has become widespread as an off-label indication [5]. In recent years, the off-label use of rFVIIa in patients with life-threatening bleeding has been reported worldwide to be effective in various clinical settings [6–13]; however, only limited numbers of case reports documenting the use of rFVIIa for Japanese patients with postpartum haemorrhage (PPH) have been published to date.

Therefore, data on Japanese cases with severe PPH in which rFVIIa had been administered were collected. In this initial report, our experience with the use of rFVIIa for PPH management between August 2005 and February 2010 is reported.

Materials and methods

This multicentre case series was initiated by the Japan Society of Obstetrical, Gynecological and Neonatal Hematology, as part of research programs supported by the Ministry of Health, Labour and Welfare (MHLW) of Japan.

The data collection started in 2007. Data of obstetric haemorrhage patients treated with rFVIIa were collected at participating institutions throughout Japan. Participating institutions have obtained approval from their ethics committees to retrospectively collect relevant patient data without disclosure of personally identifiable information to third-party organizations.

Data were obtained by retrospective review of patients' case records. A data collection sheet was sent to institutions in which the use of rFVIIa for the treatment of PPH had been reported. The data collection sheet initially developed for the ANZHR (Australian and New Zealand Hemostasis Registry) [14] was modified for use in this case series. Background information including age, weight, pregnancy and delivery details, medical history, and complications was collected. For treatments to control bleeding, rFVIIa administration (dosage and timing), other medical treatments, and physical interventions were collected. Transfusion information was also collected for the 24 h preceding and after each administration of rFVIIa, and laboratory test results including haematologic parameters such as fibrinogen and platelets were collected for the closest tests taken before and after each dose of rFVIIa. The decision to give rFVIIa to a patient and its administration (dosage and timing) rested with the treating clinicians. Treating clinicians were asked to subjectively assess the effect of rFVIIa on bleeding at each administration using four categories: "Stopped", "Decreased", "Unchanged", and "Increased".

Major significant adverse events [cerebrovascular accident (CVA), transient ischaemic attack (TIA), deep vein thrombosis (DVT), pulmonary embolism (PE), acute myocardial infarction (AMI), arterial thrombosis (AT), disseminated intravascular coagulation (DIC), multi-organ failure (MOF), acute respiratory distress syndrome (ARDS), and allergic reaction], and other events considered clinically important were recorded for up to 28 days from the first administration of rFVIIa. The outcomes of these events were also recorded if the patients were followed. Data were entered and processed by an independent clinical research organization. A data manager sent inquiries about incomplete data or further information to treating clinicians when needed.

Statistical calculations were performed in an exploratory manner because this was not a confirmatory study. The demographic characteristics of registered patients were summarized using descriptive statistics. For continuous variables, the medians were calculated by considering the distribution profile. For categorical variables, the proportion of each category was calculated. Efficacy parameters including blood product requirement and blood loss volume were compared before and after the administration of rFVIIa using the Wilcoxon signed rank sum test.

The statistical package SAS Release 9.13 was used for the analyses. All statistical tests were two-tailed, and the level of statistical significance was set at 5%.

Results

Twenty-five women received rFVIIa for the treatment of obstetric haemorrhage in 18 institutions between August 2005 and February 2010. The details are shown in Tables 1 and 2. The median age was 33 years (range 23–43 years). Ten patients (40%) were 35 years or older. The primary causes of massive bleeding for which rFVIIa was given included uterine atony (13 patients) and placental abruption (5 patients). The median number of rFVIIa doses was 1 (range 1–5). Thirteen patients (52%) received a single dose, and 7 (28%) received two doses. The median dosage per single dose was 84.0 μg/kg (range 21.0–105.0 μg/kg). The median total dose was 97.9 μg/kg (range 55.0–358.9 μg/kg) in 20 patients whose weight was measured. Of these, 10 patients (50%) received a total dose of <100.0 μg/kg.

After the first administration, bleeding was "stopped" in 8 patients (32%), "decreased" in 10 patients (40%), and "unchanged" in 7 patients (28%). After the final administration, bleeding was "stopped" in 16 patients (64%), "decreased" in 8 patients (32%), and "unchanged" in one patient (4%). The non-responding case was the patient with uterine rupture who received a single dose of rFVIIa prior to uterine suture and hysterectomy.

The median total estimated blood loss was 9,985 mL (range 2,198–55,660 mL). The median estimated blood loss before rFVIIa administration, 24 h after the first administration, and 24 h after the final administration, was 7,130 (range 2,034–49,860) mL, 1,260 (range 85–11,147) mL, and 1,010 (range 30–5,500) mL, respectively. A significant

Table 1 Details of the patients treated with rFVIIa

Patient no.	Age (years)	Causes of bleeding	Surgical interventions before rFVIIa administration	Number of delivery	Gestational weeks	Number of foetus	Mode of delivery
1	36	Placental abruption, uterine atony	Hysterectomy	1	38	2	Caesarean section
2	38	Placental abruption, sepsis	Hysterectomy	3	33	1	Caesarean section
3	34	Placenta previa/placenta accreta	Hysterectomy	3	35	1	Caesarean section
4	35	Placenta previa/placenta accreta, eclampsia/ pregnancy-induced hypertension	Hysterectomy, gauze packing	2	34	2	Caesarean section
5	23	Placental abruption, uterine atony	Hysterectomy	0	28	1	Caesarean section
6	29	Uterine vessel injury, uterine atony	Suture of bleeding sites (re-laparotomy)	0	37	1	Caesarean section
7	26	Placental abruption, uterine atony	Uterine artery embolization	1	38	1	Caesarean section
8	33	Uterine atony	Uterine artery embolization	1	39	1	Instrumental vaginal delivery
9	33	Postpartum haemorrhage (unknown cause)	Gauze packing	0	40	1	Normal vaginal delivery
10	35	Uterine rupture, cervical/vaginal laceration, uterine atony	Internal iliac artery ligation	0	40	1	Instrumental vaginal delivery
11	32	Uterine rupture, cervical/vaginal laceration	Uterine artery embolization, gauze packing	0	39	1	Normal vaginal delivery
12	29	Placenta previa/placenta accreta, uterine inversion	B-Lynch procedure, hysterectomy	0	40	1	Normal vaginal delivery
13	34	Thrombasthenia	Gauze packing	0	36	1	Caesarean section
14	33	Eclampsia/pregnancy-induced hypertension, uterine atony	Gauze packing, haematoma evacuation	0	39	1	Caesarean section
15	27	Uterine atony	Hysterectomy, gauze packing, uterine artery embolization	1	41	1	Normal vaginal delivery
16	43	Acute fatty liver of pregnancy	Uterine artery embolization, gauze packing	1	36	1	Caesarean section
17	38	Uterine rupture, cervical/vaginal laceration, uterine atony	Hysterectomy, gauze packing	1	37	1	Normal vaginal delivery
18	29	Uterine atony	Hysterectomy, gauze packing	0	40	1	Instrumental vaginal delivery
19	39	Uterine vessel injury	Uterine artery embolization, hysterectomy	1	39	1	Caesarean section
20	32	Amniotic fluid embolism, eclampsia/ pregnancy-induced hypertension	Gauze packing	0	36	2	Caesarean section
21	33	Placental abruption	None	1	28	1	Caesarean section
22	39	Uterine atony	Uterine artery embolization	1	30	1	Normal vaginal delivery
23	40	Uterine rupture, cervical/vaginal laceration, uterine atony	Uterine artery embolization, gauze packing	3	40	1	Normal vaginal delivery
24	33	Placenta previa/placenta accreta	Hysterectomy	2	38	1	Caesarean section
25	36	Uterine atony	Uterine artery embolization, gauze packing	1	39	1	Normal vaginal delivery

