no glucocorticoid treatment to determine the effect of glucocorticoids during pregnancy. Because all of these patients had autoimmune disease or nephrotic syndrome complications, we used an animal model of glucocorticoid exposure to determine the pure glucocorticoid effect *in vivo*. Finally, to observe the direct effect of glucocorticoids on the amnion, we used primary human amnion cells.

Materials and Methods

Study 1: clinical data

Measurement of fetal membrane thickness in humans

Nineteen pregnant women treated with glucocorticoids (>6.5 mg of prednisolone per day) and 19 control pregnant women who were not treated with glucocorticoids were compared. Patient characteristics are shown in Table 1. Most women treated with a large dose of glucocorticoids because of systemic lupus erythematosus or nephrotic syndrome had complications such as hypertension, renal dysfunction, preeclampsia, or fetal growth restriction during pregnancy, and termination of pregnancy midgestation was required, resulting in these patients experiencing preterm delivery. To match the gestational weeks of the control group to those of the glucocorticoid group, control cases were selected from preterm delivery cases without glucocorticoid treatment. To avoid disease bias in the control group, patient background was heterogeneous. Fetal membranes that were taken from two or three different locations were fixed in 10% formalin and stained with hematoxylin and eosin. In each section, five different parts were randomly selected, and images were captured using an Olympus BX51 microscope and DP-2 BSW software system. The thickness of the epithelial and mesenchymal layers in the amnion, chorion, and decidua was measured using Image J software; measurement was blind to the treatment group to avoid bias. Informed consent was obtained from each patient. All procedures were approved by the ethics committee of the Kyoto University Graduate School of Medicine (G0325).

Study 2: glucocorticoid-treated mice

Mice

C57BL/6 female mice were crossed with male mice overnight, and pregnancy was determined by the presence of a vaginal plug. From 12.5 days post coitum (dpc) to 18.5 dpc, corticosterone dissolved in hydroxypropyl- β -cyclodextrin

(vehicle) was subcutaneously injected into eight pregnant mice each morning at 8:00 AM (40 mg/kg/d). For the vehicle group, only hydroxypropyl- β -cyclodextrin was injected into eight pregnant mice. At 10:00 AM on 18.5 dpc, mice were euthanized, and fetal membranes were collected after washing with PBS, and the tissues were quick-frozen in liquid nitrogen and stored at -80°C . The end of the uterus that included the myometrium, fetal membranes, and fetus was cut out and fixed in 10% formalin for immunofluorescence. Using the end of the uterus enabled us to avoid the gestational sac slipping out of the myometrium. All procedures were approved by the Animal Research Committee of the Graduate School of Medicine of Kyoto University (Med Kyo 11610).

Hydroxyproline assay

Pulverized mouse fetal membrane samples were lyophilized overnight. Dried samples were rehydrated with 1 M NaCl buffer containing proteinase inhibitor cocktail (Complete Mini; Roche), and samples were extracted at 4°C for 24 hours overnight with agitation. Samples were centrifuged at 21,000g for 30 minutes at room temperature. The supernatant (Fraction A: soluble collagen = uncross-linked or immature cross-linked collagen) was frozen at -20°C . Pellets were subsequently extracted using 0.5 M acetic acid with a protease inhibitor cocktail at 4°C for 24 hours and centrifuged at 21,000g for 10 minutes at room temperature. The resulting supernatant (Fraction B: denatured collagen) and pellet (Fraction C: insoluble collagen = mature cross-linked collagen) were lyophilized and stored at -20°C .

The amount of collagen present in each fraction was measured using a colorimetric hydroxyproline assay (10). The lyophilized supernatants and pellets were hydrolyzed in 6 M of HCl at 100°C for 16 hours, 2 μL of sample was added to wells in a 96-well microplate, and the plate was placed under a hood. After HCl had completely evaporated, 80 μL of water was added to sample wells. Serial hydroxyproline standards (80 μL) were added to the microplate, and 40 μL of chloramine-T solution was added to each well and incubated for 15 minutes at room temperature (10 mL of chloramine-T solution includes 0.141 g of chloramine-T, 2 mL of water, 3 mL of ethylene glycol, and 5 mL of $10\times$ sodium acetate buffer; 1 L of $10\times$ sodium acetate buffer contains 50 g of citric acid monohydrate, 120 g of sodium acetate trihydrate, 34 g of sodium hydroxide, and 12 mL of acetic acid, pH 6). Perchloric acid (PCA) solution (40 μL) was then added to each well (10 mL of PCA includes 2.7 mL of 70% PCA and 7.3 mL of water), and then 40 μL of (dimethylamino)benzaldehyde solution [0.2 g (dimethylamino)benzaldehyde in 10 mL of ethylene glycol] was added. The plate was placed into an incubator at 60°C for 30 minutes, and the absorbance was read at 560 nm using a microplate reader.

Quantitative RT-PCR

Total RNA was extracted from mouse fetal membranes using Sepasol-RNA (Nacalai Tesque) or from human amnion cells using a PureLink RNA Mini kit (catalog no. 12183025; Ambion) according to the manufacturer's instructions. Reverse transcription was performed using a ReverTra Ace qPCR RT Kit (catalog no. FSQ-101; TOYOBO). Quantitative RT-PCR was used to determine the relative levels of gene expression using the ABI Prism 7000 Sequence Detection System (Applied

Table 1. Study 1 Patient Characteristics

	Weeks	Age, y	Parity	Mode of Delivery	Maternal Complication	PSL, mg	Indication of Delivery	Epi, μm	Mes, μm	Cho, μm	Deci, μm
Control	29	36	P	CS	N/A		HELLP	6	27	154	210
	29	40	P	CS	N/A		Labor onset	9	41	181	125
	30	36	P	CS	N/A		Fetal pleural effusion	10	34	159	129
	31	37	P	CS	N/A		PE	8	52	150	129
	32	37	M	CS	N/A		PE, HELLP	6	23	88	112
	32	29	P	CS	N/A		Hydrops fetalis	11	37	188	155
	33	37	P	CS	N/A		PE, HELLP	5	27	201	129
	34	40	P	CS	N/A		PE, fetal distress	9	36	123	252
	34	37	M	VD	N/A		PE, FGR	8	28	125	178
	34	22	P	VD	N/A		Labor onset	9	49	123	77
	34	34	P	VD	N/A		PROM	9	36	94	144
	35	35	M	CS	N/A		PE, HELLP	8	49	146	68
	36	24	P	CS	Chronic hypertension		Hypertension	7	33	122	85
	36	30	P	VD	N/A		Labor onset	7	31	120	134
	36	35	M	CS	N/A		Chorioamnionitis	8	38	146	81
	36	40	M	CS	N/A		Placenta previa	8	48	168	61
	36	39	M	CS	N/A		Placenta previa	9	28	139	135
	36	32	M	VD	N/A		Hypochondroplasia	7	42	186	164
	36	35	M	CS	N/A		Placenta previa	7	60	166	97
Average	33.6 $\pm$ 0.6	34.5 $\pm$ 1.2				0		7.9 $\pm$ 0.3	37.8 $\pm$ 2.3	146.3 $\pm$ 7.2	129.7 $\pm$ 11.2
Glucocorticoid	30	36	M	CS	Nephrotic syndrome	30	FGR, fetal distress	7	22	133	69
	30	41	M	VD	SLE	6.5	HELLP	7	34	127	148
	30	28	P	CS	SLE	40	HELLP	7	26	218	118
	32	41	P	CS	SLE	10	PE, fetal distress	7	32	117	54
	32	33	M	CS	SLE	20	FGR, fetal distress	8	38	227	77
	32	26	M	CS	SLE	30	Worsening of SLE	8	19	110	88
	33	38	M	CS	SLE	7.5	Placenta previa	9	20	135	306
	35	31	M	CS	SLE	55	Spontaneous labor onset	12	30	225	128
	35	30	P	VD	SLE	30	Worsening of SLE	7	15	190	107
	35	34	P	VD	SLE	8	Spontaneous labor onset	19	35	196	424
	36	33	P	VD	SLE	20	PROM	14	26	128	64
	36	36	P	CS	SLE	7.5	PROM	7	22	188	187
	36	37	P	CS	SLE	10	Worsening of SLE	8	29	135	126
	37	30	P	VD	SLE	20	PROM	8	23	108	97
	37	32	M	CS	SLE	20	PROM	11	21	171	263
	37	36	M	CS	SLE	7	FGR	6	25	147	218
	37	35	M	CS	Churg-Strauss syndrome	7	PE	6	35	126	188
	38	44	P	VD	Nephrotic syndrome	40	FGR	19	23	157	43
	38	39	M	CS	SLE	8	Elective CS	9	28	175	207
Average	34.5 $\pm$ 0.6	34.7 $\pm$ 1.1				19.8 $\pm$ 3.3		9.3 $\pm$ 0.9	26.5 $\pm$ 1.5	158.6 $\pm$ 9.0	153.3 $\pm$ 22.5

