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Comparison of Angiogenic, Cytoprotective, and Immunosuppressive Properties of Human Amnion- and Chorion-Derived Mesenchymal Stem Cells

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Abstract

Although mesenchymal stem cells (MSCs) can be obtained from the fetal membrane (FM), little information is available regarding biological differences in MSCs derived from different layers of the FM or their therapeutic potential. Isolated MSCs from both amnion and chorion layers of FM showed similar morphological appearance, multipotency, and cell-surface antigen expression. Conditioned media obtained from amnion- and chorion-derived MSCs inhibited cell death caused by serum starvation or hypoxia in endothelial cells and cardiomyocytes. Amnion and chorion MSCs secreted significant amounts of angiogenic factors including HGF, IGF-1, VEGF, and bFGF, although differences in the cellular expression profile of these soluble factors were observed. Transplantation of human amnion or chorion MSCs significantly increased blood flow and capillary density in a murine hindlimb ischemia model. In addition, compared to human chorion MSCs, human amnion MSCs markedly reduced T-lymphocyte proliferation with the enhanced secretion of PGE2, and improved the pathological situation of a mouse model of acute graft-versus-host disease. Our results highlight that human amnion- and chorion-derived MSCs, which showed differences in their soluble factor secretion and angiogenic/immuno-suppressive function, could be ideal cell sources for regenerative medicine.

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Introduction

Mesenchymal stem cells (MSCs) residing within various tissues, including bone marrow [1] and adipose tissue [2], are reported to differentiate into various types of cells including osteoblasts, chondrocytes, and adipocytes. This multipotency renders MSCs an attractive therapeutic source for regenerative medicine. However, because an invasive procedure is required to obtain autologous bone marrow or adipose tissue-derived MSCs, an alternative source of MSCs that can be obtained non-invasively is desirable.

Appendages of the fetus, which consist of the placenta, umbilical cord, and fetal membrane (FM), are normally discarded after delivery as medical waste. A large quantity of MSCs could be obtained without harm from the human FM because of its size (> 40×40 cm), which represents an advantageous characteristic as a source of cell therapy. We have previously reported the therapeutic potential of rat FM-derived MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Although the FM is composed of the amnion and chorion, and both layers contain MSCs [9], it is technically difficult to separate these membranes as well as their MSCs in rat.

Thus, the purposes of this study were: 1) to isolate and characterize MSCs from human amnion and chorion; 2) to examine their differences in the expression profile of growth factors and cytokines; and 3) to investigate the therapeutic potential and difference of these MSCs using murine hindlimb ischemia and acute graft-versus-host disease (GVHD) models.

Materials and Methods

Ethics Statement

The study protocol and informed consent procedure were approved by the ethics committee of the National Cerebral and

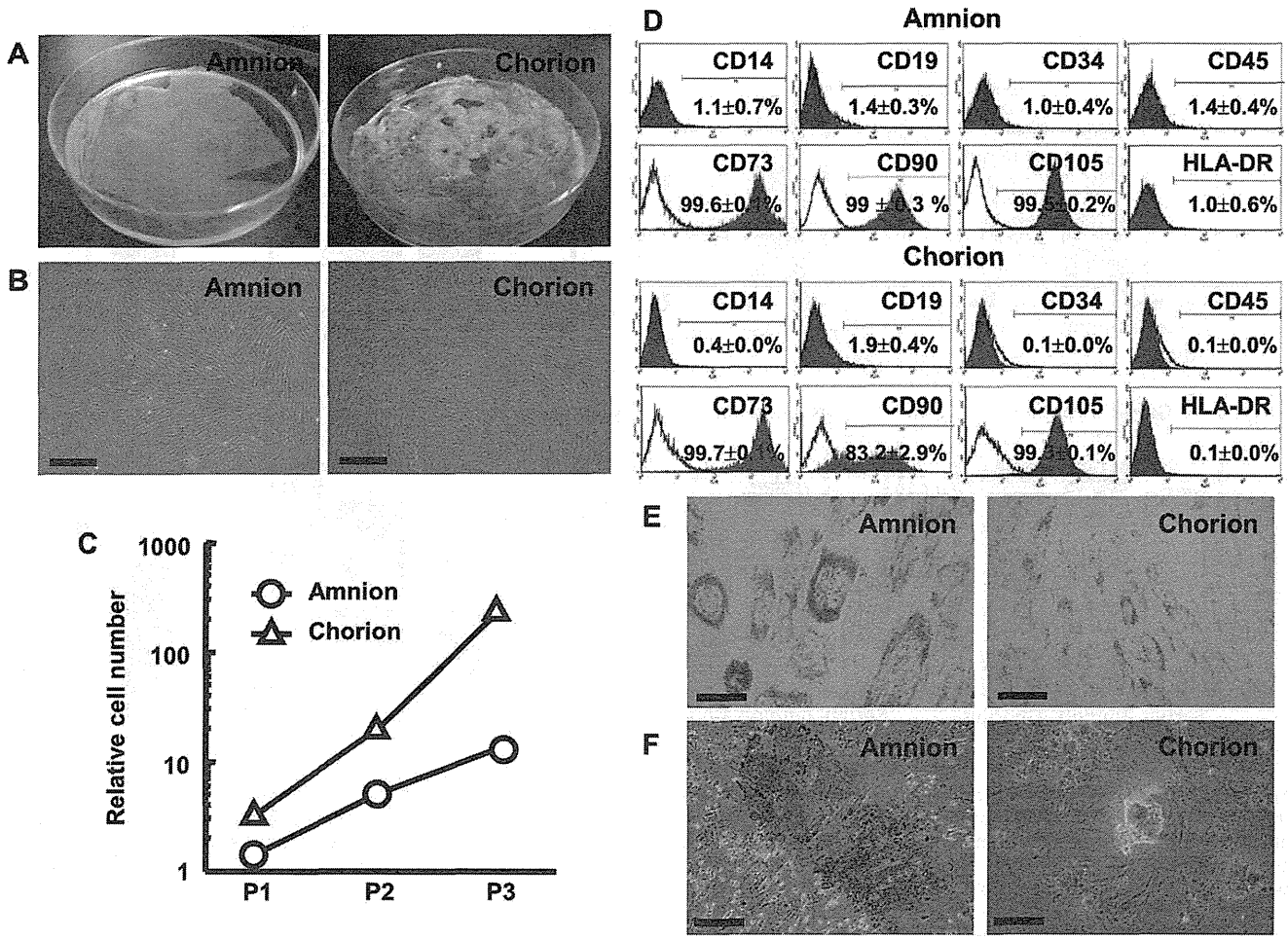

Figure 1. Characterization of human amnion- and chorion-derived MSCs. (A) Representative photographs of human amnion and chorion. (B) Photographs of cultured MSCs obtained from human amnion and chorion at passage 3. Scale bars = 500 μm. (C) Relative cell number of amnion- and chorion-derived MSCs at each passage. (D) FACS analysis of amnion and chorion MSCs. (E, F) Differentiation of amnion and chorion MSCs into adipocytes (E) and osteocytes (F). Scale bars = 100 (E) and 50 (F) μm.
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Cardiovascular Center (Permit Number: M18-042-4). Animal protocols were approved by the Animal Care Committee of the National Cerebral and Cardiovascular Center Research Institute (Permit Number: 13052). Animal studies were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

Isolation and Expansion of Amnion- and Chorion-derived MSCs from Human FMs

After obtaining written informed consent, FMs were obtained following cesarean section of healthy donor mothers. Amnion and chorion were separated by mechanical peeling of the FM, and digested with type-II collagenase solution (5 ml/g tissue and 300 U collagenase/mL, Worthington Biochemicals, Lakewood, NJ) for 1 h at 37°C in a waterbath shaker. After filtration with a mesh filter, cells were suspended in α-minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen), and incubated at 37°C with 5% CO_2 after plating on a dish. The adherent, spindle-shaped MSCs developed visible symmetric colonies by days 1 to 2.

Characterization of Human Amnion and Chorion MSCs

For defining FM-MSCs, we referred to the criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [10].

Cultured MSCs were analyzed by FACSCalibur (BD Biosciences). Cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal against human CD14 (clone M5E2), CD19 (clone HIB19), CD34 (clone 581), CD45 (clone HI30), CD73 (clone AD2), CD90 (clone 5E10), CD105 (clone 266), or HLA-DR (clone G46-6 (L243)), all purchased from BD Biosciences. Isotype identical antibodies served as controls.

To induce differentiation into osteocytes, MSCs were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions. After 14–17 days of differentiation, cells were fixed and stained with Alizarin Red S (Sigma-Aldrich, St. Louis, MO).

To induce adipocyte differentiation, MSCs were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methyl-