Table 2 Effect of rFVIIa, estimated blood loss, and required blood products

Patient no.	No. of doses	Initial response	Final response	Before rFVIIa				Within 24 h after initial rFVIIa				Total blood loss (mL)
				Blood loss (mL)	RBC (U)	FFP (mL)	PC (U)	Blood loss (mL)	RBC (U)	FFP (mL)	PC (U)	
1	1	Stopped	Stopped	14,678	62	5,120	60	2,270	8	1,280	0	16,948
2	1	Decreased	Decreased	7,130	4	840	30	2,077	4	800	20	9,207
3	2	Decreased	Decreased	49,860	126	7,200	155	5,800	12	1,200	25	55,660
4	1	Decreased	Decreased	30,534	94	4,160	75	1,412	8	480	0	31,946
5	1	Stopped	Stopped	6,520	28	3,840	15	85	8	0	10	6,605
6	2	Decreased	Decreased	4,848	12	960	0	1,890	12	1,200	40	6,738
7	1	Stopped	Stopped	4,537	6	160	0	1,891	20	1,920	20	6,428
8	2	Decreased	Stopped	4,400	14	0	0	965	4	960	0	5,365
9	1	Stopped	Stopped	3,304	4	480	0	768	8	960	0	4,072
10	1	Stopped	Stopped	2,034	10	1,080	0	164	2	0	0	2,198
11	1	Decreased	Decreased	5,990	10	1,920	10	1,010	12	3,840	20	7,000
12	5	Unchanged	Stopped	18,000	70	5,520	20	11,147	40	3,600	20	42,650
13	3	Unchanged	Stopped	2,500	4	0	90	150	0	0	20	2,650
14	4	Decreased	Stopped	3,600	24	2,160	40	900	4	480	0	4,600
15	2	Unchanged	Stopped	7,230	42	3,120	20	1,649	12	2,400	20	14,720
16	2	Unchanged	Decreased	13,595	49	15,720	80	5,156	20	4,440	40	18,751
17	1	Stopped	Stopped	19,642	58	5,760	60	2,008	10	3,840	20	21,650
18	1	Decreased	Decreased	9,357	30	5,280	60	628	18	1,680	20	9,985
19	1	Decreased	Decreased	12,706	25	960	30	1,032	8	0	0	13,738
20	2	Stopped	Stopped	2,582	22	9,000	0	1,121	8	1,200	0	11,943
21	1	Stopped	Stopped	5,800	22	3,360	40	750	6	1,440	0	6,550
22	4	Unchanged	Stopped	14,533	70	7,440	13	2,480	37	3,000	10	18,788
23	1	Unchanged	Unchanged	5,576	12	1,800	10	4,000	6	2,400	20	9,576
24	2	Decreased	Stopped	11,850	26	2,400	0	600	6	0	20	12,450
25	3	Unchanged	Stopped	16,700	12	480	0	1,260	21	2,880	20	17,210
Median	1	–	–	7,130	24	2,400	20	1,260	8	1,200	20	9,985
Min	1	–	–	2,034	4	0	0	85	0	0	0	2,198
Max	5	–	–	49,860	126	15,720	155	11,147	40	4,440	40	55,660

reduction in blood loss between before administration and the first/final administration was observed ($P < 0.0001$, respectively). The median blood loss before administration was 6,520 mL in 13 women who only received a single dose and 9,540 mL in 12 women who received two or more doses.

Use of blood products before rFVIIa administration and 24 h after the first administration is shown in Table 3. A reduction in the requirement of blood products was observed following the first rFVIIa administration.

Regarding haematologic parameters before rFVIIa administration, the median fibrinogen level was 144 mg/dL (range 50–422 mg/dL) in total. The median platelet level was 5.6 × 10^9/L (range 0.8–20.8 × 10^9/L) in total. The patients whose bleeding was "stopped" with a single dose showed higher fibrinogen and platelet levels than those whose bleeding was "decreased" in a single dose, although no significant differences were observed between the

Table 3 Blood products requirement in the 24 h before and after rFVIIa administration (median and range)

	RBC (U)	FFP (U)	PC (U)
Before rFVIIa	24 (4–126)	2,400 (0–15,720)	20 (0–155)
After rFVIIa	8 (0–40)	1,200 (0–4,440)	20 (0–40)
P value*	<0.0001	0.0034	0.0290

* *P* values are calculated using Wilcoxon signed rank sum test

Table 4 Fibrinogen and Platelet level by an effect on bleeding at an initial administration of rFVIIa (median and range)

rFVIIa effect on bleeding	Fibrinogen (mg/dL)	Platelet (10^9/L)
Stopped ($n = 8$)	144.5 (50–209)	8.5 (3.2–18.8)
Decreased ($n = 10$)	122 (50–422)	5.4 (1.9–10.4)
P value*	0.8938	0.2288

* *P* values are calculated using Wilcoxon signed rank sum test

"stopped" and "decreased" groups (Table 4). Similarly, the effect of rFVIIa on bleeding at its first administration did not differ significantly between the groups of fibrinogen of <100 mg/dL and ≥100 mg/dL. Arterial pH at the time of rFVIIa administration was lowest in the patients whose bleeding was "unchanged" (median 7.26, range 7.05–7.50, $n = 4$) and highest in the "stopped" group (median 7.40, range 7.24–7.46, $n = 6$).

Hysterectomy was required in 13 patients (52%). Two patients (15.4%) underwent hysterectomy after rFVIIa administration. Nine patients (36%) experienced 11 adverse events within 28 days of last rFVIIa administration. These were exanthem ($n = 1$), fever ($n = 1$), hypopituitarism ($n = 1$), AMI ($n = 1$), ileus ($n = 1$), asymptomatic DVT ($n = 2$), asymptomatic PE ($n = 2$), and allergic reaction ($n = 2$). The case histories for the patients with DVT or PE are detailed below.

A 34-year-old woman with placenta previa was treated for massive bleeding following an emergency Caesarean delivery (case 3). She received 126 U of RBCs, 90 U of FFP, and 155 U of PC for 3 days after the delivery, but bleeding continued, and total blood loss reached 55,660 mL. Bleeding stopped following the administration of two doses of rFVIIa (4.8 mg at each administration) combined with 1 g of tranexamic acid every 6 h. Asymptomatic iliofemoral DVTs were detected in both legs by ultrasonography. The administration of rFVIIa was considered to be related to thrombus formation. She successfully recovered and was discharged home.

Another patient with thrombotic event was a 32-year-old woman who received rFVIIa for bleeding due to uterine rupture (case 11). She had a disturbance of consciousness after placental delivery and developed cardiac arrest when transferred to a major hospital for intensive care. After an improvement in her consciousness level was observed, she received a single dose of rFVIIa (4.8 mg) and uterine artery embolization to treat bleeding, resulting in a decrease of bleeding. CT indicated asymptomatic pulmonary embolism and asymptomatic DVT in the right external iliac vein. The role of rFVIIa in thrombus formation was judged as "relationship is undeniable". She also successfully recovered and was discharged home.