Abbreviations: Cho, chorion; CS, cesarean section; Deci, decidua; Epi, epithelial layer of amnion; FGR, fetal growth restriction; HELLP, hemolysis, elevated liver enzymes, low platelet count syndrome; M, multiparous; Mes, mesenchymal layer of amnion; N/A, not applicable; P, primiparous; PE, preeclampsia; PROM, premature rupture of membranes; PSL, prednisolone; SLE, systemic lupus erythematosus; VD, vaginal delivery.

Biosystems). Primer sequences for amplifications are shown in Table 2. SYBR Green (THUNDERBIRD SYBR qPCR Mix; catalog no. QPS-201; TOYOBO) was used to detect amplification, and gene expression was normalized to that of the indicated housekeeping gene.

MMP2 and MMP9 activity assay

MMP2 and MMP9 activity in mouse fetal membranes was assayed using a Sensolyte[®] 520 MMP-2 Assay Kit (Fluorimetric, AS-71151; Anaspec) and a Sensolyte[®] 520 MMP-9 Assay Kit (Fluorimetric, AS-71155; Anaspec) according to the manufacturer's instructions.

Prostaglandin E₂ ELISA

Prostaglandin E₂ (PGE₂) concentration in mouse fetal membranes was assayed using a Parameter PGE₂ immunoassay (KGE004B; R&D Systems) according to the manufacturer's instructions.

Immunofluorescent staining

After treatment of corticosterone from 12 to 18 dpc, the mouse uterus was collected at 18 dpc. The uterus was fixed in 4% paraformaldehyde overnight and kept in 70% ethanol until paraffin embedding. After sections (5 μ m) were deparaffinized, antigen retrieval was performed by incubation with proteinase K (P8107S; New England Biolabs; working concentration, 0.6 U/mL) for 1 minute at 37°C. Sections were then preincubated with 10% normal goat serum (50062Z; Life Technologies) with 0.3% Triton X-100 (Nacalai Tesque) for 30 minutes at room temperature. Subsequently, tissue sections were incubated with a primary antibody in PBS with 1% BSA (01860-07; Nacalai Tesque) and 0.3% Triton X-100 at 4°C overnight. Primary antibodies used and their concentrations were as follows: F4/80 [clone BM8, catalog no.14-4801; eBioscience; 1:50; RRID: [AB_467559](#) (11)] and IL-1 β [catalog no.9722; Abcam; 1:100; RRID: [AB_308765](#) (12)].

Thereafter, sections were incubated with Alexa Fluor 488-, 549-, or 594-conjugated secondary antibodies (Ig, heavy and light chains; Invitrogen; 1:500 dilution) in 10% normal goat serum for 1 hour at room temperature. Next, samples were mounted on slides using ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (P36935; Molecular Probes). Images were obtained by Leica SP8 confocal microscopy. Image J software was used to generate individual images.

For counting F4/80-positive macrophages, three to five different parts in the section were randomly selected and the macrophage count in the sections was averaged. To avoid bias, the number of macrophages was counted blinded to the treatment group.

Study 3: primary human amnion cells

Isolation and culture of amnion epithelial and mesenchymal cells

Separation and isolation of amnion epithelial and mesenchymal cells was performed as previously described (13). Briefly, amnion tissue was separated by blunt dissection and then minced. Cells were dispersed via enzymatic digestion with trypsin (Trypsin 1:250; catalog no.27250-018; Gibco) and then collagenase (Collagenase B; catalog no.11088831001; Roche) and DNase I (10104159001; Roche). Isolated amnion cells were suspended in DMEM/F12 (08460-95; Nacalai Tesque) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Anti 100X; catalog no.15240-062; Gibco). Cells were plated in plastic culture dishes, maintained at 37°C in a humidified atmosphere of 5% CO₂ in the air, and allowed to replicate in a monolayer to confluence.

Statistical analysis

Values are expressed as the mean $\pm$ SD in the amnion cell experiments or as the mean $\pm$ SEM in the mice experiments. Data were analyzed by unpaired Student *t* test unless indicated