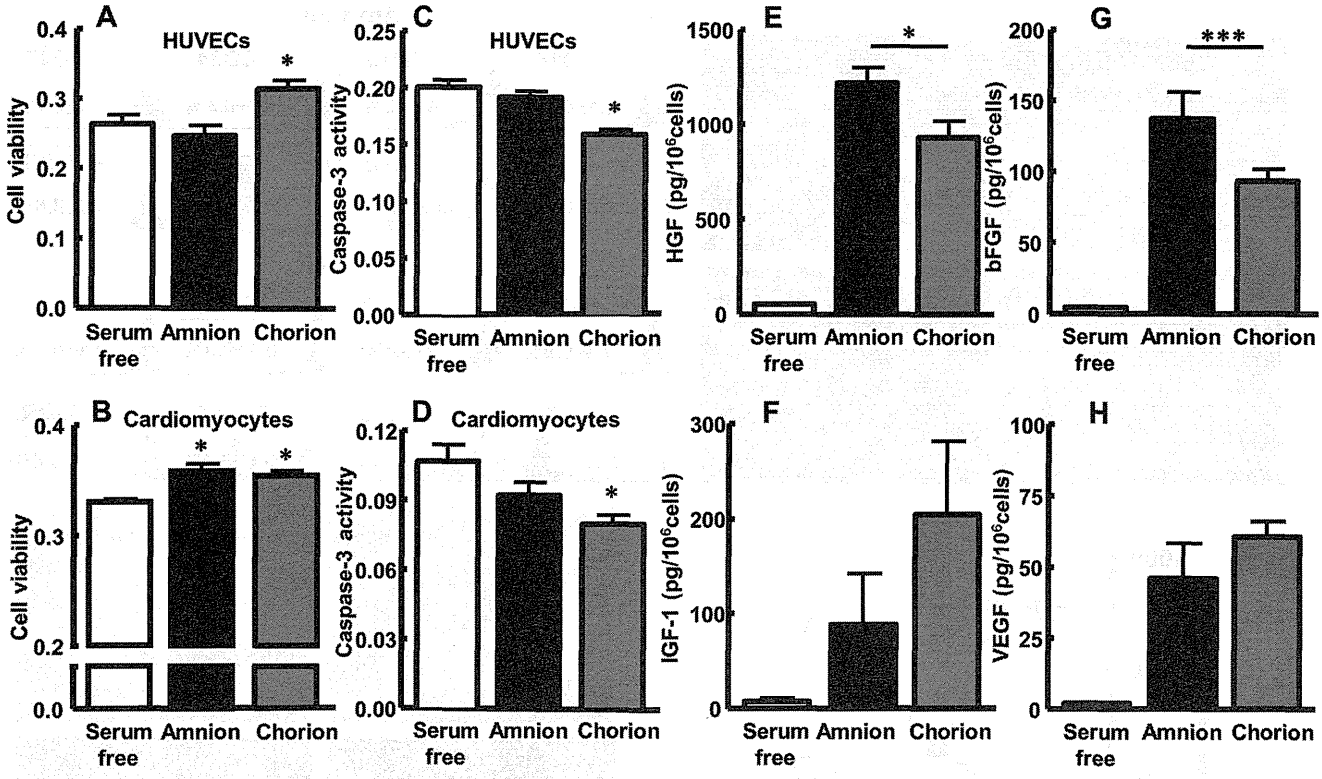

Figure 2. Growth factor secretion and the cytoprotective effect of amnion and chorion MSCs. (A–D) Cytoprotective effect of FM MSC-derived conditioned medium was analyzed by the MTS assay (A, B) and caspase-3 activity (C, D) in HUVECs (A, C) and cardiomyocytes (B, D). Values are mean ± SEM. *$p<0.05$ vs. serum-free. (E–H) Conditioned medium obtained from FM-derived MSCs was collected after incubation for 24 h. The concentration of HGF (E), IGF-1 (F), bFGF (G), and VEGF (H) in serum free conditioned medium was measured by ELISA. *$p<0.05$ and ***$p<0.001$.
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xanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako), 50 μM indomethacin (Wako), and 10 μg/mL insulin (Sigma-Aldrich) in α-MEM supplemented with 10% FCS. After 21 days of differentiation, adipocytes were stained with Oil Red O (Sigma-Aldrich).

Conditioned Medium Analysis of FM-MSC-associated Cytoprotective Function

Human umbilical vascular endothelial cells (HUVECs; Lonza, Basel, Switzerland) were seeded onto a collagen-coated plate and incubated in medium 199 (Invitrogen) supplemented with 20% FCS for 24 h. Neonatal rat cardiomyocytes were isolated from Lewis rats on postnatal day 1, as described previously [11], and seeded onto a laminin-coated plate followed by incubation in α-MEM supplemented with 10% FCS for 24 h. Cells were then subjected to serum deprivation with/without hypoxia (1% O_2) by culturing with serum-free medium or serum-free conditioned medium obtained from FM-MSCs cultured for 24 h. The cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium (MTS), indicative of cell viability, as well as caspase-3 activity, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI) and a CaspACE™ Assay System Kit (Promega), according to the manufacturer's instructions.

Analysis of FM-MSC Production of Growth Factors and Prostaglandin E2

Conditioned media were collected from MSCs cultured in α-MEM with/without 10% FCS for 24 h ($n=4–6$). The concentrations of the following growth factors were measured using ELISA kits: hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE2), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

FM-MSC Transplantation in the Hindlimb Ischemia Model

Six-week-old male KSN nude mice were anesthetized with pentobarbital, and the right common iliac artery was resected. After surgery, amnion MSCs (1×10^6 cells/50 μL PBS), chorion MSCs (1×10^6 cells/50 μL PBS), or PBS (50 μL PBS) was injected into the ischemic muscle with a 30-gauge needle at five different sites ($n=15$ in each group). A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) was used to measure serial hindlimb blood flow for 7 days, as previously described [12].

Five and seven days after MSC transplantation, ischemic hindlimb tissues were obtained and snap-frozen. Frozen tissue sections were stained with anti-mouse CD31 antibody (BD Biosciences) to detect capillary endothelial cells. Ten fields were randomly selected to count the number of capillaries. The adjusted capillary number per muscle fiber was used to compare the differences in capillary density between the three groups.

In vitro CD4+ T cell Proliferation Assay

Peripheral blood mononuclear cells were prepared from buffy coats obtained from healthy donors by centrifugation through Ficoll-Paque (GE healthcare, Uppsala, Sweden). CD4+ T cells were isolated by magnetic bead depletion of CD8, CD14+,

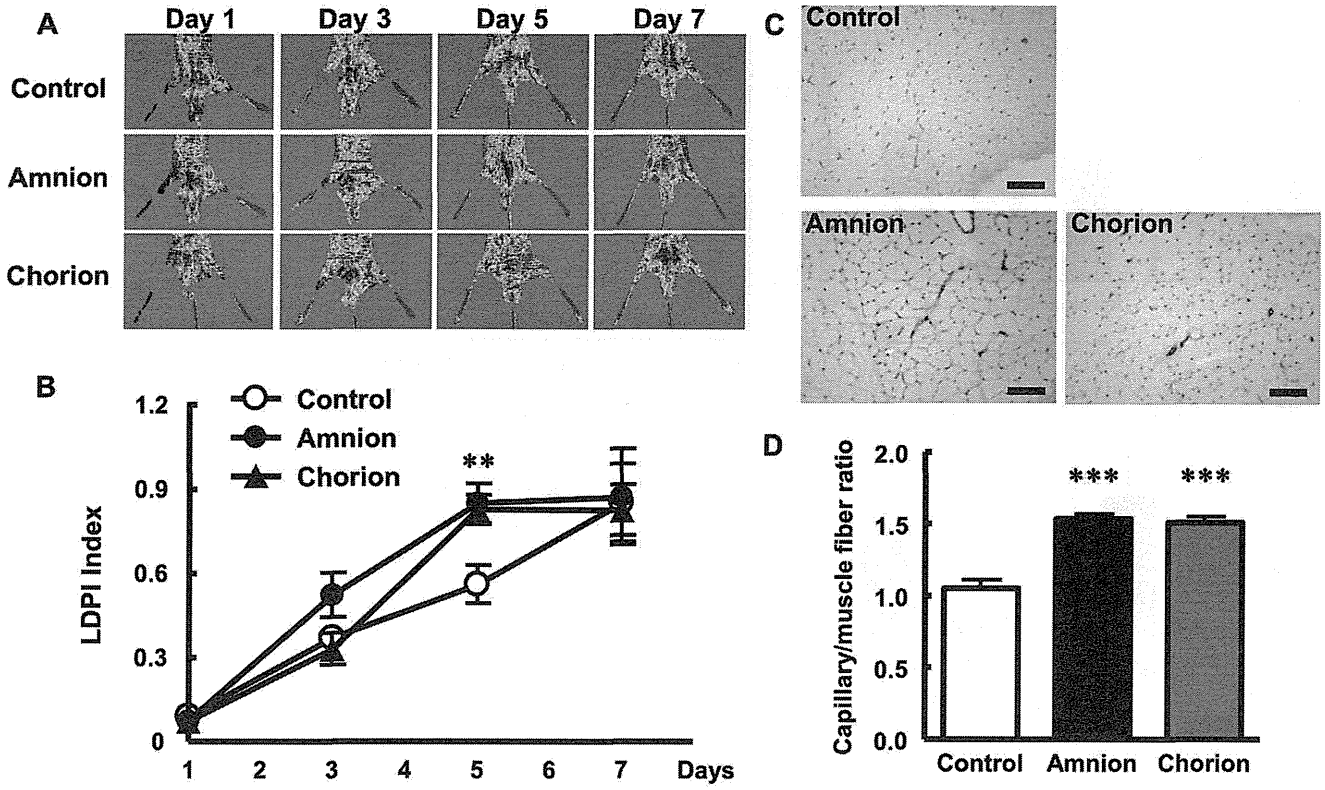

Figure 3. Angiogenic potential of amnion and chorion MSCs against hindlimb ischemia. (A) Representative images of serial hindlimb blood perfusion. Blood perfusion of ischemic hindlimb increased in the amnion and chorion MSC groups at day 5. (B) Quantitative analysis of hindlimb blood perfusion with the LDPI index, the ratio of ischemic to non-ischemic hindlimb blood perfusion. (C) Representative photographs of immunohistochemistry with anti-CD31 antibody. Scale bars = 100 μm. (D) Quantitative analysis of capillary density in ischemic hindlimb muscle at day 5 among the control, amnion, and chorion MSC groups. Capillary density is shown as the capillary-to-muscle-fiber ratio. Data are mean ± SEM. **p< 0.01 and ***p<0.001 vs. control.
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CD15+, CD16+, CD19+, CD36+, CD56+, CD123+, T cell receptor-gamma/delta, and glycophorin A–positive cells (CD4+ T Cell Isolation Kit) on an AutoMACS instrument (Miltenyi Biotec). CD4+ T cells (5×10^5 cells/well) were cultured with X-VIVO medium (Lonza, Walkersville, MD) containing 2% FBS and 5 μg/ml anti-CD28 antibody (clone CD28.2, BioLegend, San Diego, CA) in anti-CD3-precoated 24-well culture plates (clone OKT3, BioLegend). During in vitro proliferation of CD4+ T cells, human amnion-, chorion-, or bone marrow-derived (Lonza) MSCs were co-cultured at 5×10^4 cells/well. After 5 days of co-culturing, T cells were separated from the monolayer MSCs and counted with an automated cell counter (Countess, Invitrogen).

FM-MSC Transplantation into the Acute GVHD Model

Seven- to eight-week-old female B6C3F1 (recipient; C57BL/6×C3H/He; H-$2^{b/k}$) and BDF1 (donor; C57BL/6×DBA/2; H-$2^{b/d}$) mice were purchased from Japan SLC (Shizuoka, Japan). Recipient mice were lethally irradiated with 15 Gy total body irradiation (TBI; X-ray) split into two doses separated by 2 h. On the following day, donor-derived cells (1×10^7 bone marrow cells and 3×10^7 spleen cells) were suspended in 0.2 mL RPMI-1640 medium (Invitrogen) and transplanted via the tail vein into the post-irradiation recipient mice. On days 14, 17, 21, and 25 after hematopoietic stem cell transplantation, 1×10^5 amnion or chorion MSCs in 0.1 mL RPMI medium were transplanted via the tail vein. In the control group, the same amount of RPMI was infused via the tail vein. The severity of GVHD was evaluated by measuring the body weight of mice.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (S.E.M). Comparisons of parameters for more than three groups were made by one-way analysis of variance (ANOVA) followed by the Newman-Keuls' test. Comparisons of the time-course of the LDPI index were made by two-way ANOVA for repeated measures, followed by Bonferroni tests. A p value <0.05 was considered statistically significant.