A 33-year-old woman was treated for bleeding following emergency Caesarean delivery (case 14). Massive bleeding was observed in the abdominal wall. Bleeding stopped after surgical interventions and administration of four doses of rFVIIa (4.8 mg at each administration) combined with tranexamic acid. Development of a small pulmonary embolus thrombus was suspected on CT 2 days after the last administration of rFVIIa. The role of rFVIIa in thrombus formation was judged as "relationship is undeniable".

One patient died due to hypoxia-induced cerebral oedema developing before rFVIIa administration, but no causal relationship was attributed to rFVIIa. The other 24 patients recovered from haemorrhage and its complications and were finally discharged from hospitals.

Discussion

Although certain risk factors for obstetric haemorrhage can be identified in the antenatal period [15], for the most part, haemorrhage occurs unpredictably and suddenly. Once it occurs, it can progress rapidly and may cause serious complications leading to death. Early control of bleeding and correction of the complications are essential to achieve a better patient outcome. As a haemostatic agent, rFVIIa is widely used to manage massive haemorrhage in non-haemophiliac patients as an off-label use [16–19]. Since the action of rFVIIa is limited to the site of tissue injury and tissue factor exposure, administration of rFVIIa is considered to be particularly useful in an obstetric setting where there is often bleeding from a large raw area of exposed tissue [20]. Despite numerous reports demonstrating successful off-label use of rFVIIa in obstetric haemorrhage, to the best of our knowledge, this is the first case series in Japan to examine the use of rFVIIa as a haemostatic agent in the management of Japanese women with peripartum haemorrhage.

In this case series, three patients (12%) experienced thrombotic adverse events. The incidence in the present study is relatively higher than in major case series [21, 22], though a larger sample size is necessary to assess the safety profile more precisely. All thrombotic adverse events, however, were asymptomatic and were detected by careful examination using CT or ultrasonography after cessation of bleeding. Since tranexamic acid is an antifibrinolytic agent, administration of rFVIIa combined with tranexamic acid might be a risk factor for thrombotic adverse events. Excessive use of tranexamic acid should be avoided. Furthermore, based on the possibility of thrombotic adverse events induced by rFVIIa, its usage should be limited to institutions that can appropriately address these events. In addition, rFVIIa may not be needed in patients with mild to moderate bleeding, since the risk of these events may be greater than the therapeutic benefit. On the other hand, although thrombotic complications are significant concerns, the potential for thrombotic complications must be weighed against the immediate risk of death.

An initial response ("stopped" or "decreased") to rFVIIa was seen in 18 patients (72%), suggesting the benefit of rFVIIa. Only one patient did not show a clinical response at the final administration. The present result is comparable to previously reported response rates that were documented in a number of case series of PPH. Although it should be noted that the effect on bleeding was based on

subjective assessment by a treating clinician, the present results appear to indicate that rFVIIa contributed effectively to bleeding reduction.

In this case series, the blood product requirement was reduced followed by the administration of rFVIIa. Reducing exposure to blood products in the early phase is important since massive transfusion can lead to hypothermia, DIC, excessive fibrinolysis, dilutional coagulopathy, and metabolic acidosis, which may further exacerbate bleeding and morbidity [6]. However, importantly, it should be noted that a reduction in RBC requirement does not always confer a therapeutic effect of a given intervention because adequate red cell replacement to a patient with massive haemorrhage may take time despite an observed decrease in bleeding [23].

To achieve haemostasis of target tissues with rFVIIa, an adequate number of circulating platelets and adequate fibrinogen concentration levels are required [24, 25]. In Japan, use of fibrinogen concentrate and cryoprecipitate is not approved for massive haemorrhage, and FFP is the only measure for replacement of fibrinogen. In this case series, patients with higher fibrinogen and platelet levels seemed to respond better to rFVIIa administration, though there were no significant differences in fibrinogen and platelets level at the first administration of rFVIIa between the "stopped" and "decreased" groups. Since both groups responded to rFVIIa, clarifying an obvious difference in these parameters is difficult. Also, Phillips et al. [14] have pointed out that, in a rapidly evolving environment such as obstetric haemorrhage, physiological conditions change within minutes, and it is probable that tests taken >30 min before the administration of the dose do not accurately reflect the patient's condition in this respect.

Of 25 patients, 13 underwent hysterectomy. Eleven had a hysterectomy before rFVIIa administration. When standard medical therapies and surgical interventions fail to control the bleeding, hysterectomy is usually considered as a last resort to control the bleeding. However, peripartum hysterectomy is a radical procedure that has the undesirable side effects of infertility and physical and psychological trauma [23]. Also, emergency hysterectomy is reported to increase the risk of long-term maternal morbidity [25]. Whether conducting hysterectomy or not should be carefully considered in the perspective of preservation. For most cases, the use of rFVIIa is considered when it becomes absolutely necessary or when all other measures have failed. In fact, most hysterectomy surgeries in this case series were conducted before rFVIIa administration. However, the equilibrium may have shifted to cases where rFVIIa is given to prevent hysterectomy rather than as a desperate measure when nothing else seems to work [22]. We believe that usage of rFVIIa might be considered prior to hysterectomy.

To date, no consensus has been established about the specific timing of rFVIIa administration, though most of the reported use was in cases where all conventional measures were failing. Conceptually, early administration or rFVIIa will reduce blood product requirement and prevent hysterectomy. According to Ahonen et al. [12], rFVIIa should be considered when total blood loss reaches 1.5 times of a patient's total blood volume; however, hesitation to use rFVIIa until severe complications such as multiorgan failure occur will result in failure of haemostasis or even death. From the results in this case series, administration of rFVIIa should be considered in patients with total blood loss of around 5,000 mL when conventional surgical intervention is ineffective and a patient has DIC. Considering the rapid coagulation effect of rFVIIa and its half-life of 3.5 h [26], when no effect on bleeding is observed, the second dose should be administered within 1 h under conditions of maintenance of adequate body temperature, correction of acidosis, and correction of fibrinogen and platelet levels.

There are limitations that need to be acknowledged and addressed regarding the present study. Since there was no control group that was not treated with rFVIIa, interpretation of data must be limited. Also, it is important to note that the patients received multiple therapeutic interventions, which limit the assessment of the effectiveness and safety of rFVIIa. Although a randomized control trial (RCT) is a desirable method to assess the true effectiveness of a given drug, due to ethical issues, it is difficult to perform an RCT involving cases of massive obstetric haemorrhage that needs emergent treatment. We therefore must rely on data collected from case series; however, this type of study has a significant role in establishing a hypothesis that could lead to future better treatment [27].

The present results suggest that rFVIIa is a beneficial therapeutic option that could reduce blood loss and contribute to prevention of hysterectomy in Japanese patients with massive obstetric bleeding. Further assessment is necessary to identify the appropriate dosage and timing of administration to maximize the therapeutic benefit of rFVIIa.

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Conflict of interest Dr. Kobayashi has financial relationships with Novo Nordisk, CSL Behring, and Benesis. Dr. Nakabayashi has a financial relationship with CSL Behring, and he is a committee member of the blood institution of the Japanese Red Cross Society. Dr. Yoshioka has financial relationships with Baxter and Novo Nordisk.