Table 2. Primer Sequences Used for Quantitative PCR

Gene	Forward	Reverse
Mouse		
<i>CPHA</i>	5'-GCTTTTCGCCGCTTGCT-3'	5'-TCGTCATCGGCCGTGAT-3'
<i>COL1A1</i>	5'-GCCTTGAGGAACTTTGCTT-3'	5'-GCACGGAACTCCAGCTGAT-3'
<i>COL3A1</i>	5'-CACAGCAGTCCAACGTAGATGAA-3'	5'-TGACATGGTTCTGGCTTCCA-3'
<i>IL1B</i>	5'-AAGATGAAGGGCTGCTTCCA-3'	5'-TGAGTGATACTGCCTGCCTGAA-3'
<i>IL6</i>	5'-CCACGGCCTTCCCTACTTC-3'	5'-TGCACAACCTCTTTCTCATTTCCA-3'
<i>IL10</i>	5'-GGACCAGCTGGACAACATACTG-3'	5'-GCAACCCAAGTAACCTTAAAGTC-3'
<i>MMP2</i>	5'-GGACTATGACCGGGATAAGAAATATG-3'	5'-GGGCACCTTCTGAATTTCCA-3'
<i>MMP8</i>	5'-CACACACAGCTTGCCAATGC-3'	5'-TTTCGTAGGTAATTCTCAGCAGTTTTT-3'
<i>MMP9</i>	5'-AGACCAAGGGTACAGCCTGTTC-3'	5'-GCACGCTGGAATGATCTAAGC-3'
<i>MMP13</i>	5'-GGCAAAAGCCATTTTCATGCT-3'	5'-TCTTCATCGCCTGGACCATAA-3'
<i>LOX</i>	5'-TGCCAGTGGATTGATATTACAGATGT-3'	5'-AGCGAATGTACAGCGTACAA-3'
<i>TIMP2</i>	5'-GGTGGATTCCGGGAATGAC-3'	5'-TTTGAACATCTTTATCTGCTTGATCTC-3'
<i>COX2</i>	5'-AGTTTGTTGAGTCATTCACCAGACA-3'	5'-CCACTGCTGTACAGCAATTGG-3'
Human		
<i>GAPDH</i>	5'-GGAGTCAACGGATTTGGTCGTA-3'	5'-CAACAATATCCACTTTACCAGAGTTA-3'
<i>MMP1</i>	5'-AGATGAAAGGTGGACCAACAATT-3'	5'-CCAAGAGAATGGCCGAGTTC-3'
<i>MMP9</i>	5'-CCACCACAACATCACCTATTGG-3'	5'-GCAAGGCGTCGTCAATCA-3'
<i>COL1A1</i>	5'-ACGAAGACATCCCACCAATCA-3'	5'-CGTTGTCGACAGCGCAGAT-3'
<i>COX2</i>	5'-GCTCAACACCGGAATTTTGA-3'	5'-TCGAAGGAAGGGAATGTTATCA-3'

otherwise. In Study 1, parity, cesarean birth rate, and rate of chorioamnionitis were analyzed by χ^2 test. P values <0.05 were regarded as statistically significant. Correlation between gestational weeks and fetal membrane thickness was analyzed by Pearson correlation (R = Pearson correlation coefficient).

Results

Study 1: thickness of fetal membranes in women taking glucocorticoids

We compared the thickness of fetal membranes between patients treated with glucocorticoids and those without glucocorticoid treatment (Table 1). The average gestational ages in the control and glucocorticoid groups were 33.6 ± 0.6 weeks and 34.5 ± 0.6 weeks, respectively (ranges, 29 to 36 weeks and 30 to 38 weeks, respectively), and there was no statistical difference. Maternal age, parity, cesarean birth rate, rate of ruptured membranes, and pathological chorioamnionitis did not differ between the control and glucocorticoid groups (Table 1).

The average amnion epithelial layer thickness was $7.9 \pm 0.3 \mu\text{m}$ in the control group and $9.3 \pm 0.9 \mu\text{m}$ in the glucocorticoid group ($P = 0.164$) (Fig. 1A, left). However, the amnion mesenchymal layer was significantly thinner in pregnant patients treated with glucocorticoids than in control patients (26.5 ± 1.5 and $37.8 \pm 2.3 \mu\text{m}$, respectively; $P = 0.00018$) (Fig. 1A, right). Representative images of the effects on fetal membranes are shown in Fig. 1B. The chorion thickness was $146.3 \pm 7.2 \mu\text{m}$ in the control group and $158.6 \pm 9.0 \mu\text{m}$ in the glucocorticoid group; that of decidua was $129.7 \pm 11.2 \mu\text{m}$ in the control group and $153.3 \pm 22.5 \mu\text{m}$ in the glucocorticoid group. The thickness of the chorion and decidua was not changed by glucocorticoids. We also assessed the physiological change in fetal membrane thickness over the course of pregnancy. The thickness of the amnion epithelial and mesenchymal layers, chorion, and decidua was not changed after 29 weeks of pregnancy in our control group (Fig. 1C) and glucocorticoid group (data not shown). These clinical data include premature rupture of membrane (PROM) cases (one case in the control group and four cases in the glucocorticoid group) (Table 1). Even when we excluded PROM cases, there remained a significant difference in amnion mesenchymal thickness ($37.9 \pm 11.7 \mu\text{m}$ in the control group vs $27.5 \pm 1.6 \mu\text{m}$ in the glucocorticoid group, $n = 18$ and 15 , respectively; $P = 0.0020$). Thus, the amnion mesenchymal layer, which contains abundant collagen fibers and supports the mechanical integrity of the fetal membranes, was thinned by glucocorticoids.

Study 2

Mouse fetal membranes exposed to glucocorticoids

Next, we used pregnant mice injected with corticosterone or vehicle to investigate the effect of glucocorticoids *in vivo*. Neither vehicle-injected nor glucocorticoid-injected mice delivered preterm. Representative images of the fetal membranes by trichrome stain are shown in Fig. 2A and 2B. Although the thickness of the amnion epithelial layer did not differ between vehicle and corticosterone (3.10 ± 0.35 and $3.24 \pm 0.32 \mu\text{m}$, respectively), the mesenchymal layer was significantly thinner in corticosterone-injected mice ($1.39 \pm 0.13 \mu\text{m}$) than in vehicle-injected mice ($2.91 \pm 0.47 \mu\text{m}$) (Fig. 2C). There was less blue-stained collagen in the amnion layer of corticosterone-injected mice (Fig. 2B). In mice, the visceral yolk sac surrounds the fetus beneath the amnion, and this yolk sac performs several functions that are assigned to trophoblasts in the human placenta (14). The decidua disappears on approximately embryonic day 17 (15). The yolk sac thickness was $21.76 \pm 2.52 \mu\text{m}$ in the vehicle group and $16.26 \pm 1.93 \mu\text{m}$ in the corticosterone group, which was not significantly different. The thickness of the myometrium was slightly thinner in the corticosterone group than in the vehicle group, although this was not statistically different (14.8 ± 0.9 and $17.4 \pm 1.7 \mu\text{m}$, respectively, $P = 0.22$). This data indicates that glucocorticoid administration thinned the amnion mesenchymal layer in mice.

Next, we analyzed collagen content in fetal membranes using a hydroxyproline assay. Collagens were divided by their acid solubility into uncross-linked (immature cross-linked), denatured, and mature cross-linked collagen groups. The uncross-linked and denatured collagen content did not change with glucocorticoid administration, but the mature cross-linked collagen content was significantly decreased in the glucocorticoid group compared with the control group (Fig. 2D). This suggests that the glucocorticoid-induced thinner membrane resulted from reduced collagen content in the mesenchymal layer of the amnion.

