Table 1. Yearly changes of species distribution of 3,832 fungi detected at the National Cerebral and Cardiovascular Center from 2007 to 2011

Species	Number. of isolates					
	2007	2008	2009	2010	2011	Total (%)
C. albicans	461 (70.4)	457 (72.9)	350 (65.2)	555 (71.2)	725 (58.8)	2,548 (66.5)
C. glabrata	128 (19.5)	133 (21.2)	119 (22.2)	131 (16.8)	265 (21.5)	776 (20.3)
C. parapsilosis	23 (3.5)	24 (3.8)	31 (5.8)	46 (5.9)	115 (9.3)	239 (6.2)
C. tropicalis	30 (4.6)	12 (0.9)	34 (6.3)	36 (4.6)	100 (8.1)	212 (5.5)
C. krusei	6 (0.9)	1 (0.2)	1 (0.2)	2 (0.3)	13 (1.1)	23 (0.6)
C. lusitaniae	1 (0.2)	—	—	7 (0.9)	11 (0.9)	19 (0.5)
C. guilliermondii	6 (0.9)	—	2 (0.4)	2 (0.3)	5 (0.4)	15 (0.4)
Total	655	627	537	779	1,234	3,832

All data are shown as a number with the percentage for each year shown in parentheses.

an urban area in Japan, and specializes in surgical treatment of cardiovascular diseases, including cerebrovascular and internal injuries. The facility also manages pregnancy and delivery for women with maternal cardiac diseases. The NCVC has 612 beds and about 10,000 new hospital stays each year. The average hospital stay is 17 days, and 650 and 190 heart surgeries are performed annually for adults and infants, respectively. Ten heart transplantations are performed each year.

Culture media

CHROMagar Candida (CHROMagar, Paris, France) was purchased as a powder. CHROMagar is composed (per liter) of 10 g peptone, 20 g glucose, 15 g agar, 0.5 g chloramphenicol, and 2 g chromogenic mix. The medium was prepared according to the manufacturer's instructions and dispensed in petri dishes (20 m*l* in a 90-mm diameter dish).

Identification of fungus species

Clinical specimens from cases with suspected myconal infections were inoculated onto CHROMagar and incubated at 37℃ for 48 h. Macroscopic identification was performed based on the color and shape of the grown colonies. Strains without typical characteristics on the CHROMagar were identified with a ID 32℃ Yeast Identification System (bioMerieux S. A.), using colonies on the CHROMagar prepared using the solution provided with this system.

Determination of sensitivity to antifungal drugs

The microdilution method was used to study drug sensitivity, using an Antifungal Susceptibility Test for Yeast (Kyokuto Pharmaceutical Industrial Co.) that complied with Clinical and Laboratory Standards Institute (CLSI) criteria. M27-A3 was used to determine the minimum inhibitory concentration (MIC) of amphotericin B (measurable concentration range 0.03-16 μg/m*l*), flucytosine (0.125-64 μg/m*l*), fluconazole (0.125-64 μg/m*l*), micafungin (0.03-16 μg/m*l*), itraconazole (0.015-8 μg/m*l*), and voriconazole (0.03-16 μg/m*l*). Sensitive (S), sensitive dose-dependent (S-DD), intermediate (I), and resistant (R) responses to flucytosine, fluconazole, and itraconazole were evaluated using CLSI M27-S3 criteria[6].

The study was exempted from Committee on Human Research approval (National Cerebral and Cardiovascular Center) because there no longer exists a key or code sheet relating the individuals' identities to their private health information.

Results

A total of 3,832 patients had a detected fungal infection in the 5-year period from 2007 to 2011 in the NCVC, including 2,548 patients with *C. albicans* (66.5%), 776 with *C. glabrata* (20.3%), 239 with *C. parapsilosis* (6. 2%), and 212 with *C. tropicalis* (5.5%) (Table 1). Non-*albicans* infections accounted for 33.5% of cases. The location and materials of isolated *Candida* species are shown in Table 2.

The number of blood culture performed were 2,819, 3,306, 2,900, 3,797, 4,239 in 2007, 2008, 2009, 2010, and 2011, respectively. The number and percentages of patients with fungemia caused by *C. albicans, C. parapsilosis, C. glabrata, C. tropicalis, Candida lusitaniae, Candida guilliermondii*, and *Candida krusei* were 55 (42.0%), 36 (27.5%), 21

Table 2. Location and materials of isolated *Candida* species

Species	Sputum	Urogenital	Stool	Intra-body material	Blood	Skin	Others	Total
C. albicans	1,309 (72.8)	800 (62.7)	150 (56.0)	113 (58.9)	55 (42.0)	81 (79.4)	40	2,548
C. glabrata	291 (16.2)	333 (26.1)	87 (32.5)	25 (13.0)	21 (16.0)	10 (9.8)	9	776
C. parapsilosis	56 (3.1)	77 (6.0)	8 (3.0)	38 (19.8)	36 (27.5)	11 (10.8)	13	239
C. tropicalis	123 (6.8)	49 (3.8)	17 (6.3)	11 (5.7)	11 (8.4)		1	212
C. krusei	13 (0.7)	4 (0.3)	4 (1.5)		1 (0.8)		1	23
C. lusitaniae	4 (0.2)	5 (0.4)	2 (0.8)	2 (1.0)	5 (3.8)		1	19
C. guilliermondii	2 (0.1)	8 (0.6)		3 (1.6)	2 (1.5)			15
Total	1,798	1,276	268	192	131	102	65	3,832

All data are shown as a number with the percentage in parentheses. Sputum includes respiratory related materials. Intra-body materials include catheters and drainage tube. Aspiration fluid indicates ascites, pleural effusion, and pericardial effusion.