Results

Characterization of Amnion and Chorion MSCs

From each human FM, 23.5±3.7 g amnion and 37.6±2.5 g chorion could be separated (n = 5 and n = 3, respectively) (Figure 1A). By enzymatic digestion, over one million cells per gram of the amnion ($1.9\pm0.2\times10^6$/g, n = 5) or chorion ($1.3\pm0.3\times10^7$/g, n = 3) were obtained. At passage 3, cultured cells from both layers were fibroblast-like, spindle-shaped cells, and there was no difference in morphology according to the origin of layers (Figure 1B). Cell-doubling time of amnion MSCs (32.2±1.13 h) was equal to that of chorion MSCs (34.1±1.94 h) (Figure 1C).

Both amnion- and chorion-derived MSCs expressed CD73, CD90, and CD105, but not CD14, CD19, CD34, CD45, or HLA-

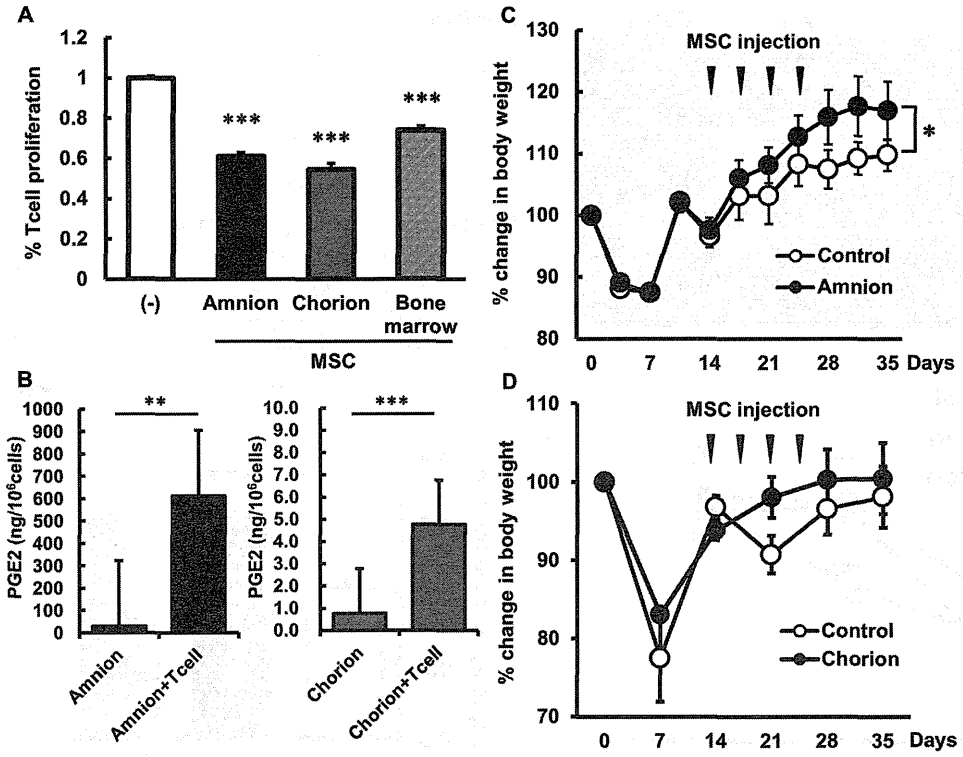

Figure 4. Immunosuppressive property of amnion and chorion MSCs. (A) Inhibition of human CD4+ T cell proliferation upon co-culture with human amnion, chorion, and bone marrow MSCs. (B) The concentration of PGE2 in FM-MSC-conditioned medium was measured by ELISA. Amnion MSCs secreted a significant amount of PGE2 compared with chorion MSCs. (C, D) Effect of human amnion (C) or chorion (D) MSC transplantation in a murine GVHD model. Treatment with amnion MSCs significantly reduced recipient weight loss in a mouse model of GVHD. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

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DR (Figure 1D), which satisfied the criteria for identifying MSCs [10]. In addition, amnion and chorion MSCs could differentiate into adipocytes and osteocytes, as demonstrated by positive Oil Red O and Alizarin Red S staining, respectively (Figure 1E and 1F).

Cytoprotective Effects of Amnion and Chorion MSCs on Endothelial Cells and Cardiomyocytes

To evaluate the cytoprotective effect of amnion and chorion MSCs, we examined cell viability and apoptosis of HUVECs and neonatal rat cardiomyocytes cultured under serum deprivation. In the MTS assay, cell viability of cardiomyocytes was significantly increased when cultured with conditioned medium obtained from amnion and chorion MSCs (absorbance value: serum-free control 0.331 ± 0.002, amnion MSCs 0.359 ± 0.006; $p<0.001$, and chorion MSCs 0.355 ± 0.004; $p<0.01$ vs. control) (Figure 2B). Cell viability of HUVECs also increased when cultured with chorion MSC-derived conditioned medium (serum-free control 0.263 ± 0.013, amnion MSCs 0.247 ± 0.014, and chorion MSCs 0.313 ± 0.012; $p<0.05$ vs. control) (Figure 2A). Similarly, conditioned medium obtained from chorion MSCs significantly decreased the caspase-3 activity of HUVECs (absorbance value: serum-free control 0.201 ± 0.006 vs. chorion MSCs 0.159 ± 0.004; $p<0.001$) and cardiomyocytes (control 0.106 ± 0.007 vs. chorion MSCs 0.079 ± 0.004; $p<0.05$) (Figure 2C, D). Amnion MSC-derived conditioned medium also showed a tendency to decrease the caspase-3 activity of these cells, but without statistical significance.

Secretion of Growth Factors from Cultured Amnion- and Chorion-derived MSCs

To investigate the secretion of major growth factors from MSCs, we performed ELISA of HGF, IGF-1, bFGF, and VEGF. The differences in the cellular expression profile of the growth factors were observed in these FM-derived MSCs (Figure 2E–H). Among these growth factors, amnion MSCs secreted significant amounts of HGF (1217.2 ± 80.2 pg/10^6 cells; $p<0.001$ vs. chorion-MSC) and bFGF (137.2 ± 18.5 pg/10^6 cells; $p<0.05$ vs. chorion-MSC) compared with chorion MSCs (HGF: 932.5 ± 85.3 pg/10^6 cells, bFGF: 93.6 ± 8.1 pg/10^6 cells) (Figure 2E, G). There was no significant difference between amnion and chorion MSCs in the level of secreted IGF-1 (88.8 ± 53.4 pg/10^6 cells and 205 ± 77.0 pg/10^6 cells, respectively) and VEGF (46.1 ± 12.3 pg/10^6 cells and 60.7 ± 5.3 pg/10^6 cells, respectively) (Figure 2F, H).

Augmentation of Angiogenesis in the Ischemic Hindlimb after Human FM-MSC Transplantation

Analysis of LDPI revealed that accelerated limb perfusion was observed in the amnion and chorion MSC-transplanted groups (Figure 3A). The LDPI index was significantly higher in the amnion and chorion MSC groups (amnion MSCs: 0.85 ± 0.07; $p<0.01$, chorion MSCs: 0.83 ± 0.05; $p<0.01$) than in the control group (0.56 ± 0.07) 5 days after transplantation (Figure 3B). At 7 days after transplantation, there was no difference between the treated and control groups.

Immunostaining with the endothelial marker CD31 showed significant augmentation of capillaries in the amnion and chorion

MSC-treated groups compared with the control group (Figure 3E). The capillaries-to-muscle-fiber ratio of ischemic muscle at day 5 after transplantation was significantly increased in the amnion and chorion MSC groups (amnion MSCs: 1.53±0.03/muscle fiber; $p<0.001$, chorion MSCs: 1.51±0.04/muscle fiber; $p<0.001$) compared with the control group (1.05±0.06/muscle fiber; Figure 3F). At day7, the capillaries-to-muscle-fiber ratio of ischemic muscle was also increased in the amnion or chorion MSC-transplanted mice (amnion MSCs: 1.67±0.17/muscle fiber, chorion MSCs: 1.43±0.09/muscle fiber) compared to the control mice (1.36±0.11/muscle fiber).

Immunosuppressive Property of Human FM-MSCs

Although the number of T cells was markedly increased under proliferating conditions of human CD4+ T cells stimulated with anti-CD3 and -CD28 antibodies, the increase was significantly suppressed when co-cultured with amnion-, chorion-, or bone marrow-derived MSCs (61.1±1.8%, 54.6±3.0%, 74.0±2.1%, respectively. $p<0.001$ vs. control) (Figure 4A).

PGE2 is a well-known immune modulator in bone marrow MSCs [13] and we confirmed that amnion MSCs in culture secreted a significant amount of PGE2 (29.7±7.8 ng/10^6 cells), particularly when co-cultured with human CD4+ T cells (613.1±139.9 ng/10^6 cells; $p<0.01$ vs. amnion MSCs) (Figure 4B). In chorion MSCs, however, the concentration of PGE2 was relatively low (0.77±0.13 ng/10^6 cells) but significantly increased in co-culture with CD4+ T cells (4.76±0.47 ng/10^6 cells; $p<0.001$ vs. chorion MSCs). The experiments were repeated with two or three independent MSC/CD4+ T cell donor pairs and the data are presented as the measured mean levels.

In addition, to evaluate the potential of FM-MSCs to suppress acute GVHD, mice underwent allogeneic hematopoietic stem cell transplantation and treatment with human FM-MSCs. As shown in Figure 4C, the loss in body weight of recipient mice after allogeneic hematopoietic stem cell transplantation was significantly reduced with concomitant transplantation of human amnion-derived MSCs. In human chorion MSC-transplanted group, however, no significant changes in body weight was observed during the observation period (Figure 4D).

Discussion

Human MSCs derived from bone marrow or adipose tissue exert a regenerative effect in animal models and human patients [14]. In addition, several reports have described the therapeutic potential of transplanted cells derived from the appendages of the fetus, including amniotic epithelium cells [15], and amniotic fluid- [16], amnion-, and chorion-derived MSCs [17,18]. We have previously demonstrated the therapeutic potential of rat FM-MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Recent studies including ours also revealed the angiogenic and immunosuppressive property of human fetal appendage-derived MSCs [14,18–20], but comparative studies of the therapeutic effects among these MSCs are lacking. Therefore, in this study, we examined the differences in the cellular function and therapeutic properties between human FM-derived amnion and chorion MSCs.