Collagen synthesis and degradation in fetal membranes exposed to glucocorticoids

We then analyzed gene expression related to collagen synthesis and degradation in the fetal membranes of corticosterone-injected mice. MMP2 is a stably expressed MMP, and although its mRNA expression and activity were not changed, MMP9 mRNA expression and enzymatic activity were increased by corticosterone (Fig. 3A and 3B). In mice, the main interstitial collagenase, MMP1, is functionally replaced by MMP8 (16–18). MMP8 mRNA expression was slightly increased by corticosterone, although this increase was not statistically significant (Fig. 3A). However, type 1

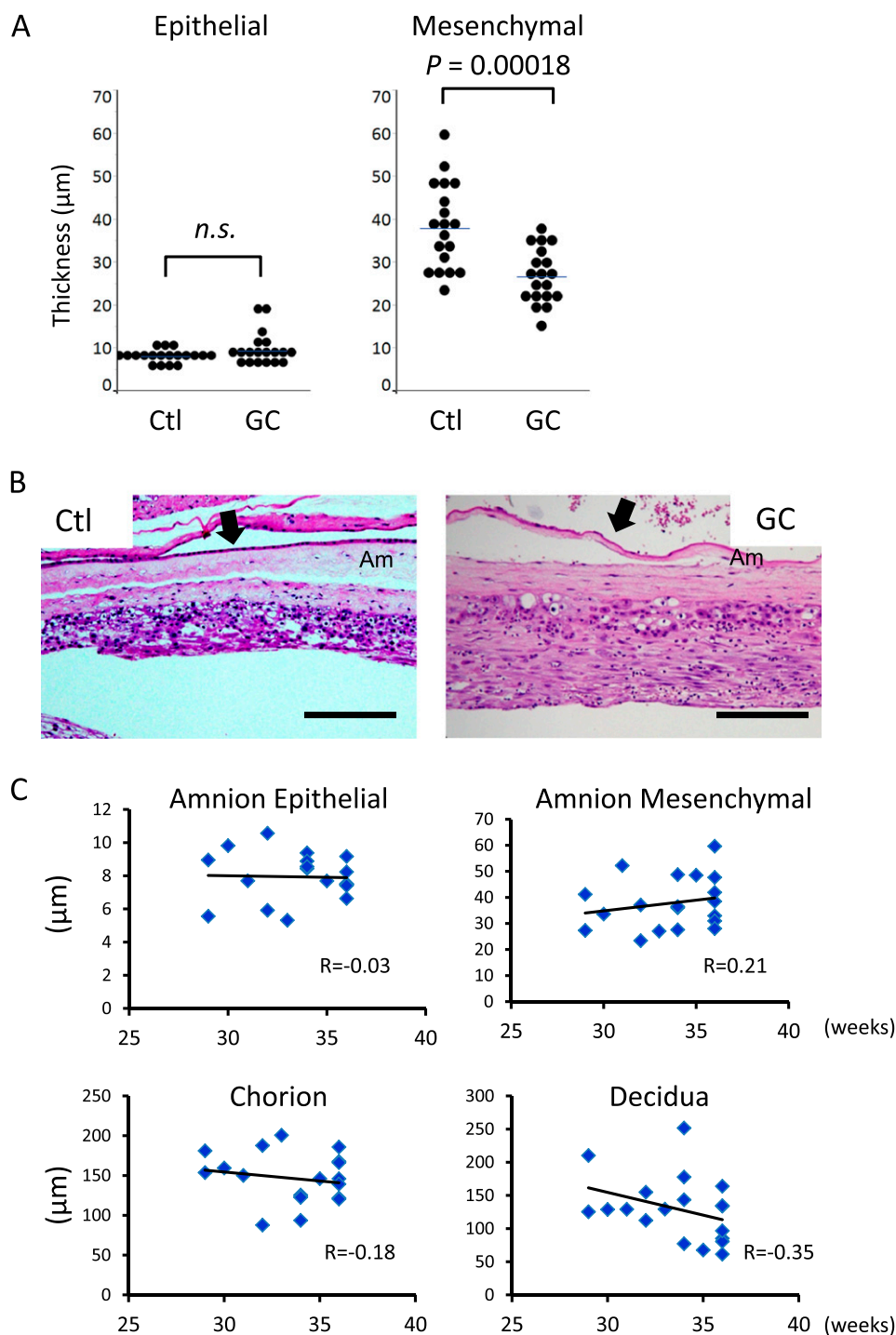


Figure 1. Thickness of the amnion in pregnant women taking glucocorticoids. (A) Thickness of the amnion epithelial and mesenchymal layer (μm) was compared between control (Ctl) women and women treated with glucocorticoid (GC); $n = 19$ in each group. (B) Representative images of the fetal membranes of the Ctl group (left; 36 weeks of gestation) and the GC treatment group (right; 35 weeks of gestation). Arrows indicate amnion (Am). Bars, 100 μm . (C) Changes in fetal membrane thickness in the Ctl group over the course of pregnancy. *n.s.*, not significant.

collagen gene (*COL1A1*) mRNA expression was decreased by corticosterone compared with the control group (Fig. 3C). mRNA expression of type 3 collagen (*COL3A1*) and lysyl oxidase (*LOX*), a collagen cross-linking enzyme, was not regulated by corticosterone (Fig. 3C). These data suggest that corticosterone suppresses *COL1A1* synthesis and increases collagen

degradation by upregulating MMP expression and activity.

PG content by corticosterone

In the fetal membranes of corticosterone-injected mice, COX2 mRNA was increased eightfold (Fig. 3D). Concomitantly, PGE₂ content in fetal membranes was also