Table 3. Yearly changes of species distribution of 131 *Candida* blood isolates detected at the National Cerebral and Cardiovascular Center from 2007 to 2011

Species	Number (%) of isolates					
	2007	2008	2009	2010	2011	Total
C. albicans	7 (41.1)	7 (43.8)	8 (44.4)	25 (59.5)	8 (25.0)	55 (42.0)
C. parapsilosis	2 (11.8)	4 (25.0)	3 (16.7)	11 (25.0)	16 (44.4)	36 (27.5)
C. glabrata	3 (17.7)	3 (18.8)	4 (22.2)	5 (11.4)	6 (16.7)	21 (16.0)
C. tropicalis	4 (23.5)	1 (6.3)	2 (11.1)	2 (4.6)	2 (5.6)	11 (8.4)
C. lusitaniae	—	1 (6.3)	—	1 (2.3)	3 (8.3)	5 (3.8)
C. guilliemondii	1 (5.9)	—	1 (5.6)	—	—	2 (1.5)
C. krusei	—	—	—	—	1 (2.8)	1 (0.8)
Total	17	16	18	44	36	131

All data are shown as a number with the percentage for each year in parentheses.

(16.0%), 11 (8.4%), 5 (3.8%), 2 (1.5%), and 1 (0.8%), respectively (Table 3), with 58% of the cases of fungemia caused by a non-*albicans* species.

Drug sensitivity

Data for the sensitivity of fungi to amphotericin B, flucytosine, fluconazole, micafungin, itraconazole, and voriconazole are shown in Table 4. Amphotericin B was not classified into S, S-DD, I, and R categories in the CLSI 2009 criteria.

The MIC_{90} of voriconazole against *C. albicans* (0.015) was the lowest among the 6 antifungal drugs, followed by micafungin (0.06), flucytosine (0.25), itraconazole (0.25), amphotericin B (0.5), and fluconazole (0.5). However, none of the 55 patients with candidemia caused by *C. albicans* showed resistance in the CLSI criteria (flucytosine $\geq$ 32, fluconazole $\geq$ 64, itraconazole $\geq$ 1, voriconazole $\geq$ 4). Of these 55 cases, 46 (83.6%) were S-DD to itraconazole and all 55 were sensitive to the other 5 antifungal drugs.

The MIC_{90} of voriconazole and against *C. parapsilosis* (0.125) was also the lowest among the antifungal drugs, followed by flucytosine (0.25), amphotericin B (0.5), itraconazole (1), micafungin (2), and fluconazole (16). The resistance rates of *C. parapsilosis* to fluconazole and itraconazole were 5.6% and 25.0%, respectively. The percentages of patients with S ($\leq$ 0.125), S-DD (0.25–0.5), and R ($\geq$ 1) responses were 27.8%, 47.2%, and 25.0%, respectively, for itraconazole. The percentages of patients with S ($\leq$ 8), S-DD (16–32), and R ($\geq$ 64) responses were 83.3%, 11.1%, and 5.6%, respectively, for fluconazole.

The MIC_{90} of micafungin against *C. glabrata*

Table 4. Antifungal susceptibilities of *Candida* blood isolates determined by microdilution method after 48 h of incubation

Species (number of isolates)	Antifungal agent	MIC (μg/ml) [a] range	50%	90%	% Resistant[b]
C. albicans (55)	Amphotericin B	0.13–1	0.5	0.5	—
	Flucytosine	< 0.13–1	0.13	0.25	0
	Fluconazole	< 0.13–2	0.25	0.5	0
	Micafungin	< 0.03–0.06	< 0.03	0.06	—
	Itraconazole	0.03–0.5	0.13	0.25	0
	Voriconazole	< 0.015–0.5	< 0.015	0.015	0
C. parapsilosis (36)	Amphotericin B	0.13–1	0.25	0.5	—
	Flucytosine	< 0.13–0.5	0.25	0.25	0
	Fluconazole	0.5–64	1	16	5.6
	Micafungin	0.25–2	0.5	2	—
	Itraconazole	0.13–2	0.25	1	25
	Voriconazole	< 0.015–1	0.03	0.125	0
C. glabrata (21)	Amphotericin B	0.13–1	0.5	1	—
	Flucytosine	< 0.13–0.25	< 0.13	0.13	0
	Fluconazole	8–> 64	16	64	19.1
	Micafungin	< 0.03–0.06	< 0.03	0.06	—
	Itraconazole	1–> 8	2	8	100
	Voriconazole	0.25–> 8	0.5	1	14.3
C. tropicalis (11)	Amphotericin B	0.13–1	0.25	0.5	—
	Flucytosine	0.13–4	0.25	0.25	0
	Fluconazole	1–> 64	8	> 64	36.4
	Micafungin	0.06–2	0.06	0.13	—
	Itraconazole	0.25–> 8	4	> 8	72.7
	Voriconazole	0.13–0.5	0.25	0.5	0