Appendix: Participating physician

Shohei Okada, Kurashiki Medical Center; Yoshikazu Goto, Kanazawa University Hospital; Tomoyoshi Nohira, Hachioji Medical Center of Tokyo Medical University; Nobukazu Sato, Kawasaki Municipal Kawasaki Hospital; Takashi Shoda, Kitasato University Hospital; Jun Murotsuki, Tohoku University Hospital; Satoshi Ohira, Shinshu University Hospital; Tomohisa Sakashita, Hiroshima University Hospital; Seiji Inoue, Okayama University Hospital; Masato Nakayama, Saitama Medical Center; Akitoshi Nakashima, Toyama University Hospital; Rei Sunai & Mayuko Komuro, University of Yamanashi; Kiyonori Miura, Nagasaki University Hospital; Ken Miyazaki, Japanese Red Cross Nagoya Daiichi Hospital; Tsutomu Shichino, National Hospital Organization Kyoto Medical Center; Ryu Matsuoka, Showa University Hospital; Takashi Ozaki, Hirosaki University Hospital; Masayuki Ito, Tottori University Hospital.

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Immunohistochemical detection of meconium in the fetal membrane, placenta and umbilical cord☆

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ABSTRACT

Objective: To develop the immunohistochemistry specific for meconium in the placenta, fetal membrane and umbilical cord.
Study design: We previously reported the specific presence of zinc coproporphyrin I (ZnCP-I) in human meconium and demonstrated the possible diagnostic use of an elevation in maternal plasma ZnCP-I levels in cases of amniotic fluid embolism. In this study, we developed a new specific monoclonal antibody for ZnCP-I and applied it to the immunostaining of meconium in the placenta, fetal membrane, and umbilical cord.
Results: Immunoreactivity of ZnCP-I clearly and specifically identified meconium in the placenta, fetal membrane, and umbilical cord. It was especially useful in cases of severe chorioamnionitis to detect meconium in the macrophages surrounded by numerous neutrophils. In more than half of the cases, meconium was detected in clear amniotic fluid at delivery, suggesting previous exposure.
Conclusions: Immunohistochemical detection of ZnCP-I is a highly sensitive histological diagnosis of meconium.

1. Introduction

Meconium is a bile-stained material present in the small bowel of fetuses long before mid-gestation, and which moves in the intestinal lumen with contractions of the intestinal wall, but is usually eliminated after birth [1]. Some investigators have reported the presence of meconium staining to be associated with an increased incidence of adverse neonatal outcome [2,3]. Others have demonstrated no association between the presence of meconium stain and neonatal levels of arterial pH, carbon dioxide pressure, or base excess [4–6]. Nathan et al. examined a large number of samples and found that the impact of meconium on neonatal morbidity and mortality is rather small and primarily related to meconium aspiration syndrome [2], especially for thick meconium [7]. However, the role of meconium as the primary factor contributing to meconium aspiration syndrome is controversial [8], because autopsy studies have suggested prenatal origins of intrauterine infection and/or chronic hypoxia [8,9]. Thus, the pathophysiological involvement of meconium in neonatal outcome is still contentious. We speculate that the unavailability of universal diagnostic criteria for meconium staining is one of the reasons for the confusion. Indeed, it is difficult to distinguish thick from thin meconium-stained amniotic fluid by gross appearance [10] and the histological detection of meconium is not always reliable, especially using the hematoxylin–eosin (HE) stain [1,11].

The aim of the present study was to develop immunohistochemistry specific for meconium. We previously reported the presence of zinc coproporphyrin I (ZnCP-I) in human meconium [12] and demonstrated the possible diagnostic use of an elevation in maternal plasma ZnCP-I levels in cases of amniotic fluid embolism in Japan [13]. In the present study, we newly developed a specific monoclonal antibody against ZnCP-I and applied it to the

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Table 1
Clinical features of subjects. Values are expressed as the mean ± standard deviation. The gross appearance of amniotic fluid at delivery was classified as clear (−), thin (+), and thick (++). PIH; Pregnancy induced hypertension. FGR; Fetal growth restriction. APS; Apgar score.

Meconium staining	Meconium in amniotic fluid at delivery by gross appearance	
	Clear (−)	Thin (+) or thick (++)
Age	33 ± 5.3	32 ± 5.4
Gestational week	37 ± 0.8	39 ± 1.2
Placental weight (g)*	540 ± 98	574 ± 124
Birth weight (g)	2815 ± 336	3085 ± 463
Intrauterine infection	0	3
PIH	0	3
FGR	0	1
Non-reassuring fetal status	1	9
APS (5 min <7)	0	0

immunostaining of meconium in the placenta, fetal membrane, and umbilical cord, comparing the findings with results of standard screening by HE, Prussian-blue staining, and the gross appearance of amniotic fluid at delivery.

2. Materials and methods

2.1. Subjects

After delivery, all specimens of umbilical cord, fetal membrane and placenta were stored at 4 °C in a refrigerator at Hamamatsu University Hospital between June 2009 and April 2011. The tissues were kept in the dark for avoiding exposure to light [14]. The tissues were then dropped in 10% formaldehyde (0.1 M sodium cacodylate buffer, pH 7.4) at room temperature until used. Two researchers (N.F. and C.Y.) retrospectively selected a total of 78 cases of placentas according to the data on the gross appearance of meconium in amniotic fluid, i.e. clear (−), thin (+), and thick (++) meconium, which was assessed by midwifes at delivery. 50 cases were selected as thin (+) or thick (++) meconium and 28 cases as clear amniotic fluid. Table 1 indicates clinical features of 78 pregnant women, whose placentas were analyzed.

The intestine was obtained at autopsy of a neonate who died one day after birth at 35 weeks of gestation.

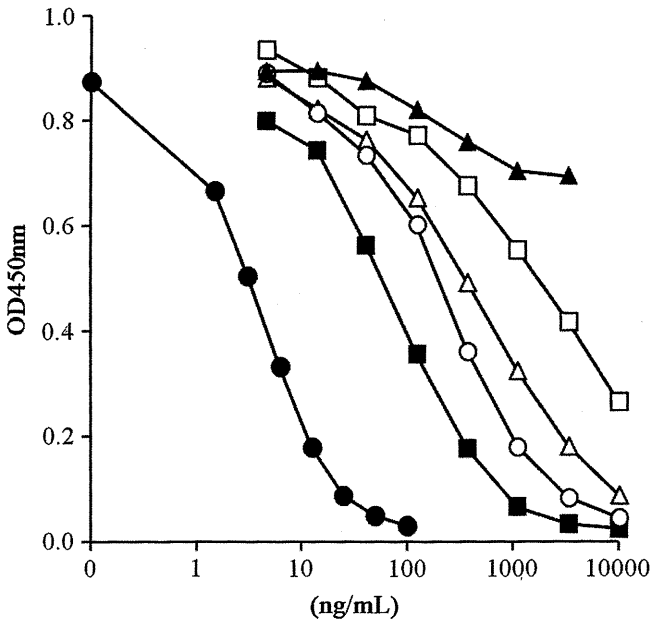

Fig. 1. Cross-reactivity of the monoclonal antibody raised against zinc coproporphyrin I (ZnCP-I) with major porphyrins. Closed circles indicate the reactivity to ZnCP-I. Open and closed triangles indicate coproporphyrin I and protoporphyrin IX, respectively. Open and closed squares indicate coproporphyrin III and uroporphyrin I, respectively. Open circles indicate uroporphyrin III.