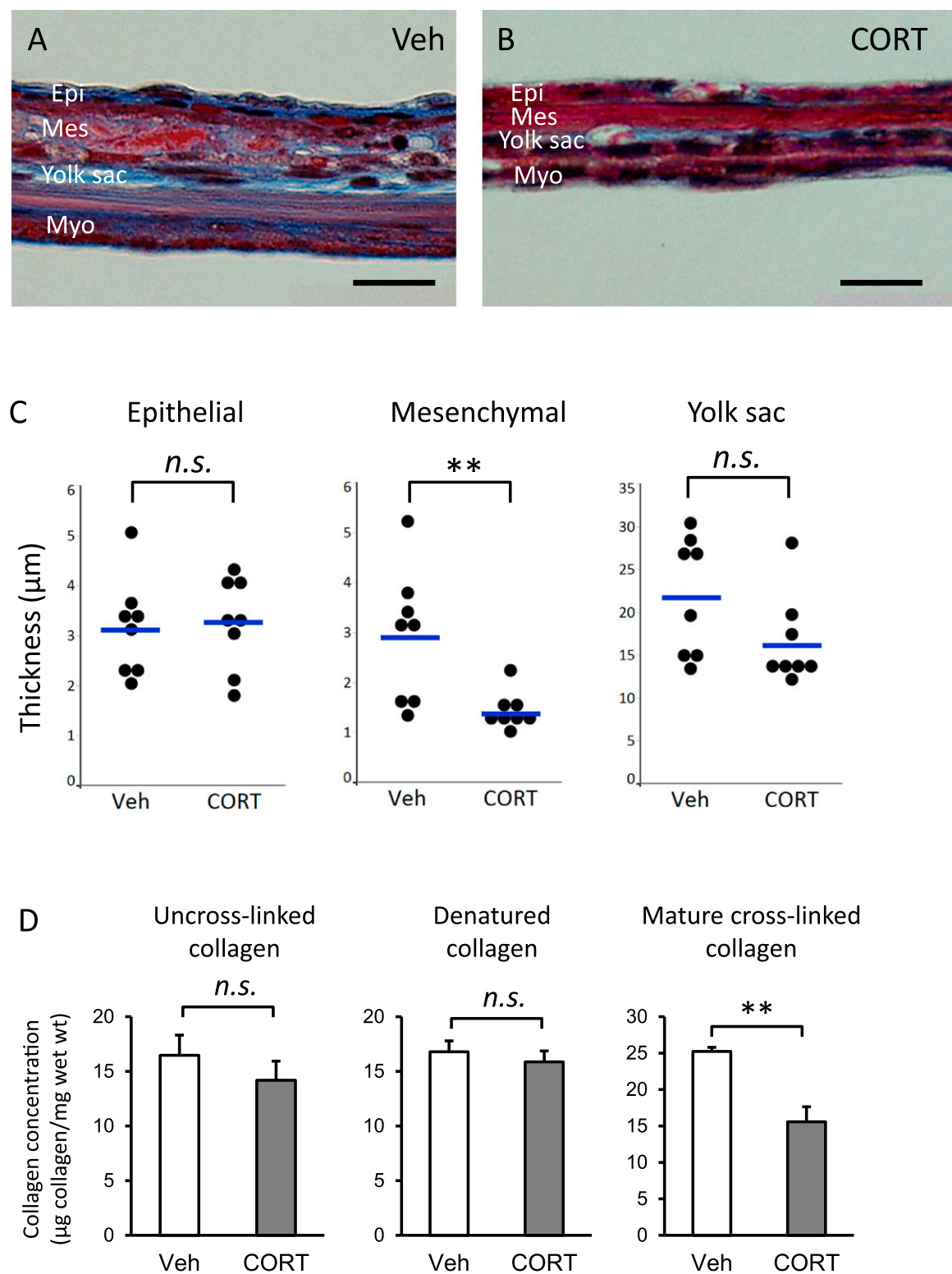


Figure 2. Fetal membranes of pregnant mice exposed to glucocorticoids. (A and B) Trichrome stain of mouse fetal membranes. A representative image of the fetal membranes of (A) a vehicle-injected mouse and (B) a corticosterone-injected mouse. Bars, 20 μm. (C) Thickness (μm) of epithelial and mesenchymal layers of the amnion and yolk sac. Bars represent average thickness; n = 8 in each group. (D) Collagen concentration measured using a hydroxyproline assay. The concentrations of uncross-linked, denatured, and mature cross-linked collagens were corrected by the wet weight of pulverized fetal membranes; n = 6 in the vehicle-injected group and n = 8 in the corticosterone-injected group. ****P < 0.01.** Error bars, SD. CORT, corticosterone; Epi, epithelial cells of the amnion; Mes, mesenchymal cells of the amnion; Myo, myometrium; n.s., not significant; Veh, vehicle.

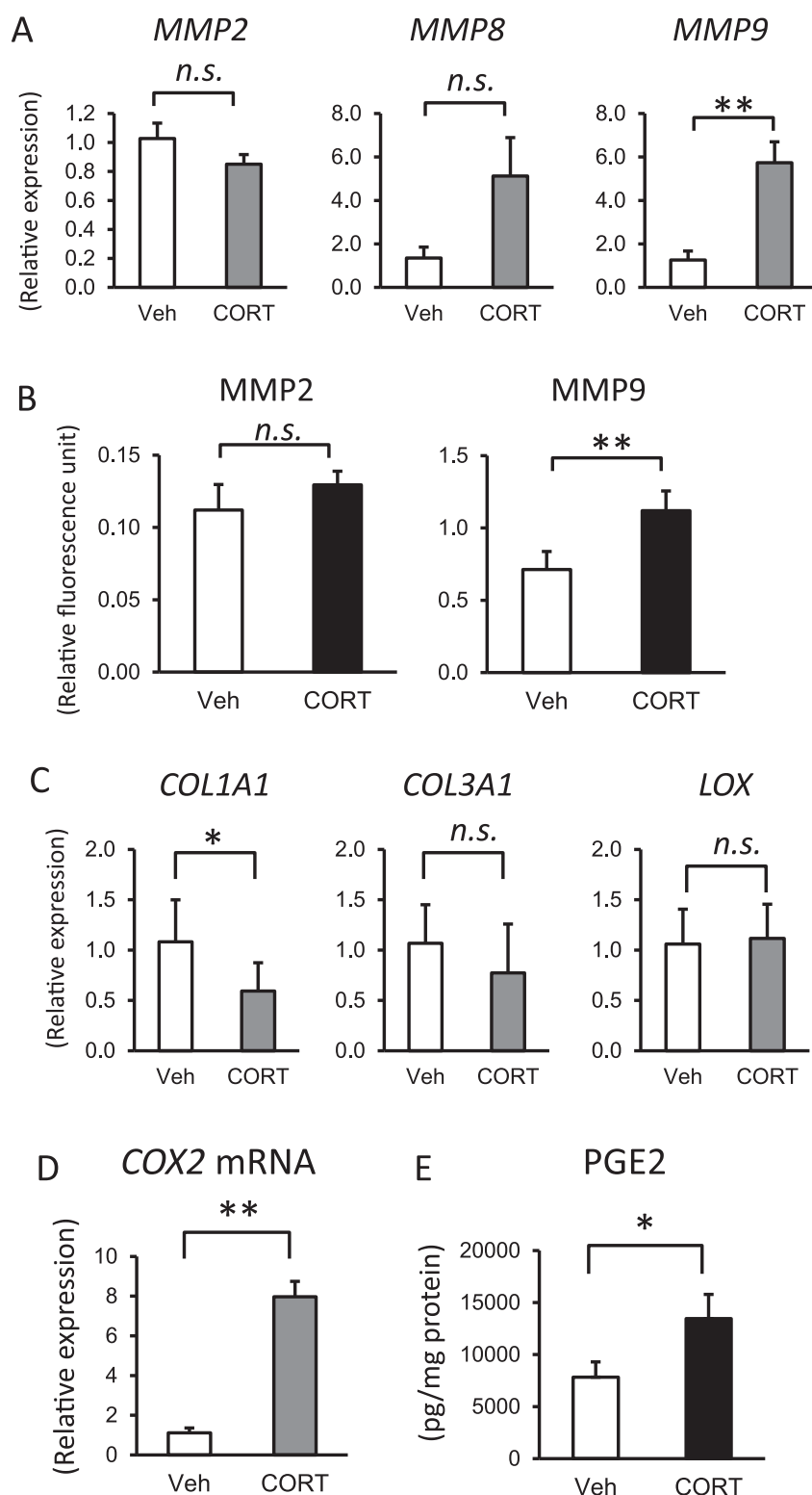


Figure 3. Changes of collagen, MMPs, and prostaglandin synthesis in fetal membranes by maternal glucocorticoid exposure. (A) MMP mRNA levels in mouse fetal membranes between vehicle (Veh) and corticosterone (CORT) injection groups; $n = 6$ in each group. (B) MMP2 and MMP9 activity assessed by the fluorescence resonance energy transfer method; $n = 6$ in each group. (C) Type 1 (*COL1A1*) and type 3 (*COL3A1*) collagen and lysyl oxidase (*LOX*) mRNA levels in the fetal membranes; $n = 6$ in each group. (D) *COX2* mRNA expression in the fetal membranes. (E) *PGE₂* synthesis in the fetal membranes; $n = 6$ in each group. * $P < 0.05$; ** $P < 0.01$. Error bars, SEM. n.s., not significant.

increased by corticosterone (Fig. 3E). These results show that glucocorticoids also upregulated PG synthesis.