It is known that MSCs exert their regenerative effects through differentiation into specific cell types, but recent studies suggest that their ability to stimulate regenerative effects is mainly induced via paracrine effects [3,4,8,21]. This theory is substantiated by several reports that MSCs secrete various growth factors and cytokines including VEGF, IGF-1, HGF, adrenomedullin (AM), and PGE2 [3–5,8,21,22]. In this study, we first confirmed that chorion MSCs as well as amnion MSCs secreted significant amount of these soluble factors, which would contribute to accelerating regenerative effects. Compared with chorion MSCs, amnion MSCs secreted significantly larger amounts of HGF and bFGF. However, amnion MSCs secreted less IGF-1 compared to chorion MSCs. We assume that these differences in the cytokine expression profile might reflect the angiogenic and cytoprotective properties of amnion and chorion MSCs, as we observed difference in the effect on endothelial cells and cardiomyocytes in our conditioned-medium analysis. However, the actual function of amnion or chorion MSC-derived cytokines should be further investigated *in vivo* because both human amnion and chorion MSC transplantation similarly induced angiogenesis in the hindlimb ischemia model.

Previous reports have shown that PGE2 is a major modulator of the MSC-induced anti-inflammatory response [13]. In this study, a noteworthy finding was a distinctly high concentration of PGE2 in amnion MSCs in comparison with chorion MSCs, particularly when co-cultured with CD4+ T cells. Because of their high PGE2 production, human amnion MSCs might be a better cell source from an immunosuppressive point of view. In fact, we proved for the first time that human amnion MSCs, but not chorion MSCs, improved the pathological situation of an acute GVHD model. Because our previous study demonstrated that human amnion MSCs markedly inhibited differentiation as well as proliferation of Th1/Th17 cells [6], human amnion MSCs could effectively suppress Th1/Th17 immunity and improve outcome in GVHD.

The merit of using FMs lies in that they are free form ethical concern and that a large number of MSCs can be obtained considering the size of FM. As more than one or ten million MSCs per gram of the amnion or chorion could be obtained, more than 10^9 or 10^{10} MSCs could theoretically be obtained at passage 3 within one month, respectively. Now we are planning to initiate clinical studies with human amnion MSCs in acute GVHD and Crohn's disease, and we need more than 10^{10} MSCs for the treatment of one patient. We are convinced that human FM-MSCs are an attractive source for cell therapy because of their easy availability compared with other somatic, embryonic stem, and iPS cells.

In conclusion, both amnion and chorion MSCs have angiogenic, cytoprotective, and immunomodulatory effects. Because of high PGE2 production and immunosuppressive properties, human amnion MSCs have the advantage for the treatment of immune-related diseases. In addition, since a large number of MSCs could be obtained from FMs, human amnion and chorion MSCs would be a useful cell source for regenerative medicine.

Author Contributions

Conceived and designed the experiments: KY AT TS HO JY MHS KK TI. Performed the experiments: KY KH MO SI SO HT KO SK JY TI. Analyzed the data: KY KH MO TI. Contributed reagents/materials/analysis tools: KY KH MO TI. Wrote the paper: KY KH MO TI.

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MATERNAL-FETAL MEDICINE

The effects of antenatal corticosteroids therapy on very preterm infants after chorioamnionitis

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Abstract
Purpose To evaluate the effectiveness of antenatal corticosteroids (AC) therapy on outcomes of very low birthweight infants with histologic chorioamnionitis.
Methods We performed a retrospective analysis of 10,935 single infants born at a gestational age between 22 + 0 and 33 + 6 weeks and birth weight <1,500 g. Clinical data were obtained from the Neonatal Research Network that included the tertiary neonatal intensive care units throughout Japan between 2003 and 2008.
Results Data of 7,896 infants were available for the period 2003–2008 and were included in the analysis. According to logistic regression analysis, AC were significantly associated with reduced mortality [odds ratio (OR) = 0.50; $p < 0.001$], lower incidence of respiratory distress syndrome (OR = 0.72; $p < 0.001$), neonatal seizure (OR = 0.65; $p = 0.003$) and intraventricular hemorrhage (OR = 0.68; $p = 0.001$) in cases after histologic chorioamnionitis compared with the cases had no AC therapy ($n = 3,271$ vs. 4,625). Antenatal corticosteroids were significantly associated with reduced mortality [odds ratio (OR) = 0.60; $p < 0.001$] among the cases without histologic chorioamnionitis.
Conclusion In the retrospective population-based study in Japan, AC exposure was significantly associated with a lower rate of death and neurological morbidity in cases with histologic chorioamnionitis. These outcome data in Japan will be important for further improvement of antenatal practice and care.

Keywords Chorioamnionitis · Antenatal corticosteroids · Outcome · Preterm infants · Very low birthweight infants

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Introduction

Maternal administration of antenatal corticosteroids (AC) is effective to decrease respiratory distress syndrome (RDS) and it improves neurological morbidity and mortality in preterm infants [1–4]. AC therapy has become one of common interventions for threatened preterm delivery [1]. However, most obstetricians are concerned about AC administration and fear adverse effects in cases of suspected infection represented by chorioamnionitis [3, 5]. Major guidelines have indicated that the benefits of AC outweigh the infection risk and recommend AC therapy for pregnant women as long as they show no clinical evidence of infection [4, 5]. However, no evidence has been reported that AC make maternal or fetal infections worse [5]. Pregnant women with signs suggestive of chorioamnionitis have been excluded from randomized control trials [1, 6–9] according to the recommendation. To the best of our knowledge, a randomized clinical trial about the effects of

AC therapy in cases of chorioamnionitis does not exist; only small observational studies or cohort studies are available on the efficacy of AC therapy in cases of chorioamnionitis [10–14].

Chorioamnionitis is associated with the incidence of preterm birth, especially in spontaneous preterm birth at very low gestation (<30 weeks) [15], and this association increases with decreasing gestational age [13]. The presence and severity of chorioamnionitis have been diagnosed by pathological examinations of the placenta (histologic chorioamnionitis). Although accurate, this histological diagnosis can only be made after birth. Antenatal infection can be diagnosed in various ways from clinical signs such as elevated temperature, increased white blood cell (WBC) counts, and uterine tenderness. Clinicians are concerned about AC therapy, particularly in cases where the mother already has chorioamnionitis.

The most significant morbidities such as intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), and chronic lung disease (CLD) are associated with infants weight <1,500 g [10, 16]. In previous studies, AC therapy for women in preterm labor at <34 weeks of gestation decreases neonatal problems, including RDS, IVH, and death [2, 3]. Therefore, the efficacy of AC is much more expected for very low birthweight (VLBW) infants.

The purpose of the study was to evaluate the effectiveness of AC therapy for women we could get postnatal information regarding histologic chorioamnionitis on outcomes of VLBW infants by analyzing a large database (the Neonatal Research Network Japan) and to accumulate one of the largest body of evidence at present.

Materials and methods

This was a retrospective analysis of 7,896 single infants born at a gestational age between 22 + 0 and 33 + 6 weeks and birth weights <1,500 g. Clinical data between 2003 and 2008 were obtained from the Neonatal Research Network Japan which collects data on >50 % of neonates born in Japan. All tertiary neonatal units designated by the government participate in this database. 63 Level III perinatal centers out of the 73 participating facilities were registered for the Neonatal Research Network Japan in the year 2008. This database contains information on morbidity and mortality of VLBW infants <1,500 g and born in participating hospitals.

Data for infants who were born alive but died in the delivery room were included. The clinician's perspective on active treatment or withdrawal of care to preterm infants born at 22 and 23 weeks of gestation depended on the status of infants. After 23 weeks of gestation, most clinicians make an effort to save infants [17].

Data were analyzed according to presence of histologic chorioamnionitis. We analyzed the effect of AC therapy on mortality as the main outcome and studied secondary outcomes with neurological morbidities such as neonatal seizure, IVH, PVL, respiratory morbidities such as RDS, CLD, and adverse effects such as sepsis, late-onset adrenal insufficiency, patent ductus arteriosus (PDA), and necrotizing enterocolitis (NEC).

Non-reassuring fetal status (NRFS) was diagnosed when there is persistent bradycardia or recurrent decelerations. The presence and severity of histologic chorioamnionitis were examined on the basis of Blanc's criteria [18, 19]. IVH was reported according to the classification of Papile et al. [20]. PVL was diagnosed by either cranial ultrasonography or head magnetic resonance imaging scan, performed at 2 weeks or later. RDS was diagnosed on the basis of clinical presentation and characteristic radiographic appearance. CLD was diagnosed on the basis of dependency on oxygen supplementation at a corrected age of 28 days. Neonatal sepsis was documented by a positive blood culture. Late-onset adrenal insufficiency was clinically diagnosed by systematic hypotension with oliguria, hyperkalemia, and myocardial dysfunction [21]. PDA was defined as persistence of an open ductus arteriosus after birth with clinical symptoms. NEC was defined according to Bell classification stage II or greater [22]. Neonatal mortality was defined as death of an infant before discharge.

Statistical analysis

Data are presented as means ± standard deviations for continuous data and median and range for ordinal data. Differences between the AC and no-steroid groups were tested using a Chi-square test and *t* test, as appropriate. Multivariable logistic regression analyses were performed to assess the effect of AC therapy on neonatal mortality and morbidity. Odds ratios or coefficients adjusted for confounding variables and 95 % confidence intervals were calculated. Multivariate logistic regression analysis was performed after adjusting for maternal age, parity, diabetes, preeclampsia, premature rupture of the membranes (PROM), non-reassuring fetal status (NRFS), mode of delivery, gestational age at delivery, and sex of the infant.

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical tests were considered significant at a p value of <0.05.