[a] 50% and 90% minimum inhibitory concentrations: MIC_{50} and MIC_{90}, respectively.
[b] Percentage of resistant strains according to CLSI breakpoints (CLSI M27-S3 2009)
CLSI: Clinical and Laboratory Standards Institute; "–" indicates break point is not established in CLSI M27-S3

(0.06) was the lowest among the antifungal drugs, followed by flucytosine (0.13), amphotericin B (1), and voriconazole (1). The resistance rate to voriconazole was 14.3%. The drug resistance rates of itraconazole and fluconazole were 100% and 19.1%, respectively.

The MIC_{90} of micafungin against *C. tropicalis* (0.13) was significantly lower than those for other drugs, followed by amphotericin B and voriconazole (both 0.5). The resistance rate of *C. tropicalis* against voriconazole was 0%.

Discussion

131 *Candida* strains isolated from blood at the NCVC from 2007 to 2011 showed species distribution, *C. albicans* 42.0%, *C. parapsilosis* 27.5%, *C. glabrata* 16.0%, *C. tropicalis* 8.4%, and *C. krusei* 0.8%. Our data and the results of a national surveillance study indicate that *C. albicans* is still the major causal fungus of candidemia in Japan. In *C. albicans* no isolate with resistance to fluconazole ≥ 64 μg/ml was found in this study[7]. The 90%MIC was 0.5 μg/ml and the isolate with

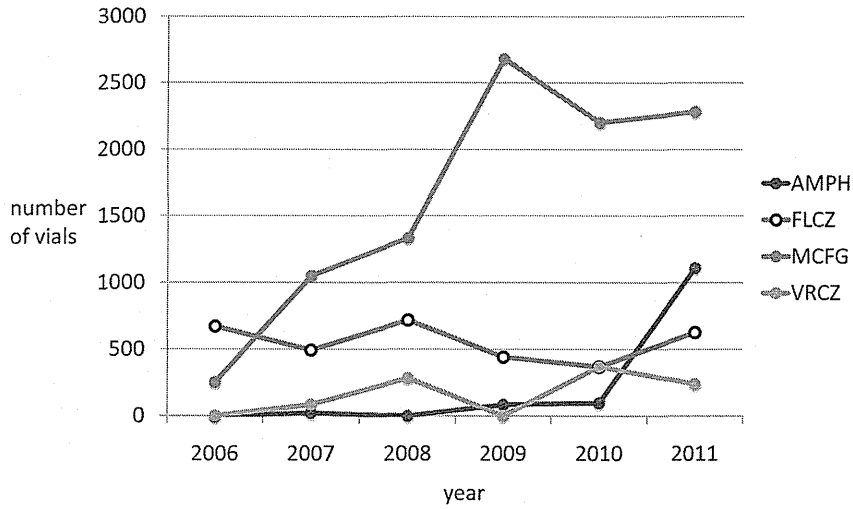

Fig. 1. Use of antifungal agents. Fluconazole was the most commonly used antifungal drug in 2006, whereas micafungin was most commonly used from 2007 to 2011. Use of AMPH increased in 2011, which reflects the increase of fungemia caused by *C. parapsilosis*. AMPH: amphotericin B, FLCZ: fluconazole, MCFG: micafungin, VRCZ: voriconazole.

lowest susceptibility required 2 μg/ml. These findings led us to use an antifungal susceptibility-based management strategy in NCVC for treatment for known *C. albicans* infection in which fluconazole is the first line antifungal drug.

The rates of resistance of *C. glabrata* to itraconazole and fluconazole (100% and 20%) were greater than those (56.3% and 5.2%) in Takakura et al[7]. High rates of resistance to itraconazole for *C. glabrata* detected in the bloodstream were also found by Myoken (100%, 8/8) [8] and St-Germain et al. (83.3%, 65/78) [9]. In our study, none of the 25 *Candida* isolates with reduced susceptibility to fluconazole (MIC $\geq$ 16 μg/ml) was susceptible to itraconazole (MIC $\leq$ 0.12 μg/ml). However, Pfaller et al. suggested that MICs of $\leq$ 1 μg/ml may better reflect 'susceptibility' in invasive candidiasis, due to the higher serum concentrations achievable with the new nanocrystal intravenous formulation of itraconazole[10]. Given this new threshold, 40.0% of our isolates with reduced susceptibility to fluconazole would be considered susceptible to itraconazole. Furthermore, our observations are similar to those of Pfaller et al., with all four of our *C. glabrata* isolates that were resistant to fluconazole also showing resistance to itraconazole.