Study 3

Glucocorticoids did not increase MMP mRNA expression in human amnion mesenchymal cells

To observe the direct glucocorticoid effect on the fetal membranes, we next used primary human amnion cells. The physiological concentration of cortisol in amniotic fluid from 25 to 35 weeks was 28 to 112 nM (19), whereas amniotic fluid cortisol concentration increased to 138 to 1380 nM in PROM cases at 30 to 34 weeks of gestation (20). Although we do not have data on the amniotic fluid cortisol level of prednisolone-treated patients, we tested 0.1 to 10 μ M of cortisol as a pathological condition in the *in vitro* study. MMPs, collagens, and COX2 are synthesized mainly in the mesenchymal cells of the amnion (21). In human amnion mesenchymal cells, COX2 mRNA expression was increased and *COL1A1* mRNA expression was suppressed by cortisol (Fig. 4). Unexpectedly, cortisol treatment did not increase *MMP9* mRNA expression (Fig. 4). *MMP1* mRNA expression was decreased by cortisol (Fig. 4). These data suggest that *MMP9* transcription is not directly regulated by glucocorticoids and that the expression of *MMP1* mRNA is slightly decreased by glucocorticoids.

Macrophages were recruited to amnion in glucocorticoid-injected mice (Study 2)

We examined whether other cells or tissues were involved in regulating MMPs and COX2 expression in our mouse model. Tissue samples of the murine uterus, including the gestational sac, were directly fixed in formalin so the histology of the entire environment surrounding the fetal membranes, such as the fetus and amniotic fluid, could be observed. Histology of corticosterone-injected mice showed many immune cells aggregated around the amnion

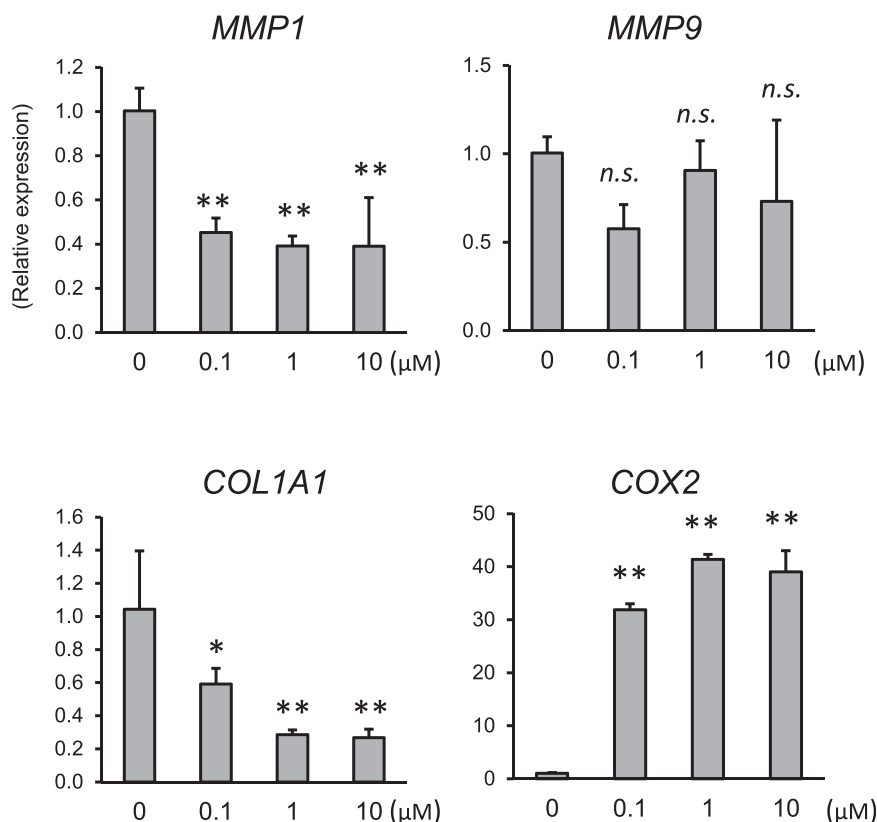


Figure 4. Glucocorticoids did not increase *MMPs* mRNA expression in human amnion mesenchymal cells. Relative mRNA expression of *MMP1*, *MMP9*, *COL1A1*, and *COX2* was assessed using quantitative PCR ($n = 3$ in each group). Primary human amnion mesenchymal cells were treated with different doses of cortisol for 48 hours. $n = 3$ in each group; * $P < 0.05$, ** $P < 0.01$, compared to 0 μM , ANOVA. Error bars, SD. n.s., not significant.

(Fig. 5B) compared with histology of vehicle-injected controls (Fig. 5A). In immunofluorescence, these cells were F4/80-positive macrophages (Fig. 5D). These macrophages were rarely observed in control mice (Fig. 5C), suggesting that macrophages proliferated and migrated around the amnion of corticosterone-injected mice. Quantification of F4/80-positive macrophages revealed that there were more in the amnion of corticosterone-injected mice (Fig. 5E). In contrast, the number of macrophages in the myometrium was minimal in both groups, and there was no statistically significant difference (3.8 ± 1.4 per section in the vehicle group and 2.0 ± 0.7 in the corticosterone group; $P = 0.30$).

Macrophages are roughly divided into two phenotypes: the M1-inflammatory and the M2-anti-inflammatory phenotypes (22). The proinflammatory cytokines *IL1B* and *IL6* showed high mRNA expression in the fetal membranes of corticosterone-injected mice (Fig. 5F), whereas *IL10*, an anti-inflammatory cytokine, showed no change in its mRNA expression (Fig. 5F). Immunofluorescence results show that *IL-1 β* was located in the macrophages of corticosterone-injected mice but not in the fetal membranes of vehicle-injected control mice (Fig. 5G). The amount of *IL-1 β* -releasing macrophages was significantly higher in corticosterone-injected mice than in control mice (Fig. 5H). Overall, maternal glucocorticoids

induced inflammatory macrophages in the amnion, which released proinflammatory cytokines *IL-1 β* and *IL-6*.

IL-1 β increased MMP and COX2 expression in human amnion mesenchymal cells (Study 3)

Finally, we investigated the role of *IL-1 β* in amnion mesenchymal cells because *IL-1 β* was significantly released from amniotic macrophages in corticosterone-injected mice. *IL-1 β* is usually undetected in amniotic fluid in normal women without complications, whereas amniotic fluid *IL-1 β* levels in cases of preterm birth with and without infection are between 100 and 2000 pg/mL (23, 24). Because we do not know the *IL-1 β* concentration in amniotic fluid treated with prednisolone, we tested serial doses of *IL-1 β* from 0.1 to 10 ng/mL in amnion cells. In primary human amnion mesenchymal cells, *MMP1* mRNA expression was increased fivefold by 0.1 to 10 ng/mL of *IL-1 β* compared with controls (Fig. 6). *MMP2* was stably expressed in the amnion, and its expression was not changed by *IL-1 β* (data not shown), but *MMP9* mRNA expression was dose-dependently increased twofold by *IL-1 β* (Fig. 6). These results suggest that the upregulation of *MMP1* and *MMP9* was mediated by *IL-1 β* that was released from macrophages and not by the direct effect of the glucocorticoids.