Meconium was obtained from a neonate delivered at 38 weeks of gestation, which was expelled approximately 2 min after birth.

2.2. Measurement of ZnCP-I level in the meconium

ZnCP-I level in the meconium was measured as previously described [12]. In brief, 100 mg wet weight meconium was dissolved 1.6 mL distilled water and after

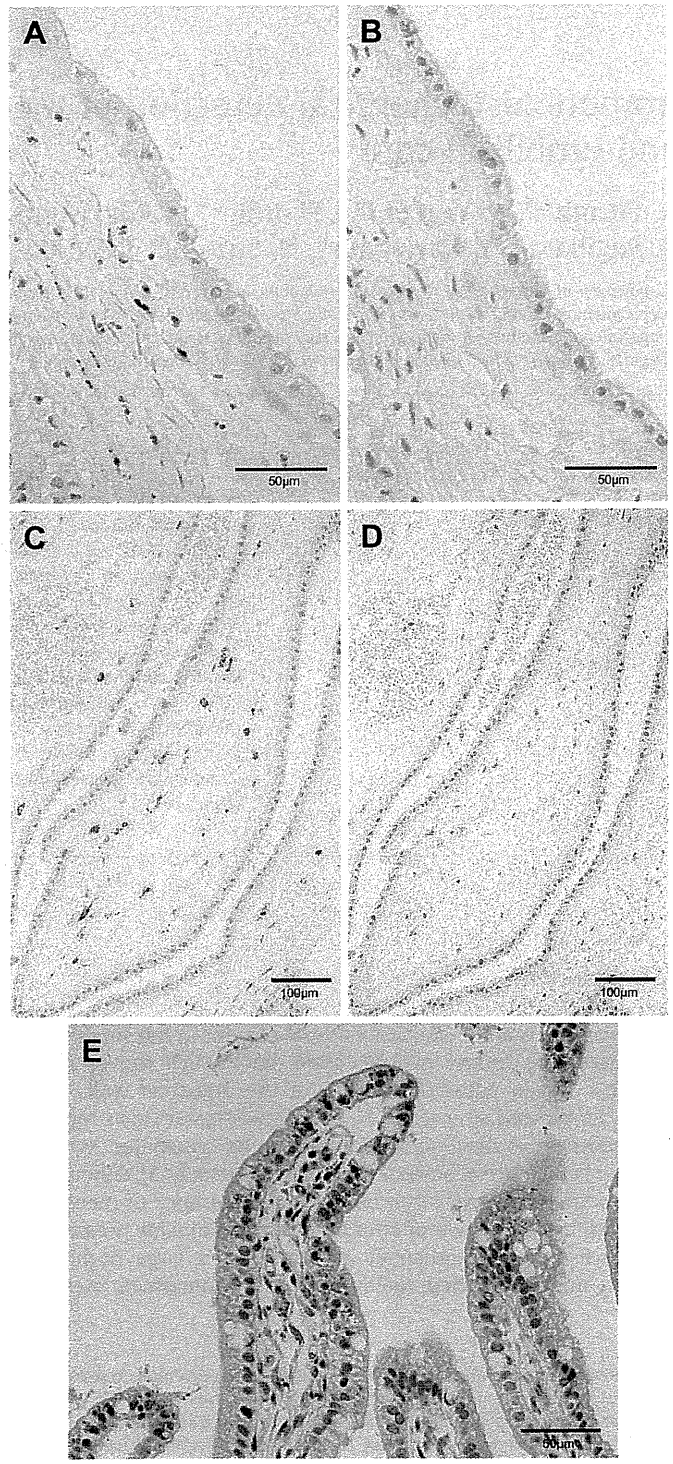

Fig. 2. Immunohistochemical detection of ZnCP-I in term placenta (A, B), term fetal membrane (C, D) and neonatal intestine (E). Brown cytoplasmic granules in macrophages indicate ZnCP-I immunoreactivity (A, C and E), which completely disappeared with the pre-absorption of 0.1 μM ZnCP-I (B) or 15.7 mg wet weight/L meconium containing 0.1 μM ZnCP-I (D). Black bars indicate 50 μm (A, B and E) and 100 μm (C, D).

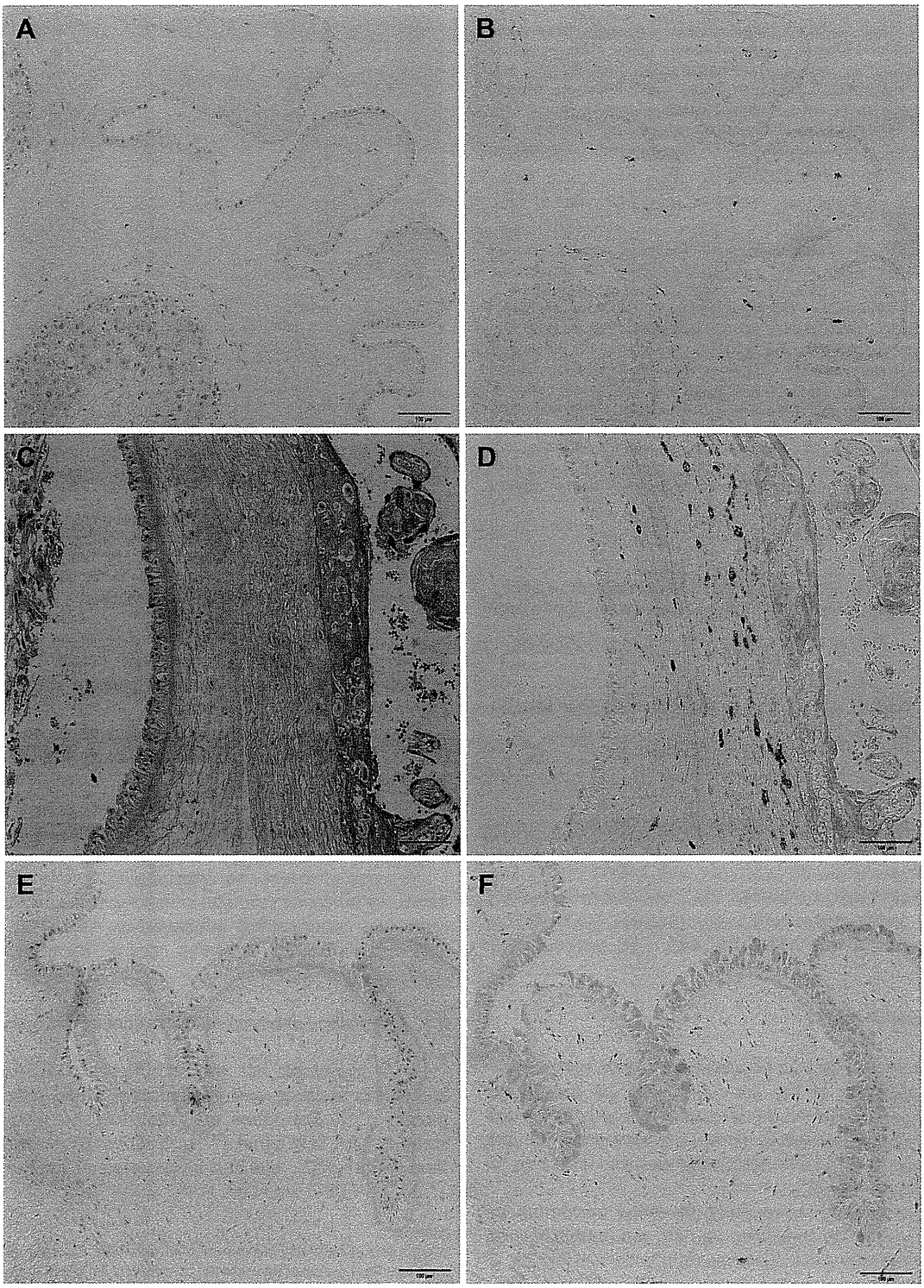

Fig. 3. Comparison of HE staining of (A, C and E) with immunostaining of ZnCP-I (B, D and F) in fetal membrane (A, B), placenta (C, D), and umbilical cord (E, F). Black bars indicate 100 μm.