The higher resistance rate of *C. tropicalis* to fluconazole (36.4%) compared to reports from the USA (6.2%) and Spain (16.6%) is another characteristic of non-*albicans* candidemia in this study[11, 12]. We attribute this high resistance to the consistent and high frequency use of fluconazole in our facility. Interestingly, for each case of fluconazole-resistant non-*albicans* candidemia (4 isolates of *C. glabrata*, and 4 isolates of *C. tropicalis*), micafungin showed high sensitivity and can be regarded as the first choice for treatment of fluconazole-resistant *C. glabrata* and *C. tropicalis*. Voriconazole showed no resistance to *C. tropicalis* and may be used as the second choice for these isolates in our hospital; however, voriconazole showed a resistance rate of 43.5% in a national survey[7]. This discrepancy suggests that the susceptibility of each species of *Candida* differs from hospital to hospital, due to the different disease backgrounds and treatments at each center. This indicates that antifungal drug susceptibility at each facility should be considered in the selection of antifungal drugs.

In this study, the greatest number of fungi in the bloodstream was detected in 2011 and the incidence of the disease caused by *C. parapsilosis* (n = 16) was the highest in the same year. The incidence of candidemia caused by *C. albicans* gradually decreased in the study period. We attribute this increase of *C. parapsilosis* to the increase in operations for candidates for heart transplantation and for neonates with congenital heart diseases. These immunologically compromised patients underwent treatments including central line management, which is a known risk

factor for *C. parapsilosis*. This increase in *C. parapsilosis* caused a temporary increase in use of amphotericin B in 2011 (Fig. 1). The selection of this drug for *C. parapsilosis* has turned out to be appropriate because in this study fluconazole showed a resistance rate of 5.6% and a MIC_{90} with micafangin that was as high as 2.0 μg/ml. The MIC_{90} of voriconazole was 0.125, which makes this drug the second choice for *C. parapsilosis* in the NCVC.

We introduced micafungin for treatment of deep mycosis in 2004. By 2006, micafungin accounted for 27% of all antifungal drugs used in the NCVC and from 2009 to 2011 this rate reached 70%. This increased use has occurred because micafungin is an echinocandin that has a broad antifungal spectrum and exhibits good activity against azole antifungal drug-resistant strains. Micafungin is effective in fungal cell lines and several reports have shown excellent tissue penetration and clinical effects[13, 14]. Thus, micafungin has been most commonly used at the NCVC since 2007, including preservational use for immunocompromised patients, such as those undergoing cardiac transplantation or in extremely low-birthweight infants in the NICU. However, several clinical isolates of *Candida* with low resistance to echinocandin antifungal drugs have been described and care is taken regarding this issue at the NCVC[15–18]. The mechanism of this reduced sensitivity involves a mutation in FkslP, which is a 1,3β-D-glucan synthase subunit of the target enzyme of echinocandins[15–18]. No strains with reduced sensitivity to micafungin were found in this study. However, as clinical use of the drug continues to increase, particular attention should be paid to the sensitivity of clinical isolates to micafungin.

C. lusitiniae is an infrequent cause of fungemia, but the rate obtained in this study (3.8%) was 6.8 times higher than that in Takakura[7] and Minari et al[19]. The reported underlying conditions for patients with deep seated *C. lusitaniae* infections are malignancy 53%, neuropenic 35%, receiving broad-spectrum antibiotics 27%, receiving long-term corticosteroid therapy 16%, and having a central venous catheter 27%[20]. Although fungemia is the most common type of *C. lustaniae* infection (80%), primary infection focuses were identified in 20% of cases[20]. These included endocarditis, infection of a left ventricular device, meningitis, chorioamnionitis, peritonitis, abdominal abscess, and cutaneous infection, and most of these diseases are treated at our center. These facts may be related to the higher detected rate of *C. lustaniae* fungemia at our institution. *C. tropicalis* and *C. krusei* are likely to cause deep mycosis in patients with hematologic tumors undergoing digestive tract surgery, but this type of surgery is not performed at the NCVC. This may explain the low incidence of fungemia at the NCVC due to these species[2–4].

In summary, it is important to comprehend the susceptibility for antifungal drug and distribution of each *Candida* isolate of each hospital in selection of antifungal drug, due to the different disease backgrounds and treatments at each center.

Limitations

The sample population was small in this study. In particular, only 11 patients had *C. tropicalis*, which is the minimum required to calculate MIC, and further validation of this result is required. Drug sensitivity may vary depending on the actual treatment in medical institutions, in particular regarding use of the antifungal drug. The NCVC is a specialized center for internal medicine and cardiovascular surgery in patients with cardiovascular disorders or cerebrovascular accident, in contrast to the roles of secondary or tertiary hospitals for general patients. Therefore, it is important to study the drug sensitivity of fungi and measures to be taken against infections in centers such as the NCVC, in which immunocompromised patients are treated, including those undergoing cardiac transplantation, even if the study population is small.

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Conflict of Interest Statement

None of the authors has a conflict of interest regarding the work in this study.