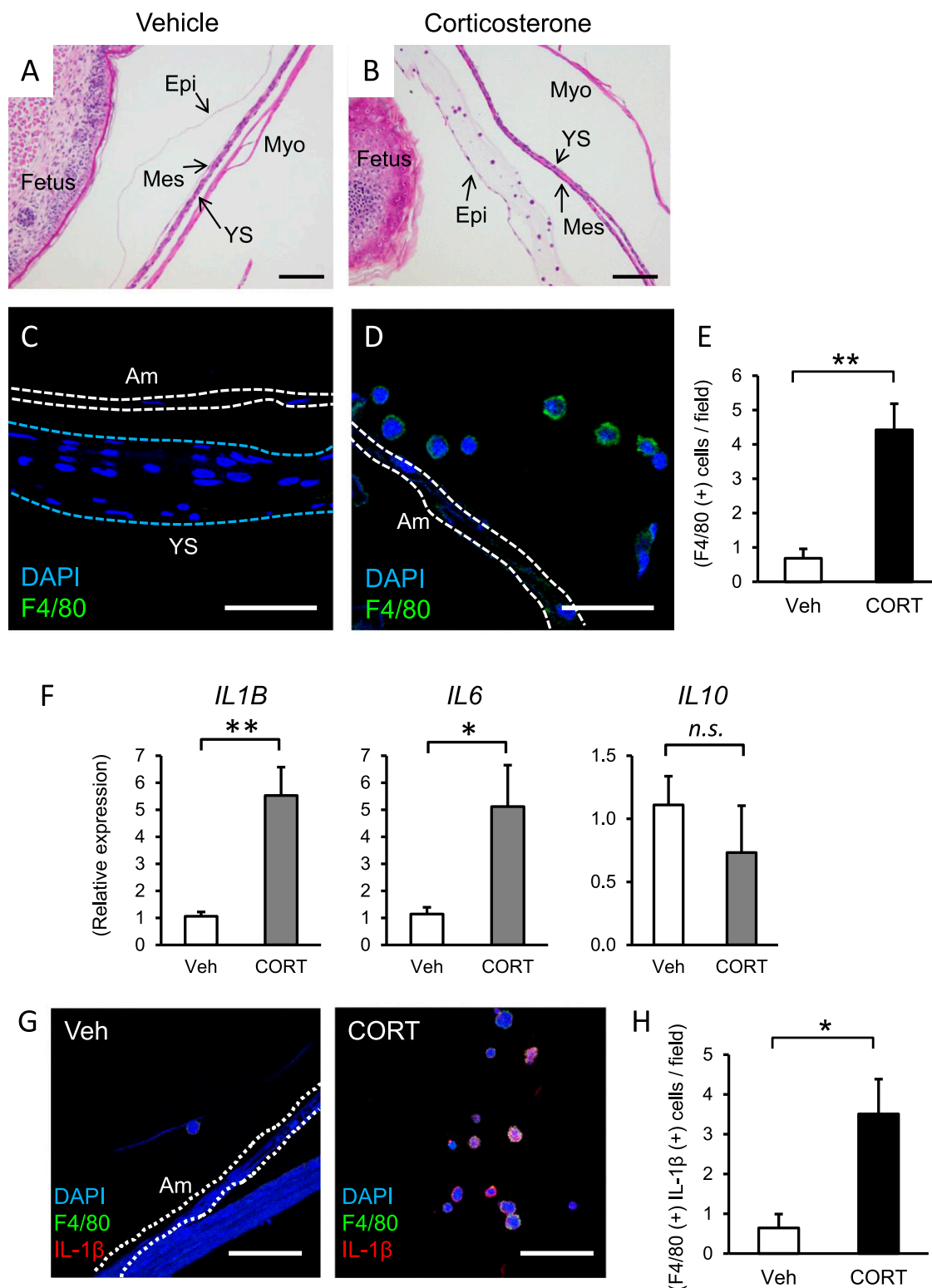


Figure 5. Amniotic fluid macrophages were involved in weakening of the fetal membranes in mice. (A and B) Hematoxylin and eosin staining of mouse fetal membranes at 18.5 dpc. (A) Vehicle (Veh) or (B) corticosterone (CORT) injection. Bars, 50 μ m. (C and D) Immunofluorescence for F4/80 (green) and DAPI (blue) of the fetal membranes from (C) Veh or (D) CORT injection at 18.5 dpc. Dashed lines indicate the Am (white) or YS (light blue). Bars, 50 μ m. (E) Average number of F4/80-positive macrophages in the field; $n = 4$ for Veh and $n = 3$ for CORT injection groups. (F) *IL1B*, *IL6*, and *IL10* mRNA expression in the fetal membranes of the Veh-injected or CORT-injected mice; $n = 6$ in each group. (G) Immunofluorescence of F4/80 (green), IL-1 β (red), and DAPI (blue) in the fetal membranes from Veh or CORT injection at 18.5 dpc. Dashed lines indicate the Am (white). Bars, 50 μ m. (H) Average number of F4/80 and IL-1 β double-positive macrophages in the field; $n = 3$ in each group. * $P < 0.05$; ** $P < 0.01$. Error bars, SEM. Am, amnion epithelial layer; DAPI, 4',6-diamidino-2-phenylindole; Epi, epithelial cells of the amnion; Mes, mesenchymal cells of the amnion; Myo, myometrium; n.s., not significant; YS, yolk sac.

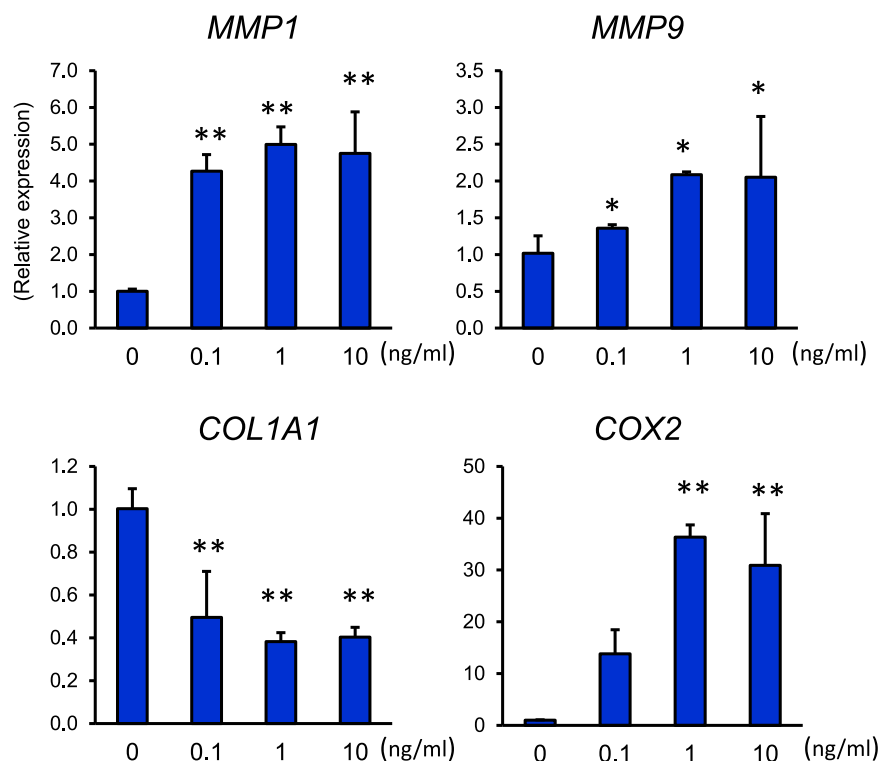


Figure 6. IL-1 β increased *MMP* and *COX2* mRNA expression in human amnion mesenchymal cells. Relative *MMP1*, *MMP9*, *COL1A1*, and *COX2* mRNA expression by quantitative PCR ($n = 3$ in each group). Primary human amnion mesenchymal cells were treated with different doses of recombinant human IL-1 β for 48 hours. For *COX2*, cells were treated for 24 hours. * $P < 0.05$; ** $P < 0.01$. Error bars, SD.