Results

A total of 19,226 infants were registered in the database between 2003 and 2008 (Fig. 1). In total, 5,156 infants

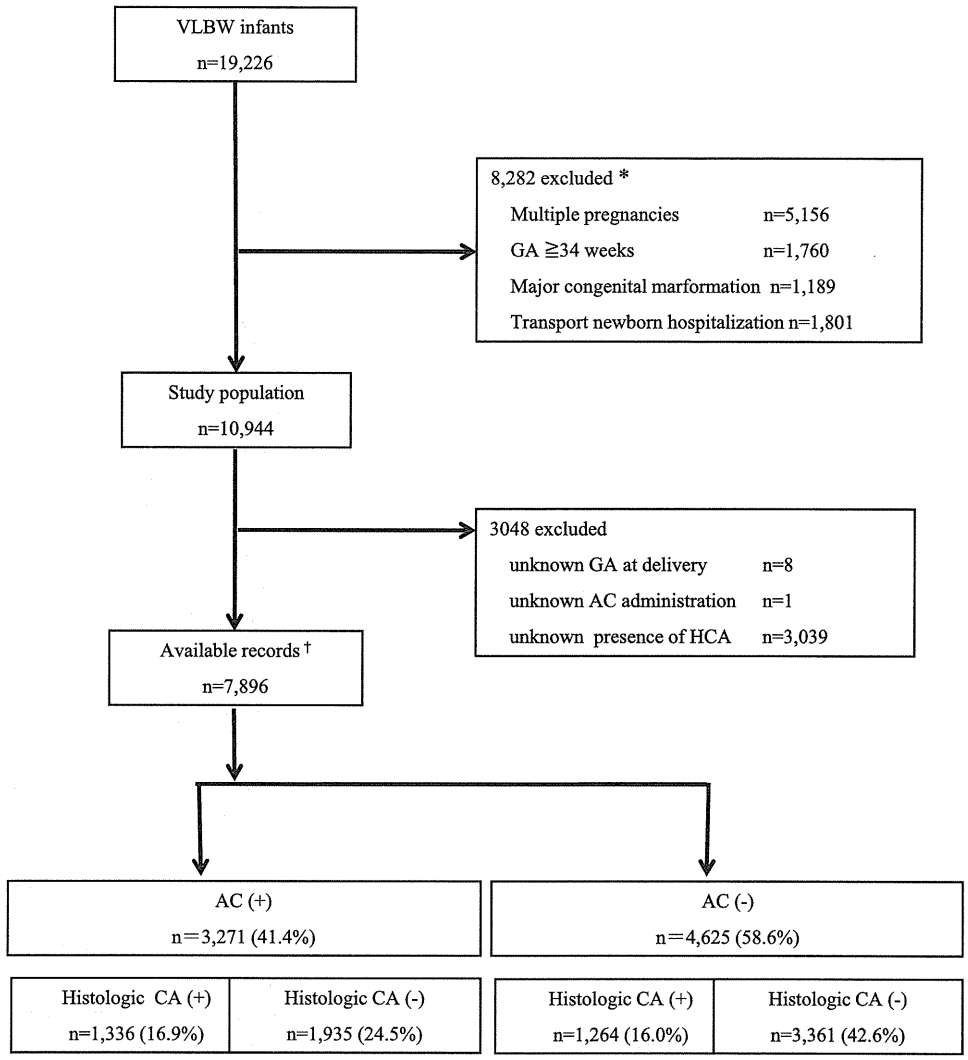

Fig. 1 Flow of study population. *Asterisk* some terms are crossover

were excluded because of multiple pregnancies, 1,189 infants were excluded because of major congenital malformation, and 1,801 infants were excluded because they were born outside participant centers. In study population, 3,039 infants were excluded due to lack of the information about the presence and severity of histologic chorioamnionitis. Data of 7,896 (72.1 %) single infants born at gestational ages between 22 + 0 and 33 + 6 weeks were available, of which only 3,271 (41.4 %) received AC therapy.

Maternal and delivery characteristics for the groups are described in Table 1. The AC group had significantly higher rates of PROM ($p < 0.001$), and lower rates of NRFS ($p = 0.02$), higher rates of cesarean delivery ($p = 0.001$), lower gestational age at delivery ($p < 0.001$), and lower birthweight ($p < 0.001$).

As the main outcome, the effect of AC therapy on neonatal mortality is shown in Table 2. The mortality rate was 6.2 and 13.3 % in the AC and no-steroid groups, respectively, in pregnant women with histologic chorioamnionitis. Similarly, the mortality rate was 5.7 and 9.1 % in the AC and no-steroid groups, respectively, in pregnant women with no histologic chorioamnionitis. In the logistic regression analysis, AC therapy was associated with a significant decrease in mortality of infants whose mothers had histologic chorioamnionitis [adjusted odds ratio (OR), 0.50; 95 % confidence interval (CI); 0.37–0.68; $p < 0.001$].

Regarding neurological morbidities, AC therapy resulted in a significant decrease in neonatal seizures (adjusted OR 0.65; CI 0.44–0.95; $p = 0.03$), IVH (adjusted OR 0.72; CI 0.58–0.89; $p = 0.002$), but no significant association with PVL (adjusted OR 0.74; CI 0.52–1.11; $p = 0.15$) in infants of mothers with histologic chorioamnionitis (Table 2).

The effect of AC therapy on respiratory outcome is shown in Table 2. AC therapy was associated with a significant decrease in RDS in cases of histologic chorioamnionitis (adjusted OR 0.72; CI 0.60–0.85; $p < 0.001$).

AC therapy was associated with a significant decrease in neonatal sepsis in women with histologic chorioamnionitis (adjusted OR 0.72; CI 0.56–0.93; $p = 0.01$). No significant

Table 1 Demographics and baseline characteristics

	Steroid ($n = 3,271$)	No steroid ($n = 4,625$)	P value
Maternal age (years)[a]	31.1 ± 5.2	31.1 ± 5.4	0.67
Parity[a]	0.68 ± 0.86	0.67 ± 0.89	0.77
Diabetes	1.1 %	1.8 %	0.006
Preeclampsia	17.5 %	22.7 %	<0.001
PROM	41.8 %	26.2 %	<0.001
NRFS	25.8 %	27.7 %	0.02
Mode of delivery			0.001
Vaginal	26.8 %	28.5 %	
With manipulation	0.5 %	1.0 %	
Cesarean section	72.7 %	70.5 %	
GA at delivery (weeks)[a]	27.6 ± 2.6	27.8 ± 3.0	<0.001
Birth weight (g)[a]	970.2 ± 290.6	995.7 ± 306.3	<0.001
Male sex	52.8 %	51.2 %	0.11

PROM preterm rupture of membranes, *NRFS* non-reassuring fetal status

[a] Mean ± SD

differences were seen for late-onset adrenal insufficiency, PDA, or NEC in women with histologic chorioamnionitis in the logistic regression analysis (Table 2).

Discussion

The current study was an extremely large population-based cohort study that sought to determine the effectiveness of AC therapy in pregnant women who were diagnosed as histologic chorioamnionitis after delivery.

AC therapy was administered to only 41.4 % of women delivered prematurely. This is low rate of AC use in this high-risk population compared with other countries, because in Japan AC therapy was covered under National Health Insurance since 2009. AC therapy decreased neonatal mortality rate and neurological complications regardless of chorioamnionitis in pregnant women (Table 2). The effect was more apparent in pregnant women with histologic chorioamnionitis than those without them. The decrease in neurological complications with the use of AC therapy might lead to a decrease in mortality rate. Hemorrhage in cases of IVH occurs when venous pressure is elevated [23]. PVL is caused by reactive oxygen toxicity resulting from reperfusion or ischemia/hypoxia occurring in the immature fetal brain [24]. AC therapy stabilizes the hemodynamic parameters of the fetus such as blood pressure [25], prevents congestion, ischemia, or reperfusion damage, thus decreasing neurological complications [26].

We experienced decrease in RDS as a result of AC therapy in pregnant women with histologic chorioamnionitis. Fetuses are reportedly stressed by the presence of intrauterine infection/inflammation such as chorioamnionitis, which thereby accelerates lung maturity by encouraging the secretion of endogenous corticosteroids resulting in the production of surfactant [27]. However, we could find AC therapy decreased RDS in the group with histologic chorioamnionitis. In contrast, AC therapy increased RDS in the group without histologic chorioamnionitis. The finding differs from the trend reported in past reports with respect to RDS [1]. This could be because various factors such as the period from steroid administration to delivery had not been adjusted, and RDS has often been subjectively diagnosed by clinical symptoms other than chest X-ray. CLD tended to increase in infants receiving AC therapy, although not significantly. This result possibly occurred because the mortality rate decreased and a larger number of infants in serious condition required long-term ventilation management, oxygen administration, and artificial nutrition [27].

The current study had several limitations. First, the database used in this study had little information about mothers. Aspects such as the type of corticosteroid used during AC therapy, the number of doses of corticosteroids, the period from corticosteroid administration to delivery, maternal sepsis, and maternal mortality were not studied. Second, we analyzed data from multiple facilities; hence, the timing of AC therapy may have differed depending on the facility. However, since this database is very large, the neonatal information associated with AC therapy obtained in the present study is beneficial.

PROM is a higher risk condition for maternal, fetal, and neonatal infection. Intrauterine infection is strongly associated with PROM and particularly complicates 30–50 % of mid-trimester PROM in previous reports [28, 29]. There have been concerns about promoting this risk because of corticosteroid use. However, some recommendations show that AC therapy for pregnant women with PROM before 32 weeks of gestation is beneficial [4, 7, 8]. In this regard, treatment did not increase the risk of maternal death and infection in a systematic review about AC therapy in pregnancies complicated by PROM [1, 30]. As stated above, administration of corticosteroids to pregnant women who have a higher possibility of intrauterine infection is believed to be acceptable without significant maternal adverse effects.

In future, data including information about mothers must be examined, differences in the effectiveness of AC therapy depending on gestational age, and differences among different steroids, dosage, and other variations in treatments regimes must be studied. The effectiveness of AC therapy must be ascertained in a study of multiple pregnancies. In addition, the data on long-term prognosis of cases included in this study are being analyzed.