voltex-mixed for 1 min, centrifuged at 10,000 rpm for 1 min. The supernatant was filtered through a 0.20 μm filter (Millex-LG, Millipore, Carrigtwohill, Ireland). Then, ZnCP-I level was measured by fluorometry after High-performance liquid chromatography (HPLC).

2.3. Preparation of a specific monoclonal antibody against zinc coproporphyrin I (ZnCP-I)

ZnCP-I was synthesized as described previously [12]. Coproporphyrin I, coproporphyrin III, uroporphyrin I, uroporphyrin III, and protoporphyrin IX were purchased from Frontier Scientific Porphyrin Products (Logan, Utah, USA). ZnCP-I was conjugated to keyhole limpet hemocyanin (KLH). After immunization using four BALB/c mice, splenic lymphocytes were fused with mouse myeloma cells (Sp2). This fusion produced in excess of over 100 hybridomas, which were incubated in (HAT) medium and screened by an enzyme-linked immunosorbent assay (ELISA) as follows. ZnCP-I or Porphyrins, horseradish peroxidase (HRP) conjugated ZnCP-I, and monoclonal antibody were added to the microtiter wells (Nunc Brand Products, Roskilde, Denmark) coated with goat anti-mouse IgG (Cappel, West Chester, PA, USA), incubated for 2 h and washed 5 times. Then, 3,3′, 5, 5′-tetramethylbenzidine (TMB) reagent (Dako Japan, Tokyo) was added into the wells and incubated for

30 min, followed by measurement of enzyme activity using a microplate reader set to 450 nm. Five cell lines had positive response and were repeatedly cloned under conditions of limiting dilution. They were injected into the mouse abdomen, which produced tumors secreting an antibody-rich fluid in ascitic fluid. The IgG was purified by Protein A (Sigma Aldrich Japan Co., Tokyo). One cell line was selected because of high affinity for ZnCP-I and low affinity for coproporphyrin I (CP-1). The subclass of IgG was IgG1. The specificity of the antibody against ZnCP-I and it's cross-reactivity with coproporphyrin I, coproporphyrin III, uroporphyrin I, uroporphyrin III, and protoporphyrin IX were examined by ELISA.

2.4. Immunohistochemistry of ZnCP-I

The tissues were fixed in 10% formaldehyde (0.1 M sodium cacodylate buffer, pH 7.4), embedded in paraffin, and cut into 3 μm sections. Then, the antigen was retrieved in a water bath (95 °C for 40 min) and endogenous peroxidase activity was quenched by incubation in methanol with H_2O_2 (3%) for 10 min. A mouse monoclonal anti-ZnCP-I antibody (120 ng/mL) or anti-CD 68 antibody (4× dilution from initial liquid; Dako Japan, Tokyo, Japan) was applied to the sections (1 h and 30 min, respectively, room temperature). Detection was performed with a polymer detection kit (ChemMate EnVision™; Dako Japan, Tokyo, Japan) according to the manufacturer's instructions, followed by a reaction with 3,3′-diaminobenzidine and counterstaining with hematoxylin.

ZnCP-I (0.1 μM) or meconium (15.7 mg wet weight/L including 0.1 μM ZnCP-I) was used to absorb the primary antibodies for the purpose of demonstrating antibody specificity. As positive control sections, tissues obtained in recorded cases of severe meconium staining were used.

2.5. Prussian-blue staining

Prussian-blue staining was performed with an equal parts mixture of ferrocyanide (Sigma Aldrich Japan Co., Tokyo) and hydrochloric acid (Sigma Aldrich Japan Co., Tokyo) for 10 min.

2.6. Approval

The Ethics Committee of the Hamamatsu University School of Medicine approved all the procedures of this study.

3. Result

3.1. Specificity of the monoclonal antibody against ZnCP-I

The antibody showed specific reactivity with ZnCP-I (Fig. 1). The sensitivity of the ELISA was 0.16 ng/mL. The cross-reactivity against coproporphyrin I, coproporphyrin III, uroporphyrin I, uroporphyrin III, or protoporphyrin IX was 0.6%, 0.1%, 4.4%, 1.4%, and less than 0.1%, respectively.

3.2. Immunostaining of ZnCP-I

ZnCP-I immunoreactivity was indicated by brown cytoplasmic granules in macrophages in Fig. 2A and C. Fig. 2B and D are representative images of the pre-absorbance of antibody by 0.1 μM ZnCP-I and 15.7 mg wet weight/L meconium containing 0.1 μM ZnCP-I, respectively. ZnCP-I immunostaining was also indicated in the neonatal intestine (Fig. 2E).

ZnCP-I immunoreactivity successfully identified meconium in the fetal membrane (Fig. 3B), placenta (Fig. 3D), and umbilical cord (Fig. 3F), being apparently clear in comparison with HE (Fig. 3A, C and E).

3.3. Comparison between meconium staining and gross appearance of meconium in amniotic fluid

Meconium staining was classified into three stages; stage 0 (no meconium-stained macrophages), stage 1 (red arrow in Fig. 4; meconium-stained macrophages only in the amnion), and stage 2 (blue arrow in Fig. 4; meconium-stained macrophages in the chorionic plate) based on immunostaining of ZnCP-I. Among 14 cases of thick meconium, 12 cases (85.7%) were diagnosed as stage 2 by immunohistochemical analysis (Table 2). Even in the cases of clear amniotic fluid, considerable numbers of stage 1 (8/28 cases, 28.6%) as well as 2 (9/28 cases, 32.1%) meconium staining was observed (Table 2).

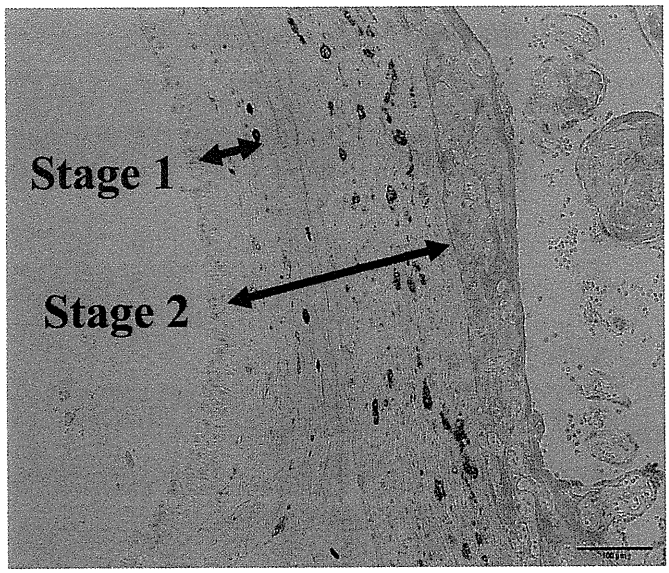

Fig. 4. Classification of meconium staining in the placenta. Meconium staining was classified into three stages, stage 0 (no detection of meconium-stained macrophages), stage 1 (red arrow; meconium-stained macrophages only in the amnion), and stage 2 (blue arrow; meconium-stained macrophages in the chorionic plate) based on immunostaining of ZnCP-I. Black bar indicates 100 μm.