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Pregnancy-associated Intracranial Hemorrhage: Results of a Survey of Neurosurgical Institutes across Japan

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Background: Pregnancy-associated hemorrhagic stroke is considered a serious complication. Although coagulopathy, pregnancy-induced hypertension, eclampsia, and other systemic complications have been emphasized, pre-existing cerebrovascular diseases (CVDs) have not been fully analyzed. To clarify the role of these vascular lesions more in detail, the Japan Neurosurgical Society conducted a nationwide survey on all the neurosurgical institutes across Japan. *Methods:* This 2-year survey focused on hemorrhagic stroke occurring in pregnancy, delivery, and puerperium. Clinical data based on retrospective chart review were obtained through a questionnaire and analyzed according to the time of onset, underlying CVDs, obstetric systemic complications, therapeutic approaches, and maternal and neonatal prognoses. *Results:* The survey identified 97 hemorrhagic strokes that were associated with pregnancy. Baseline CVDs responsible for hemorrhage were detected in 54 cases (55.7%), among which 47 lesions (87.0%) had been undiagnosed before stroke onset. The detection rate of baseline CVDs before the 32nd week of gestation was significantly higher than that after the 32nd week (90.0% versus 53.3%, $P = .0017$). Arteriovenous malformations (AVMs) were the most frequent CVDs causing intracranial hemorrhage, occurring at 1.8 times the frequency of ruptured aneurysms during pregnancy. Poor outcomes, including 10 deaths, were seen in 36.1% of the cases despite aggressive treatment. *Conclusion:* Pregnancy-associated hemorrhagic strokes frequently concealed baseline CVDs, especially when they occurred before the 32nd week of gestation. AVMs were the predominant bleeding source. For appropriate treatment, therefore, close examination for cerebral vascular lesions is essential when a pregnancy-associated hemorrhagic stroke is encountered. **Key Words:** Pregnancy—stroke—intracranial hemorrhage—arteriovenous malformation—cerebral aneurysm—moyamoya disease.

Introduction

Pregnancy-associated hemorrhagic stroke is well recognized as a serious complication.[1,2] In previous studies conducted mainly by neurologists and obstetricians, systemic obstetric complications including coagulopathies, pregnancy-induced hypertension, and eclampsia were identified as the causes of hemorrhage.[2-7] Pre-existing cerebrovascular diseases (CVDs) such as cerebral aneurysms and arteriovenous malformations (AVMs) were also reported,[4,8] but their incidence and treatments were not fully analyzed. The Japan Neurosurgical Society, therefore, set out to conduct a survey of neurosurgical institutes across Japan regarding pregnancy-associated hemorrhagic stroke with a special focus on identifying underlying CVDs.

Methods

This study is a retrospective analysis based on the clinical chart review in each neurosurgical institute and was

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conducted in 2 phases (primary and secondary surveys) in 2012 as an official project of the Japan Neurosurgical Society. The society has 109 main training institutes across Japan under which 755 affiliated local training institutes participate in providing neurosurgical services. The target of the primary survey was all strokes occurring during pregnancy, delivery, and puerperium (no later than 6 weeks after delivery) that were treated in these institutes between January 2010 and December 2011. In the primary survey, all 109 main training institutes were assigned to compile the number of pregnancy-associated strokes treated in their own hospitals or affiliated local training institutes during the earlier mentioned period. The results were e-mailed to the survey office without any clinical information, and only the e-mail address of the corresponding physician in each case was provided. In the secondary survey, a questionnaire requesting detailed clinical information on each case was e-mailed to each corresponding physician and returned to the survey office without any personally identifying information attached. The clinical information included stroke type and time of stroke onset (gestational age or time after delivery), causes of hemorrhage, types of underlying CVDs, types of obstetric systemic complications, therapeutic procedures for strokes, methods of delivery, and maternal and neonatal prognoses.

Feedback on the primary survey was obtained from 102 (93.6%) main training institutes covering 729 affiliated local training institutes. The survey office sent secondary survey questionnaires to the 126 attendant physicians who had declared their experience with pregnancy-associated stroke and received feedback from 100 physicians (79.4%). After determining the eligibility of each case and eliminating duplications resulting from patient transfer between institutes, the authors extracted 134 cases. These strokes were divided into 97 hemorrhagic strokes (intracerebral or subarachnoid hemorrhage) and 37 other strokes (eg, cerebral arterial infarction or venous infarction), and the former 97 cases were submitted for the further analysis. Intracranial hemorrhage was confirmed by computed tomography (CT) or magnetic resonance (MR) imaging in all cases, and bleeding sources were further examined by MR angiography, digital subtraction angiography, or CT angiography except for a few cases of early death that could not allow further examinations.

Statistical Methods

The data were presented as frequency or means within a standard deviation. Fisher exact probability test and Mann–Whitney *U* test were applied to categorical data. All analyses were performed with Statcel 3 software (OMS Publishing, Inc., Tokorozawa, Japan). Prognosis of the patients was expressed with the modified Rankin Scale (mRS)[9] at discharge.