Table 2 Effects of antenatal corticosteroid on neonatal outcomes after histologic chorioamnionitis

	Affected/total (%)					
	Steroid	No steroid	Crude OR	Adjusted OR[a]	95 % CI	*P* value
Neonatal mortality						
Histologic CA(+)	83/1,336 (6.2)	168/1,264 (13.3)	0.43	0.50	0.37–0.68	<0.001
Histologic CA(−)	111/1,935 (5.7)	306/3,361 (9.1)	0.61	0.60	0.47–0.78	<0.001
Overall	194/3,271 (5.9)	474/4,625 (10.2)	0.65	0.56	0.48–0.67	<0.001
Neurological outcomes						
Neonatal seizure						
Histologic CA(+)	49/1,336 (3.7)	80/1,264 (6.3)	0.56	0.65	0.44–0.95	0.03
Histologic CA(−)	49/1,935 (2.5)	122/3,361 (3.6)	0.69	0.69	0.48–0.97	0.03
Overall	98/3,271 (3.0)	202/4,625 (4.4)	0.66	0.56	0.45–0.71	<0.001
IVH						
Histologic CA(+)	224/1,336 (16.8)	294/1,264 (23.3)	0.67	0.72	0.58–0.89	0.002
Histologic CA(−)	217/1,935 (11.2)	433/3,361 (12.9)	0.85	0.87	0.72–1.05	0.14
Overall	441/3,271 (13.3)	727/4,625 (15.7)	0.87	0.74	0.65–0.83	<0.001
PVL						
Histologic CA(+)	56/1,336 (4.2)	66/1,264 (5.2)	0.79	0.74	0.52–1.11	0.15
Histologic CA(−)	61/1,935 (3.2)	125/3,361 (3.7)	0.84	0.80	0.56–1.10	0.18
Overall	117/3,271 (3.6)	191/4,625 (4.1)	0.95	0.83	0.67–1.03	0.09
Respiratory outcomes						
RDS						
Histologic CA(+)	686/1,336 (51.3)	762/1,264 (60.3)	0.70	0.72	0.60–0.85	<0.001
Histologic CA(−)	1,121/1,935 (57.9)	1,759/3,361 (52.3)	1.25	1.17	1.03–1.33	0.02
Overall	1,807/3,271 (55.2)	2,521/4,625 (54.5)	1.16	0.92	0.84–1.00	0.06
CLD						
Histologic CA(+)	727/1,256 (57.9)	621/1,122 (55.3)	1.11	1.05	0.84–1.31	0.66
Histologic CA(−)	685/1,810 (37.8)	866/2,972 (29.1)	1.48	1.38	1.18–1.61	<0.001
Overall	1,412/3,066 (46.1)	1,487/4,094 (36.3)	1.55	1.16	1.04–1.28	0.01
Other outcomes						
Sepsis						
Histologic CA(+)	135/1,336 (10.1)	183/1,264 (14.5)	0.66	0.72	0.56–0.93	0.01
Histologic CA(−)	120/1,935 (6.2)	223/3,361 (6.6)	0.93	0.98	0.76–1.25	0.85
Overall	255/3,271 (7.8)	406/4,625 (8.8)	0.97	0.86	0.74–0.99	0.05
Late-onset adrenal insufficiency						
Histologic CA(+)	163/1,295 (12.6)	149/1,180 (12.6)	0.99	1.05	0.81–1.36	0.70
Histologic CA(−)	156/1,873 (8.3)	241/3,146 (7.7)	1.10	0.92	0.73–1.15	0.47
Overall	319/3,168 (10.1)	390/4,326 (9.0)	1.27	1.02	0.88–1.19	0.77
PDA						
Histologic CA(+)	515/1,336 (38.5)	465/1,264 (36.8)	1.08	1.16	0.97–1.39	0.09
Histologic CA(−)	639/1,935 (33.0)	950/3,361 (28.3)	1.25	1.17	1.02–1.33	0.02
Overall	1,154/3,271 (35.3)	1,415/4,625 (30.6)	1.34	1.13	1.04–1.24	0.01
NEC						
Histologic CA(+)	26/1,336 (1.9)	25/1,264 (2.0)	0.98	1.13	0.63–2.02	0.68
Histologic CA(−)	28/1,935 (1.4)	39/3,361 (1.2)	1.25	1.35	0.81–2.25	0.25
Overall	54/3,271 (1.7)	64/4,625 (1.4)	1.23	1.13	0.82–1.57	0.45

CA chorioamnionitis, *OR* odds ratio, *CI* confidence interval

[a] Adjusted for maternal age, parity, diabetes, preeclampsia, preterm rupture of membrane (PROM), non-reassuring fetal status (NRFS), mode of delivery, gestational age of delivery, birthweight, and sex of the infant

Conclusion

Based on this body of the evidence and the results of our study, we propose that active use of AC therapy is recommended for women with a single pregnancy at the very preterm and even those with chorioamnionitis. These outcome data in Japan will be important for further improvement of antenatal practice and care.

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Conflict of interest The authors declare no conflict of interest.

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C1 Esterase Inhibitor Activity in Amniotic Fluid Embolism

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AQ3 **Introduction:** Amniotic fluid embolism exhibits activation of the complement system and the kallikrein-kinin and coagulofibrinolytic systems. C1 esterase inhibitor is a major inhibitor of C1 esterase and can inhibit plasma kallikrein and also factors XIIa and XIa. Its activity has been shown to be significantly lower in pregnancy and labor than in the nonpregnant state. The purpose of this study was to determine C1 esterase inhibitor activity levels in amniotic fluid embolism.

Methods: This study was retrospectively conducted on 194 singleton pregnant women. One hundred six cases of amniotic fluid embolism had applied to the Japan amniotic fluid embolism registration center in Hamamatsu University School of Medicine between January 2010 and December 2011. In amniotic fluid embolism cases, 85 cases were nonfatal and 21 cases were fatal. Eighty-eight women who delivered without amniotic fluid embolism were regarded as a control. C1 esterase inhibitor activity levels at the onset of amniotic fluid embolism in amniotic fluid embolism cases and at the completion of labor in control cases were measured and compared using the Mann-Whitney *U* test.

Results: C1 esterase inhibitor activity levels were significantly lower in amniotic fluid embolism patients (30.0% ± 1.8%) than in control women (62.0% ± 2.0%) ($p < 0.0001$). C1 esterase inhibitor activity levels in fatal amniotic fluid embolism cases (22.5% ± 3.4%) were significantly lower than those in nonfatal amniotic fluid embolism cases (32.0% ± 2.1%) ($p < 0.05$).

Conclusions: These results demonstrated that low C1 esterase inhibitor activity levels were closely associated with the pathogenesis of amniotic fluid embolism suggesting that C1 esterase inhibitor activity levels have potential as a prognosis factor of amniotic fluid embolism. (*Crit Care Med* 2014; XX:00–00)

Key Words: amniotic fluid embolism; C1 esterase inhibitor; disseminated intravascular coagulopathy; kallikrein; postpartum hemorrhage; serpin

Amniotic fluid embolism (AFE) is one of the most serious complications of obstetrics, anesthetics, and critical care. Despite earlier recognition and intensive critical care, the mortality of AFE remains high and has been estimated at between 5% and 15% of all maternal deaths (1). Maternal mortality rates due to AFE have been estimated at between 37% and 80% (2, 3). Maternal death has been decreasing year by year in Japan; however, the prevalence of maternal death due to AFE has remained unchanged. The maternal mortality rate due to AFE has increased to 24.3% in Japan (4). AQ4

AFE is recognized as a kind of syndrome characterized by the abrupt onset of hypoxia, hypotension, and disseminated intravascular coagulopathy (DIC) (5). Benson et al (6) reported that maternal complement levels were significantly decreased in AFE. These findings suggested a disorder in the coagulofibrinolytic system as well as the complement system that may play important roles in the pathogenesis of AFE.

We developed the Japan AFE registration system in 2003 and collected clinical data, maternal serum, and uterine tissue from nearly all cases of fatal AFE in Japan (4, 7). Under the system, maternal serum has been applied to determine mainly the levels of specific amniotic fluid complements such as Sialyl Tn and zinc coproporphyrin 1 (8, 9). These clinical and histopathological observations demonstrated that AFE was frequently associated with uterine atony due to angioedema (unpublished data). AQ5

C1 esterase inhibitor (C1INH), belonging to the serpin group/family, is a major inhibitor not only of C1 esterase but also of kallikrein and factors XIIa and XIa (10–12). Its deficiency has been known to be a direct cause of hereditary angioedema (HAE) as well as acquired angioedema (13). Since

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C1INH has the potential to regulate the coagulofibrinolytic system, complement system, and kallikrein-kinin system, we have become greatly interested in C1INH activity levels in AFE in Japan.

MATERIALS AND METHODS

Definition of AFE

AFE was defined based on the Japan consensus criteria for the diagnosis of AFE based on the United States/United Kingdom criteria as shown in **Figure 1** (7, 14). A pathological diagnosis was determined when fetal debris was found in the maternal pulmonary arteries. The diagnosis of nonfatal AFE depended on clinical manifestations and was done when factors B1–B3 were all present, but more than one of the signs and symptoms listed in B1 needed to be present. Consumptive coagulopathy/DIC due to evident etiologies such as abnormal placentation (placental abruption, etc.), trauma during labor and delivery, and severe preeclampsia/eclampsia should be excluded from the criteria.

Patients

The Japan AFE registration system was started in 2003 (7). This system has included the procedure of consent to apply and analyze their clinical data and blood samples. Consent was obtained from patients or patient's family, when physicians regarded their patients with significant symptoms as AFE based on the diagnostic criteria of AFE. Clinical data and serum from nearly all cases of AFE have accumulated in Hamamatsu University School of Medicine. Subjects of the present study were extracted from entry cases in the Japan AFE registration center in Hamamatsu University School of Medicine, Shizuoka, between January 2010 and December 2011. Women with multiple pregnancies, preeclampsia, thrombophilia, preterm labor, uterine disorder such as uterine myoma, and a history of systemic disease were excluded from this study. Cesarean section was carried out due to obstetrical indications, such as breech presentation, history of cesarean section, arrest of labor, and nonreassuring fetal status. Women, who delivered at Hamamatsu University Hospital between April 2011 and September 2011, without AFE and any medical intervention other than general birth and surgical assistances were analyzed as the control subjects. One hundred six cases of AFE and 88 cases of control were defined (**Table 1**). Among the AFE cases, 85 cases survived and 21 cases died due to AFE.

The Japan consensus criteria for the diagnosis of AFE

A. Pathological confirmation; A diagnosis is made on the basis of clinical presentation after excluding differential diagnosis and at autopsy in the event of death of the parturient. The diagnosis is confirmed by histochemical studies.

B. Clinical manifestation; The patients has the hallmark clinical manifestations of AFE following 1, 2, and 3:
1. Signs and symptoms: Cardiac arrest/ Respiratory arrest/ Consumptive coagulopathy
2. Onset of all of the signs and symptoms during pregnancy, labor, or cesarean section or within 12 hours of delivery
3. Absence of other illness that could explain the signs and symptoms described above

Figure 1. The Japan consensus criteria for the diagnosis of amniotic fluid embolism (AFE). A pathological diagnosis was determined when fetal debris was found in the maternal pulmonary arteries. The diagnosis of nonfatal AFE depended on clinical manifestations and was done when factors B1–B3 were all present, but more than one of the signs and symptoms listed in B1 needed to be present.