Clear immunostaining was observed in the umbilical cord (Fig. 3F); however, we could not classify the pattern because of heterogeneity.

3.4. Immunostaining of ZnCP-I in chorioamnionitis

In cases of severe chorioamnionitis, it was difficult to identify brown cytoplasmic granules in macrophages by HE staining due to numerous surrounding inflammatory cells (Fig. 5A). Immunostaining of ZnCP-I, however, clearly identified meconium in macrophages (Fig. 5B).

3.5. Comparison between ZnCP-I immunostaining and iron staining

It is important, but rather difficult, to distinguish between meconium staining and degenerated blood products in macrophages [11]. The latter are identified by iron (Prussian-blue) staining [11]. Low panel of Fig. 5 shows HE staining (C), Prussian-blue staining (D), and immunostaining of ZnCP-I (E) in a case of

Table 2
A comparison between meconium staining of the placenta and meconium in the amniotic fluid assessed by midwifes at delivery. Numbers indicate cases diagnosed by immunostaining of ZnCP-I. The gross appearance of amniotic fluid at delivery was classified as clear (−), thin (+), and thick (++). Meconium staining was classified into three stages; stage 0 (no detection of meconium-stained macrophages), stage 1 (meconium-stained macrophages only in the amnion), and stage 2 (meconium-stained macrophages in the chorionic plate) based on using immunostaining of ZnCP-I.

Meconium staining	Meconium in amniotic fluid at delivery by gross appearance		
	Clear (−)	Thin (+)	Thick (++)
Stage 0	11	1	0
Stage 1	8	5	2
Stage 2	9	30	12

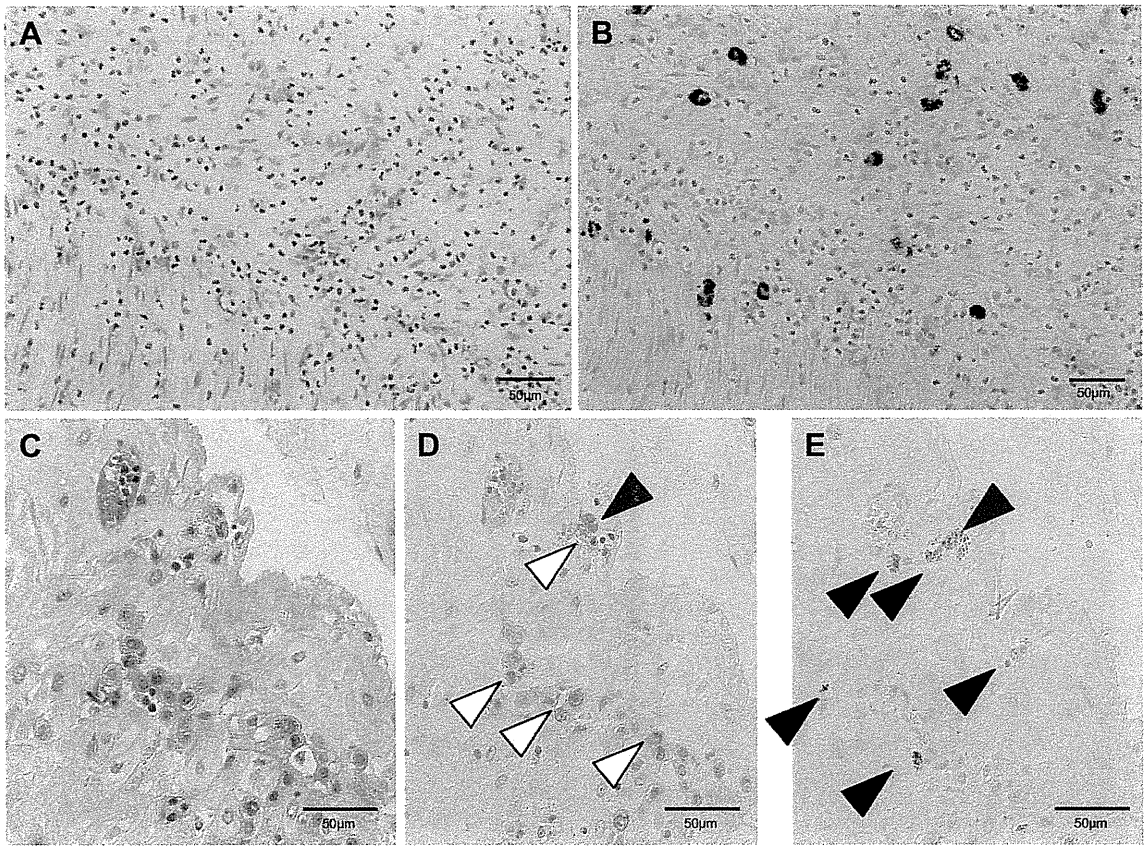

Fig. 5. Assessment of chorioamnionitis (A, B) and subchorionic hematoma with thick meconium (C, D and E). Comparison between HE staining (A) and immunostaining of ZnCP-I (B) in a case of chorioamnionitis. Comparison between HE staining (C) and Prussian-blue staining (D) and immunostaining of ZnCP-I (E) in a case of subchorionic hematoma with thick meconium. White and black arrowheads indicate hemosiderine and ZnCP-I, respectively. The red arrowheads suggest macrophages with both hemosiderine and ZnCP-I. Black bars indicate 50 μm.

subchorionic hematoma with thick meconium. The immunostaining of ZnCP-I (black arrowheads, Fig. 5E) was clearly distinguishable from Prussian-blue positive macrophages with hemosiderine (white arrowheads, Fig. 5D). Interestingly, some macrophages showed both staining with Prussian blue and immunostaining of ZnCP-I (red arrowheads, Fig. 5D and E).

3.6. Comparison between immunostaining of CD68 and macrophage-specific ZnCP-I

Some CD68-positive macrophages (Fig. 6A) showed positive immunostaining of ZnCP-I (Fig. 6B). While others (Fig. 6C) showed negative immunostaining of ZnCP-I (Fig. 6D).

4. Comment

By developing a monoclonal antibody against ZnCP-I, we have established an immunohistochemical means of detecting meconium, as an objective diagnostic marker. The almost complete disappearance of brownish pigmentation on absorbance of the antibody with 0.1 μM ZnCP-I (Fig. 2A and B) as well as meconium (Fig. 2C and D) supports the specificity of the method. Indeed, positive immunostaining was also observed in the neonatal intestine (Fig. 2E).