COL1A1 mRNA expression was also decreased by IL-1 β (Fig. 6). *COX2* mRNA expression increased 40-fold in response to 1 ng/mL of IL-1 β (Fig. 6). Thus, glucocorticoids can suppress collagen synthesis by both a direct glucocorticoid effect and through IL-1 β release from macrophages. Although we did not perform IL-1 β inhibitory experiments in corticosterone-treated mice, a glucocorticoid-induced increase in *MMP* and *COX2* expression appears to be mediated at least in part by IL-1 β from recruited amniotic macrophages.

Discussion

In this study, we found that glucocorticoid use during pregnancy thinned the mesenchymal layer of the amnion in humans. In the mouse model of glucocorticoid treatment, the mesenchymal layer of amnion cells was thinned, upregulating *MMP9* and *COX2* mRNA and suppressing *COL1A1* mRNA. We initially considered that glucocorticoids had directly caused these changes. To observe the direct glucocorticoid effect on the amnion, we used human amnion cells. As expected, glucocorticoids suppressed *COL1A1* expression and increased *COX2* expression. However, *MMP1* expression was unexpectedly downregulated, and *MMP9* was not regulated by glucocorticoid in amnion cells. Because we observed the

release of IL-1 β from migrated macrophages around the amnion in glucocorticoid-treated mice, we suspected that released IL-1 β might upregulate *MMP* expression. These data were supported by previous reports that IL-1 β increased *MMPs* and *COX2* in human amnion cells (25, 26). Thus, macrophages appear to be the main mediator of *MMPs* and PG synthesis in the amnion in response to glucocorticoid treatment during pregnancy. Some *MMPs*, such as *MMP9* and *MMP13*, might also be secreted from macrophages (27). Because some of the placentas were collected after vaginal delivery and were affected by labor, we could not evaluate the recruitment of macrophages by glucocorticoids in the human fetal membranes. Here, we present the possibility that amniotic inflammatory macrophages are induced by glucocorticoids, which release IL-1 β and weaken the amnion through *MMPs*, leading to pPROM.

In humans, the CRH–glucocorticoid axis regulates the timing of delivery (28, 29) by increasing PG synthesis in the fetal membranes (30, 31) and estrogen (32). Our study revealed the effect of glucocorticoids on the fetal membranes. If cortisol is increased endogenously in the mother and fetus at term, weakening of the fetal membranes can be considered physiological because rupture of the membranes at term is a natural part of the delivery process. Exposure of the fetal membranes to exogenous glucocorticoid may, however, have detrimental effects on the fetal membranes that result in PROM. Only four cases of membrane rupture occurred between 36 and 37 weeks in our study, and although delivery around this time is not previable, the risk of glucocorticoids to the fetal membranes should be recognized.

There are many differences in the mechanism of parturition between rodents and humans. At the onset of murine parturition, PGs trigger luteolysis and the withdrawal of progesterone (33). In contrast, serum progesterone levels are not decreased at term in humans (34). Instead, a functional progesterone withdrawal mediated by a shift in progesterone receptor isoforms (35) and a decline in progesterone receptor coactivator (36) or a functional estrogen activation (37) is associated with human parturition. Despite some biological differences in the mechanism of parturition, however, rodents still possess similarities to humans in the structure and function of fetal

membranes. Thus, the mouse model is useful for investigating the mechanism of membrane rupture.

Glucocorticoids suppress the immune system and increase the risk of infection. In our observation of human fetal membranes, however, pathological chorioamnionitis was observed in 4 of 19 women (21%) in the control group and in 4 of 19 women (21%) in the glucocorticoid group; the infection rate was similar in the groups. Therefore, weakening of the amnion is not necessarily caused by infection under glucocorticoid exposure.

The reduction of extracellular protein synthesis, such as collagen, following glucocorticoid treatment is widely known (38). We demonstrated herein that glucocorticoid suppressed *COL1A1* synthesis *in vivo* and *in vitro*; similarly, dexamethasone reduced fetal fibronectin synthesis in amnion epithelial cells (39). Collectively, glucocorticoid directly reduces various extracellular matrix proteins of both epithelial and mesenchymal cells of the amnion.

Sun and Myatt (40) showed that both epithelial and mesenchymal cells in the amnion expressed glucocorticoid receptors (α and β) and that glucocorticoid increased 11β -hydroxysteroid dehydrogenase type 1, which converts biologically inactive cortisone into active cortisol. This implies that externally administered glucocorticoid stimulates the synthesis of itself in the amnion in a feed-forward manner, amplifying glucocorticoid activity inside the uterus. Previous research showed that cortisol increased *COX2* mRNA in the amnion mesenchymal cells (41), and our data is compatible with those results. We suggest that *IL-1 β* that was released from amniotic macrophages enhanced PG production via *COX2* in glucocorticoid exposure *in vivo*.

During normal labor at term, inflammatory cells such as neutrophils and macrophages infiltrate the myometrium in humans (42) and the decidua in both rats and humans (43). Inflammatory cells also infiltrate the fetal membranes (44) and cervix (45) during labor in humans. These infiltrated immune cells release various inflammatory cytokines that activate the reproductive organs, resulting in membrane rupture, uterine contraction, ripening of the cervix, and eventually birth. Therefore, the infiltration of macrophages is essential for parturition (46). In our glucocorticoid-injected mice, however, no infiltration of macrophages into the myometrium was observed. This explains why these glucocorticoid-injected mice did not deliver preterm.

The mechanism by which macrophages increase and migrate to the amnion remains unclear. Although it was reported that steroid hormones such as estrogen and progesterone increased colony stimulating factor-1 and macrophages in the uterus (47), we did not identify any *CSF1* mRNA changes in glucocorticoid-treated human amnion epithelial and mesenchymal cells (data not shown). The mechanism of macrophage recruitment needs further study.

Previously, we showed that mechanical sterile rupture of the membranes in pregnant mice induced macrophage proliferation in amniotic fluid (48). These macrophages then migrated to the damaged amnion and assisted in its healing. In this study, however, macrophages migrated to the amnion to weaken or digest the fetal membranes, releasing inflammatory cytokines. Thus, there exist diverse macrophages with many functions inside the uterus: Some are inflammatory and cause parturition or weakening of the fetal membranes, and some are anti-inflammatory and aid in wound healing.

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