Blood Collection and Measurement of C1INH Activity

Blood samples from registered AFE patients were collected at the Japan AFE registration center in Hamamatsu, and serum and plasma samples were then kept at –30°C until use. Time points of blood samples obtained were at onset of and before interventions against AFE. Control blood samples were obtained at the completion of labor. The determination of C1INH activity was performed using the Berichrom C1 inhibitor kit (Siemens Healthcare Diagnostics) according to the manufacturer's instructions. The intra-assay coefficients of variation (CV) ranged between 1.8% and 7.9% and the interassay CV were between 3.2% and 6.6%. We analyzed all the samples at the same time under a blind fashion. In the present study, we demonstrated the measurement of C1INH activity in serum. Furthermore, C1INH activity was measurable in serum as well as plasma; there were no significant differences ($p < 0.0001$, $R^2 = 0.9881$) in the activity level between serum and plasma under the Berichrom C1 inhibitor kit (data not shown).

Approval

Written informed consent was obtained after full explanation of the study. The study was carried out under the approval of the Ethics Committee of Hamamatsu University School of Medicine (Number 24–130 and 25–107), which conforms to the provisions of the Declaration of Helsinki (as revised in Tokyo 2004).

Data Analysis

Values of C1INH activity (%) were presented as the median ± SE. Significant differences were assessed with the Mann-Whitney *U* test. A *p* value of less than 0.05 was considered significant.

RESULTS

As shown in **Figure 2**, C1INH activity levels in the controls and AFE cases were 62.0% ± 2.0% and 30.0% ± 1.8%, respectively. C1INH activity levels in the AFE cases were significantly lower than those in the controls ($p < 0.0001$). C1INH activity levels in fatal and nonfatal AFE cases were 22.5% ± 3.4% and 32.0% ± 2.1%, respectively. A significant difference was observed between the two groups ($p = 0.0121$).

Changes in C1INH activity levels in one survivor case and one case that died due to AFE are shown in **Figure 3**. Both cases were defined as AFE by the Japan consensus criteria for the diagnosis of AFE shown in Figure 1. C1INH activity levels were potentially very low before the onset of AFE. C1INH activity in the survivor case was at its lowest level at the onset 3 hours after the selective cesarean section due to history of cesarean section when AFE was defined due to the development of DIC. Immediate replacement therapy with FFP successfully increased the activity of C1INH. In the case that died,

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AQ6 **TABLE 1. XXX**

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	Control	Total AFE	Nonfatal AFE	Fatal AFE
No. of subjects	88	106	85	21
Age (yr)	31.0±4.8	33.8±5.8	33.3±5.4	35.6±3.8
Gravida[a]	1.27±1.02	1.64±1.77	1.74±1.82	1.23±1.47
Parity[a]	0.72±0.63	0.83±1.06	0.89±1.12	0.57±0.72
Nulliparous (%)	28 (31.8)	52 (49.0)	41 (48.3)	11 (52.4)
Multiparous (%)	60 (68.2)	54 (51.0)	44 (51.7)	10 (47.6)
Gestational period (d)	273±12	268±19	267±20	270±17
Delivery methods				
Vaginal delivery (%)	60 (68.2)	52 (49.0)	44 (51.7)	10 (47.6)
Cesarean section (%)	28 (31.8)	54 (51.0)	41 (48.3)	11 (52.4)
Blood loss at delivery (mL)				
Vaginal delivery	395±170	4,864±3,039	5,038±3,111	4,097±2,569
Cesarean section	840±279	4,270±2,988	4,314±2,657	4,107±3,961

AFE = amniotic fluid embolism.
[a]Woman without previous history of pregnancy and delivery was determined as gravida 0 and parity 0, respectively.

C1INH activity was also low before the manifestation of AFE symptoms. In this case, amniotic fluid, fetal substance, and gram-positive coccus were observed and autopsy diagnosis was AFE and bacteremia.

DISCUSSION

AFE is an unpredictable and serious disorder of pregnancy characterized by hypotension, hypoxia, and coagulopathy (5). In most pregnant women, the entry of small amounts of amniotic fluid into the maternal circulation may be innocuous; however, such exposure is associated with a fatal outcome in other women. Anaphylactic reactions have been suggested as a concept of AFE to explain such an individual difference in the response to amniotic fluid (2, 15). Benson (6, 16) reported that serum tryptase and urinary histamine increased and complement levels decreased in AFE patients, suggesting that contact and maternal immune activation played important roles in the pathophysiology of AFE.

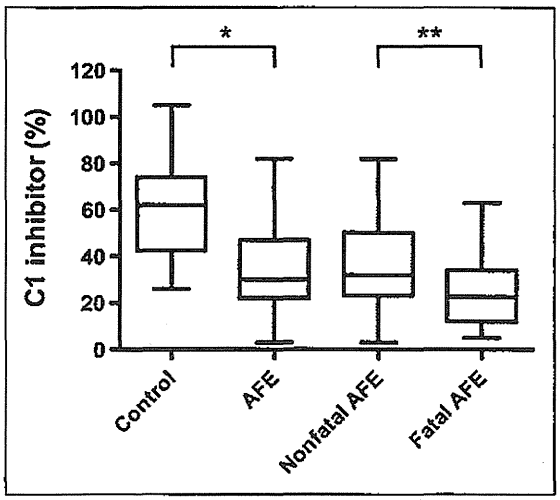

Figure 2. C1 esterase inhibitor (C1INH) activity levels in control, amniotic fluid embolism (AFE), nonfatal AFE, and fatal AFE cases. Columns indicate the medians and whiskers represent the minimum and maximum values. Significant differences were $^{*}p < 0.0001$ and $^{**}P = 0.0121$, respectively.

Clinically, DIC-type postpartum hemorrhage accompanying uterine atony is one of the recognized symptoms of AFE (4, 17). To explain this, coagulation factor XII (FXII) may be responsible for the pathological condition as it is activated by contact with various artificial or biological negatively charged surfaces, resulting not only in blood coagulation but also in the activation of the complement system and kallikrein-kinin system to produce bradykinin (18). We demonstrated that FXII inactivated plasminogen activator inhibitor 1 and enhanced fibrinolysis (19). Interestingly, bradykinin has strong vasodilation effects, a hypotensive effect, and causes an increase in vascular permeability resulting in a hypotonic uterus (20, 21). These findings suggest that FXII activation by contact triggers the subsequent catastrophic chain of AFE. We are continuing to investigate the possible role of FXII in AFE.

C1INH, which is mainly synthesized in hepatocytes and endothelial cells and belongs to serpin family, is a major inhibitor of not only C1 esterase but also FXIIa and kallikrein (11, 12). Its deficiency is known to be a specific cause of HAE (13). Since C1INH is capable of not only inhibiting the complement system but also modulating the coagulofibrinolytic and kallikrein-kinin systems (22, 23), we hypothesized that C1INH was key in the pathophysiology of AFE.

Halbmayer et al (24) reported that basal C1INH activity levels decreased markedly with pregnancy up to labor. Although the mechanism remains unclear, estradiol (E2) was shown to suppress the potential activity of C1INH (25, 26).

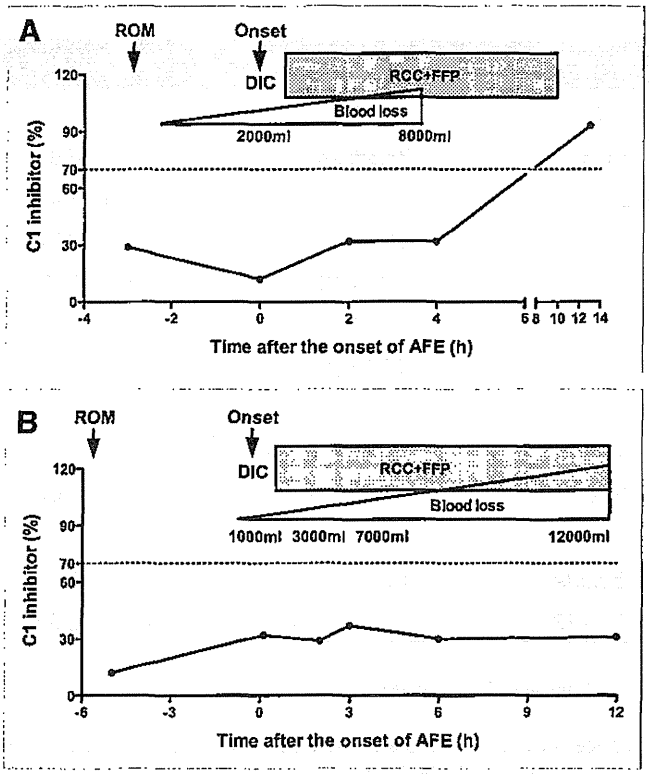

Figure 3. Chronological changes in C1 esterase inhibitor (C1INH) activity levels in amniotic fluid embolism (AFE) cases. A survivor of AFE (**A**) and a case that died due to the coexistence of AFE with bacteremia (**B**). Potential C1INH activity levels were low before the onset of AFE. In case A, the sudden onset of AFE presented disseminated intravascular coagulopathy (DIC) 3 hr after rupture of membrane (ROM) under a selective cesarean section. When AFE was recognized as abnormal coagulopathy, blood replacement therapy was immediately administrated resulting in an increase in C1INH activity levels. In case B, despite intensive care including adequate blood transfusions, C1INH activity levels did not recover. A *dotted line* indicates 70% of C1INH activity. Normal C1INH activity is more than 70%. RCC =, FFP =.

The increase in E2 levels during pregnancy may be associated with the decrease in C1INH activity levels in pregnant women. They also observed that C1INH activity levels were significantly lower in preeclampsia patients than in normal pregnant women (24). Increases in C3a and C5a have been reported not only in AFE patients but also in patients with preeclampsia and eclampsia (27), which suggests that the consumption of C1INH is due to activation of the complement system. This may explain the high risk of AFE associated with preeclampsia and eclampsia as risk factors of AFE (28, 29).

The present study demonstrated that C1INH activity levels in AFE cases were significantly lower than those of controls. Furthermore, when we compared fatal cases to nonfatal cases using Pearson chi-square test for C1INH activity less than 25% as a cutoff value almost comparable to "attack of angioedema," there was a significant difference with p equal to 0.026 (degree of freedom 1 and chi-square value 4.956). In addition, the chronological assessment of C1INH activity levels in two AFE patients indicated that their basal C1INH activity levels before delivery and onsets of AFE were also lower at 29% and 12% than that of healthy pregnant controls at 74.3% ± 15.5% during the third trimester (24). These results suggest that low C1INH activity levels before onset of AFE could be a predictive factor as well as low levels at onset and the persisting low levels of C1INH activity could be a prognostic factor of AFE.