Table 1. *Demographics of patients with pregnancy-associated hemorrhagic stroke*

	n = 97 (100%)
Mean age (y)	32.2 ± 5.4
Timing of onset	
During pregnancy	
Number of cases	60 (61.9%)
Mean gestational age at onset (wk)	27.7 ± 10.1
At delivery	
Number of cases	13 (13.4%)
Mean delivery weeks	38.4 ± 3.7
Puerperium	
Number of cases	24 (24.7%)
Time after delivery	
<24 h	8
1-3 d	4
3-7 d	3
8-42 d	8
Unknown	1

Results

Patient Demographics

Table 1 summarizes the patient demographics. Among the all 97 hemorrhagic strokes, 60 (61.9%) occurred during pregnancy, 13 (13.4%) at delivery, and 24 (24.7%) during puerperium. Mean gestational age at the onset of hemorrhage during pregnancy was 27.7 ± 10.1 weeks.

Causes of Hemorrhage in Each Period

Figure 1, A shows the causes of hemorrhagic stroke throughout all periods (pregnancy, delivery, and puerperium). Baseline CVDs responsible for hemorrhage were detected in 54 cases (55.7%). Among all vascular lesions, AVMs are the most frequent cause of hemorrhage, followed by cerebral aneurysms and moyamoya disease. Another 3 lesions were also detected, including 2 cavernous malformations and 1 hemorrhage from the vasculature of an intraparenchymal tumor. Of all the detected CVDs, only 7 lesions (13.0%) had been diagnosed before pregnancy, and 47 lesions (87.0%) including all the aneurysms, 92.0% of AVMs, and 60.0% of moyamoya diseases had remained undiagnosed before stroke onset. Fourteen obstetric complications were identified, including pregnancy-induced hypertension, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, eclampsia, and disseminated intravascular coagulation. Because 2 of these complications were accompanied by bleeding from the AVM and moyamoya disease, they were categorized as "baseline CVDs," and the other 12 cases were categorized as "obstetric complication" in Figure 1. The cause could not be determined in 31 cases (32.0%). Figure 1, B illustrates the causes of hemorrhage in each period. The CVD detection rate was

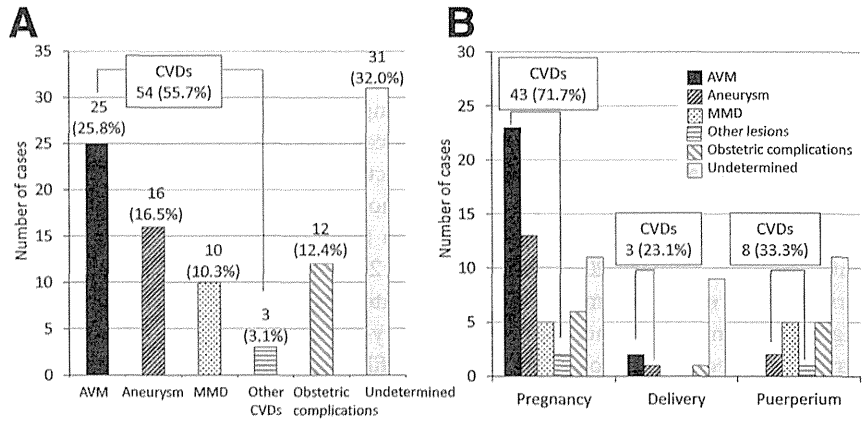

Figure 1. *(A) Causes of hemorrhagic stroke throughout all periods (pregnancy, delivery, and puerperium). (B) Causes of hemorrhagic stroke in each period. Abbreviations: AVM, arteriovenous malformation; CVDs, cerebrovascular diseases; MMD, moyamoya disease.*

71.7% for hemorrhage during pregnancy, 23.1% at delivery, and 33.5% during puerperium. Twenty-three of 25 AVM ruptures (92.0%) were detected during pregnancy, and none were detected during puerperium. Aneurysmal rupture occurred in all periods, but 13 of 16 ruptures (81.3%) were detected during pregnancy. All 6 hemorrhages related to obstetric complications were seen after the 32nd week of gestation.

Gestational Age at Onset and Cause of Hemorrhage during Pregnancy

Figure 2 shows the gestational age at onset of hemorrhagic stroke during pregnancy. Hemorrhagic strokes remarkably increased in number at a later gestational age. Although hemorrhagic strokes before and after the 32nd week of gestation were equal in number (30 cases each), the detection rate of baseline CVDs reached 90.0% (27 of 30) before the 32nd week of gestation, which was significantly higher than that after the 32nd week (53.3%, $P = .0017$ in Fisher exact probability test). Hemorrhagic stroke without baseline CVDs occurred significantly later than that with CVDs (mean 33.7 ± 8.7 weeks versus 25.3 ± 9.6 weeks, respectively; $P < .001$ in Mann–Whitney U test).

Figure 3 compares AVMs and cerebral aneurysms in terms of the gestational age at the onset of hemorrhage. The mean ages at the onset of AVM rupture and aneurysmal rupture were 24.6 ± 9.2 and 27.4 ± 10.4 weeks of gestation, respectively. More specifically, 13 of the 23 AVM ruptures (56.5%) occurred during the latter half (after the 22nd week) of pregnancy, whereas 10 of the 13 aneurysmal ruptures (76.9%) occurred during that same period. Although not statistically significant, aneurysmal rupture had a greater tendency to occur during the latter half of pregnancy than did AVM rupture ($P = .195$, Fisher exact probability test).