Meconium has been diagnosed based on brown cytoplasmic granules in macrophages by HE staining of the placenta and fetal membrane obtained at birth [11]. However, degenerated blood products that diffuse out of hematomas within or adjacent to the placenta often show similar such granules in macrophages [11]. Although Prussian blue stains hemosiderine in degenerated blood products, the diagnosis is based on the exclusion of meconium and not direct proof of the presence of meconium in macrophages. Indeed, Fig. 5 indicates that most macrophages with Prussian-blue staining (white arrowheads) showed no immunostaining of ZnCP-I (black arrowheads). Thus, immunostaining of ZnCP-I can avoid a misdiagnosis of hemosiderine deposition. Interestingly, some macrophages exhibited both Prussian-blue staining and immunostaining of ZnCP-I (red arrowheads, Fig. 5D and E), suggesting that they phagocytosed degenerated blood products as well as meconium. Moreover, some macrophages were ZnCP-I positive (Fig. 6A and B), while others negative (Fig. 6C and D), which denies the possibility of non-specific staining of vacuolated macrophages.

Pigmentation by bile is a potential marker of meconium; however, the presence of bile is not readily confirmed histologically [1]. Luna–Ishak stain [15] turns bile a greenish color and may be helpful to confirm pigmentation. Nevertheless, it was reported that brown cytoplasmic granules in macrophages in the fetal membrane sometimes show no staining with either both Prussian-blue or Luna–Ishak stain [1]. Also, Luna–Ishak staining is not easily performed as a part of routine diagnostic testing. Thus, a conventional histological methodology for staining meconium has not been established, and the reported prevalence of meconium staining ranges from 7 to 25 percent [16]. The present study showed that immunostaining of ZnCP-I specifically detects meconium and is promising for histological diagnosis.

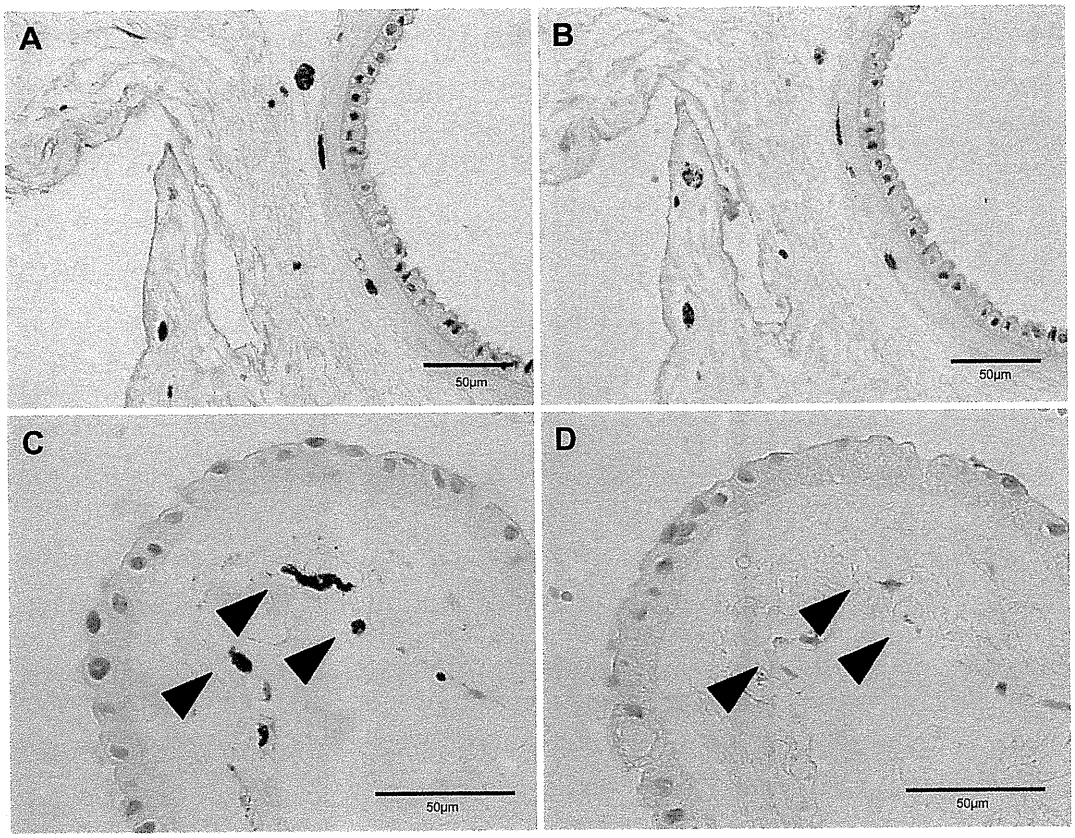

Fig. 6. Comparison between immunostaining of macrophage-specific CD68 (A, C) and ZnCP-I (B, D) in two different sections from a placenta (A v.s. B; C v.s. D). Black arrows indicate macrophages with negative immunostaining of ZnCP-I. Black bars indicate 50 μm.

Kraus et al. described chorioamnionitis to be associated with neonatal morbidity when it accompanies meconium staining [11]. Eidelman et al. demonstrated that the growth of group B streptococcus is enhanced in the presence of meconium [17]. Thus, chorioamnionitis seems to play a substantial pathological role in association with meconium, although there is a considerable evidence that meconium does not cause chorioamnionitis [11,18]. However, in cases of chorioamnionitis, the numerous neutrophils make it difficult to identify pigmented macrophages by standard screening of HE stain (Fig. 5A). Nevertheless, immunostaining of ZnCP-I can easily detect meconium phagocytosed by macrophages (Fig. 5B). Thus, immunostaining of ZnCP-I may provide a clue as to any possible pathological association between chorioamnionitis and meconium staining.

Miller et al. estimated that meconium reached the amnion and chorion within 1 and 3 h exposure, respectively [19]. Fujikura et al. estimated that it took 4–6 h for meconium to reach the chorion [20]. However, it would be difficult to make these observations prospectively *in vivo*. In the present study, using placental tissue, we classified meconium staining into stage 0 (no meconium-stained macrophages), stage 1 (meconium-stained macrophages only in the amnion, red arrow in Fig. 4), and stage 2 (meconium-stained macrophages in the chorionic plate, blue arrow in Fig. 4) based on the immunostaining of ZnCP-I. Then, we compared the different stages with the gross appearance of meconium in the amniotic fluid (Table 2). In clear amniotic fluid, by gross appearance, stage 1 (8/28 cases, 28.6%) as well as 2 (9/28 cases, 32.1%) meconium staining was observed (Table 2). This is the first evidence of a possible previous exposure to meconium in more than half of cases of term delivery with clear amniotic fluid at the time of delivery. Most cases of thin (30/36 cases, 83.3%) and thick (12/14 cases, 85.7%) meconium in amniotic fluid showed stage 2 staining (Table 2). It is yet to be clarified if the transient expelling of meconium long before delivery is physiological, pathological or true.

Recently, Incerti et al. reported histological analysis of the timing of meconium passage during labor [21]. It is the next aim of the study to investigate a possible association between quantitative analysis of meconium staining and clinical findings, such as duration of labor and presence of infection.

Falciglia et al. reported a case of meconium aspiration syndrome in a newborn weighing 610 g, as confirmed by the detection of pigmented materials in the lung autopsy [22]. Preliminary study showed positive ZnCP-I immunostaining in the lung of newborns suffering from meconium aspiration syndrome (Furuta N and Yaguchi C unpublished findings).

In conclusion, we here reported the specific immunohistochemical detection of meconium using a monoclonal antibody against ZnCP-I, as a potential diagnostic tool.

Acknowledgments

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