It has been reported that the levels of C1INH may be increased during infection as an acute phase protein, then cleaved and inactivated by neutrophil elastase and bacterial proteases under developing inflammatory conditions due to bacteremia and sepsis resulting in a functional C1INH deficiency (30–32). As demonstrated in the fatal case in **Figure 3B**, we should note here that not only C1INH consumption under AFE condition but also C1INH inactivation under inflammatory conditions due to bacteremia may be involved in the persistent low levels of C1INH activity (32).

As a treatment for DIC with AFE, the rapid administration of FFP or cryoprecipitate was sufficient to extricate the patient from a critical situation. FFP contains several essential proteins such as ATIII and fibrinogen. One hundred units of C1INH are contained in FFP derived from 200 mL blood. Our chronological assessment of C1INH activities in the AFE patient shown in **Figure 3A** demonstrated that a suited blood transfusion including FFP was able to improve C1INH activity. Clinically, the use of 500–1,500 U of human plasma–derived C1INH concentrates can revert HAE in C1INH-deficient patients (33–35). Since AFE patients certainly have significant lower levels of C1INH activity, similar to a C1INH deficiency, the clinical application of human plasma–derived C1INH concentrates may become one of the promising candidates for the treatment of AFE.

In summary, we reported here that C1INH activity levels were significantly lower in AFE cases, particularly in fatal cases. These results indicate that C1INH activity levels reflect the severity of AFE and can be a prognostic factor of AFE. We speculate that the clinical application of C1INH concentrates will be effective for the treatment of AFE. Although the chronological measurement of C1INH activity was small, our results suggest that pregnant women with potentially low C1INH activity levels may be at a high risk of the onset of AFE. Further clinical studies are required to elucidate the etiological role of C1INH in AFE and determine whether C1INH activity may be a predictive factor of AFE.

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Regular Article

Nonsynonymous mutations in three anticoagulant genes in Japanese patients with adverse pregnancy outcomes☆

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ABSTRACT

Background: Hereditary thrombophilias may associate with uteroplacental thrombosis leading to adverse pregnancy outcomes. The present study was conducted to reveal the frequency of the low-frequency thrombophilic protein S K196E mutation, as well as the frequency of very rare nonsynonymous mutations in protein S, protein C, and antithrombin genes, in patients with adverse pregnancy outcomes.
Patients and methods: We enrolled 330 Japanese patients with adverse pregnancy outcomes and divided them into 233 patients with two or more miscarriages and 114 patients with fetal growth restriction (FGR) and/or intrauterine fetal death (IUFD); 17 patients belonged to both groups. We sequenced the entire coding regions of three anticoagulant genes in all 330 patients.
Results: We found that protein S K196E mutation was identified in 4 out of 233 patients with recurrent miscarriage and in 2 out of 114 patients with FGR and/or IUFD. The frequencies of this mutation in these patient groups were not different from that in a Japanese general population. Very rare nonsynonymous mutations were identified in 3.3% (11 out of 330) of patients with adverse pregnancy outcomes.
Conclusions: Although the low-frequency protein S K196E mutation can increase the risk for venous thromboembolism, it did not increase the risk for adverse pregnancy outcomes even in Japanese.

Introduction

Approximately 1-5% of pregnant women have a serious adverse pregnancy outcome, such as severe preeclampsia, placental abruption, intrauterine fetal death (IUFD), or severe fetal growth restriction (FGR) [1]. Recurrent miscarriage, defined as two or more miscarriages, is also a significant public health problem for women, and the prevention of these adverse pregnancy outcomes is important for women's health [2]. An estimated 5% of women of reproductive age in general experience two or more miscarriages [3]. The causes of serious pregnancy outcomes are unknown, but they may be associated with abnormal placental vasculature and disturbances of hemostasis, leading to inadequate maternal-fetal circulation [4]. Therefore, in order to prevent adverse pregnancy outcomes, it will be important to identify the inherited factors related to inadequate maternal-fetal circulation.

Substantial progress has been made in the identification and understanding of inherited hypercoagulable disorders that promote thrombosis, collectively termed inherited thrombophilia [2,5,6]. These include low-frequency mutations, the factor V Leiden mutation, the prothrombin G20210A mutation, and very rare mutations of protein S, protein C, and antithrombin genes. The factor V Leiden mutation and the prothrombin G20210A mutation are modest genetic risk factors for venous thromboembolism and are widely distributed among Caucasians [7] but are not present in the Japanese population [8,9]. Instead, other authors and we have identified a low-frequency thrombophilic mutation, the protein S K196E mutation, as a genetic risk factor for venous thromboembolism with odds ratios between 3.74 and 8.56 in several Japanese populations [10–14]. The prevalence of this mutant allele in the general Japanese population was found to be about 0.009, suggesting a substantial proportion of the Japanese population carries the protein S E-allele

Abbreviations: FRG, fetal growth restriction; IUFD, intrauterine fetal death; SD, standard deviation.

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and is at risk of developing deep vein thrombosis [13–15]. This mutation seems to be specific to Japanese, because it has not so far been identified in Chinese, Koreans, or Caucasians [13,15–18].

Hereditary thrombophilia can be explained by low-frequency mutations including factor V Leiden mutation, prothrombin G20210A mutation, and protein S K196E mutation, as well as by very rare mutations causing deficiencies of protein S, protein C, and antithrombin. The former (the low-frequency mutations) have weak effects and the latter have large effects on thrombosis. It is controversial whether there is an association between low-frequency mutations, such as the factor V Leiden mutation and the prothrombin G20210A mutation, and adverse pregnancy outcomes, such as miscarriage, preeclampsia, FGR, and placental abruption [4,19–37].

The present study was conducted to reveal the hypothesis that the mutations predisposing patients to thrombosis in Japanese may be important risk factors for inadequate maternal-fetal circulation that may explain adverse pregnancy outcomes including recurrent miscarriage, FGR, and IUFD. We therefore examined the frequency of the low-frequency protein S K196E mutation, as well as those of very rare nonsynonymous mutations in protein S, protein C, and antithrombin genes, in patients with adverse pregnancy outcomes.

Patients and Methods

Patients and Diagnostic Criteria for Adverse Pregnancy Outcomes

In this prospective observational study, 330 patients who had experienced adverse pregnancy outcomes were enrolled from four tertiary perinatal centers: the National Cerebral and Cardiovascular Center, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka Medical College, and Kaizuka City Hospital. All of these centers are located in Osaka prefecture, which has the third-largest population out of the 47 prefectures in Japan.

Patients with adverse pregnancy outcomes were classified into two groups: those with recurrent miscarriage (233 patients) and those with FGR and/or IUFD that had occurred after 22 weeks of gestation (114 patients); 17 patients belonged to both groups. We excluded patients with multiple pregnancy. Patients were excluded in a case that patients and the partners had chromosomal abnormalities. Patients with antiphospholipid syndrome, diabetes mellitus, thyroid dysfunction, infectious diseases, and uterine deformity were also excluded. Antiphospholipid syndrome was diagnosed according to the revised classification criteria, requiring at least one of the clinical criteria (vascular thrombosis or pregnancy morbidity) and at least one of the following laboratory criteria: lupus anticoagulant, anticardiolipin antibody, and anti-β2-glycoprotein-I antibody [38]. Previous miscarriage was defined as pregnancy loss at a gestational age of 22 weeks or less. The definition of miscarriage included documentation of pregnancy by a positive pregnancy test and clinical manifestations of miscarriage (e.g., abdominal pain, cramps, and vaginal bleeding). Recurrent miscarriage was defined as at least two miscarriages. We diagnosed FGR if an ultrasound examination showed a fetus with an estimated fetal weight of less than −1.5 standard deviation in Japanese at more than 22 weeks of gestation. We also diagnosed IUFD as fetal death at more than 22 weeks of gestation by ultrasound examination. The plasma samples were obtained at least 3 months' postpartum and after at least 3 months without the use of warfarin or oral contraceptives. Protein S anticoagulant activity, free protein S antigen, protein C amidolytic activity, and heparin-dependent antithrombin activity were measured as previously described [39].

The protocol of this study was approved by the Ethics Review Committee of National Cerebral and Cardiovascular Center, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka Medical College, and Kaizuka City Hospital. Only those who had given written informed consent for genetic analysis were included in the genetic analysis.

DNA Sequencing of Protein S, Protein C, and Antithrombin Genes

We sequenced the entire coding regions of protein S, protein C, and antithrombin genes in 330 patients with adverse pregnancy outcomes. The method of direct sequencing using the 96-capillary 3730xl DNA Analyzer (Applied Biosystems Japan, Tokyo, Japan) was described previously [39]. We have adopted the numbering standards of the Human Genome Variation Society, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide +1 and the initial Met residue is denoted as amino acid +1 [40]. The potential impact of a missense mutation on the structure and function was examined using PolyPhen-2 (URL: http://genetics.bwh.harvard.edu/pph2/), a program to predict the functional significance of missense mutations [41].

Statistical Analysis

Statistical analysis was performed using JMP 10 (SAS Institute, Cary, NC, USA). Data are presented as mean ± standard deviation (SD) or the number of patients. Comparisons between groups were analyzed

Table 1
Baseline characteristics of 233 patients with recurrent miscarriage.

	Patients with genetic mutation (n = 13)	Patients without genetic mutation (n = 220)	P value
Age			
years, mean ± SD (range)	35 ± 5 (27–43)	34 ± 4 (25–44)	0.50†
No. ≧35 years old (%)	7 (53.8)	97 (43.9)	0.51‡
Body mass index, mean ± SD (range)	19.9 ± 1.8 (17.2-22.5)	21.1 ± 2.9 (16.6-30.5)	0.13†
Miscarriage			
No. of times, mean ± SD (range)	2.9 ± 0.5 (2–4)	3.3 ± 1.0 (2–9)	0.13†
≧3 miscarriages, no. of patients (%)	11 (84.6)	202 (91.4)	0.41‡
Number of mutation carriers			
Protein S mutation	6*	0	
(protein S K196E mutation)	(4*)	(0)	
Protein C mutation	4	0	
Antithrombin mutation	3	0	
Other complications of pregnancy or infant, no.			
FGR and/or IUFD	1	16	
Preeclampsia	0	1	
Eclampsia	0	1	
HELLP syndrome	0	2	
Placental abruption	0	1	

SD, Standard deviation; FGR, fetal growth restriction; IUFD, intrauterine fetal death; HELLP, hemolysis, elevated liver enzymes, and low platelets.
*One patient was a compound heterozygote with protein S K196E and T630I mutations. †t-test. ‡chi-square test.