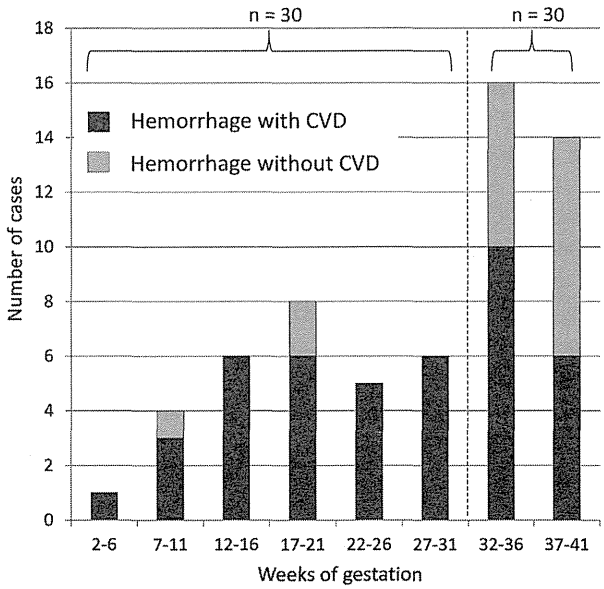

Figure 2. *Distribution of hemorrhagic strokes with and without determined baseline. CVDs by gestational age at onset. Abbreviation: CVD, cerebrovascular disease.*

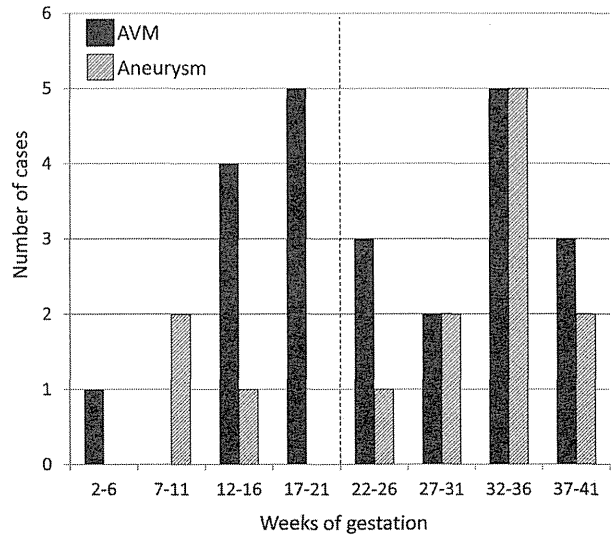

Figure 3. *Distribution of hemorrhagic strokes caused by AVMs and aneurysms by gestational age at onset. Abbreviation: AVM, arteriovenous malformation.*

Table 2. *Therapeutic approaches for pregnancy-associated hemorrhagic stroke*

AVM (n = 25)		
Emergent surgery	15	
Nidus removal		8
Hematoma removal		6
Ventricular drainage		1
Delayed surgery (nidus removal)	2	
Surgery in unknown period (nidus removal)	2	
Embolization in unknown period	1	
Nonsurgical treatment	4	
Unknown	1	
Aneurysm (n = 16)		
Emergent neck clipping	11	
Emergent embolization	4	
None (dead on arrival)	1	
Moyamoya disease (n = 10)		
Emergent surgery	3	
Hematoma removal		1
Ventricular drainage		2
Nonsurgical treatment	7	
Other CVDs (n = 3)		
Emergent hematoma removal	1	
Nonsurgical treatment	2	
Hemorrhage without baseline CVDs (n = 43)		
Emergent surgery	16	
Hematoma removal		10
Ventricular drainage		6
Nonsurgical treatment	27	

Abbreviations: AVM, arteriovenous malformation; CVDs, cerebrovascular diseases.

Therapeutic Approaches and Modes of Delivery

Table 2 summarizes the therapeutic approaches applied to hemorrhagic stroke. Among all cases, 55 (56.7%) required surgical treatment (direct surgery or endovascular surgery) and at least 50 (51.5%) were performed emergently. Eight of 25 AVMs were emergently removed by craniotomy, whereas in the other 7 cases hematoma removal or ventricular drainage was performed without nidus resection. All the aneurysms were emergently clipped or embolized except for 1 case found to be dead on arrival.

Figure 4 shows the methods of delivery adopted in 60 cases of hemorrhagic stroke during pregnancy. Before the 22nd week, induced abortion was selected in 36.8% of the cases, whereas gestation was continued until the elective delivery in 52.6%. When hemorrhage occurred after the 32nd week, 90% of the patients underwent emergent delivery, 96.3% of which were carried out by cesarean section.

Clinical Outcomes of Patients and Children

Figure 5 illustrates the maternal clinical outcomes. Poor outcome (mRS score at discharge ≥ 3) was observed in 35 patients (36.1%). Fatal cases totaled 10,

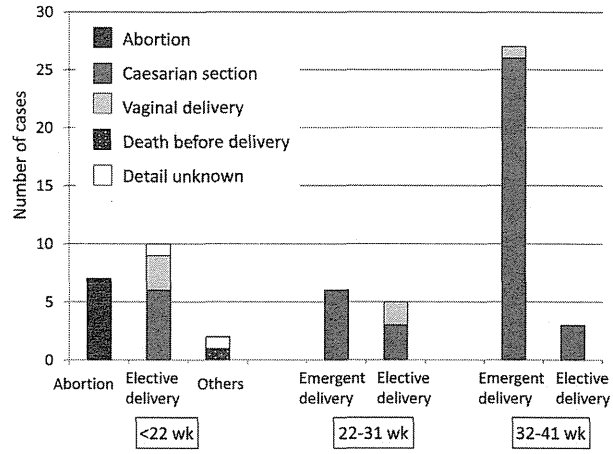

Figure 4. *Methods of delivery adopted after the onset of hemorrhagic stroke during pregnancy.*

making the mortality rate to be 10.3%. Hemorrhage without baseline CVDs showed a higher rate of poor outcome (mRS score ≥ 3) than did that associated with detected CVDs (41.9% and 32.1%, respectively), but the difference was not statistically significant (P = .22, Fisher exact probability test).

Analysis of the prognosis for the children revealed that 81 (83.5%) were normal, whereas 1 (1.0%) had some sequelae and 1 (1.0%) died with the mother. There were 8 cases of abortion (8.2%): 7 were forced abortions after stroke at an early gestational age and 1 was an elective abortion followed by a fatal hemorrhage within 24 hours postpartum. Prognosis of 6 (6.2%) children was not reported.

Discussion

Pregnancy-associated intracranial hemorrhage is a rare but potentially devastating event. A large population-

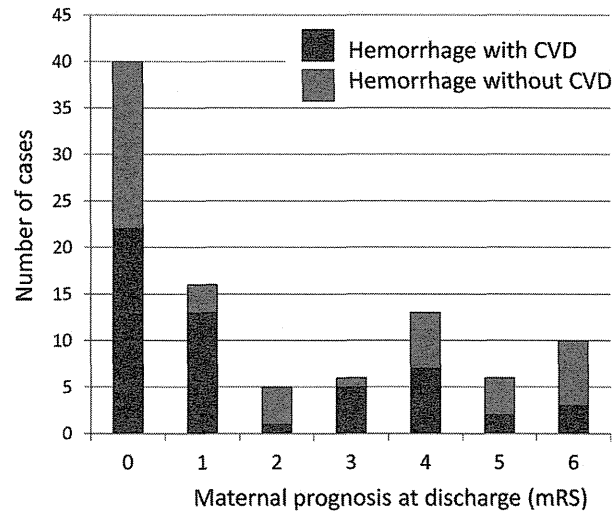

Figure 5. *Maternal outcomes after hemorrhagic stroke with and without determined baseline CVDs. Abbreviations: CVD, cerebrovascular disease; mRS, modified Rankin Scale.*

Table 3. *Recent studies on pregnancy-associated hemorrhagic stroke*

Reference	Country	Total number	Causes of hemorrhage					Rate of CVDs (%)
			AVM	AN	MMD	Other CVDs	No CVD	
Simolke et al[13]	United States	6	1	1	0	0	4	33.3
Sharshar et al[12]	France	16	2	2	0	2	10	37.5
Kittner et al[5]	United States	14	3	0	0	0	11	21.4
Witlin et al[14]	United States	6	1	2	0	0	3	50.0
Jaigobin et al[8]	Canada	13	5	3	0	0	5	61.5
Witlin et al[15]	United States	5*	1	0	0	0	4	20.0
Jeng et al[4]	Taiwan	22	5	3	0	0	14	36.4
Liang et al[1]	Taiwan	21	4	2	0	0	15	28.6
Scott et al[7]	United Kingdom	12	1	3	0	0	8	33.3
Present series	Japan	97	25	16	10	3	43	55.7

Abbreviations: AN, aneurysm; AVM, arteriovenous malformation; CVDs, cerebrovascular diseases; MMD, moyamoya disease.
*Confined to postpartum stroke.

based epidemiologic study in Sweden conducted by retrospective *International Classification of Diseases, Ninth Revision*, code analysis revealed its incidence to be 6.2 (2.4 for subarachnoid hemorrhage and 3.8 for intracerebral hemorrhage) per 100,000 deliveries.[10] A more recent survey in the United States also based on the *International Classification of Diseases, Ninth Revision*, codes reported the incidence of intracerebral hemorrhage to be 6.1 per 100,000 deliveries.[11] In the study in Taiwan, on the other hand, much higher incidence has been reported (31.4 per 100,000 deliveries for all the intracranial hemorrhage).[1]

Causes of the hemorrhage emphasized in the previous studies have been rather different between one and another. In the earlier mentioned survey in the United States, various risk factors including pre-eclampsia/eclampsia, hypertension, and coagulopathy were pointed out and emphasized,[11] but the pre-existing CVDs were not analyzed in detail. A study from France particularly emphasized eclampsia that accounted for 44% of intracerebral hemorrhage although rupture of vascular lesions was found in 37%.[12] Several studies have described CVDs as the cause of hemorrhage, with the detection rate ranging from 21.4% to 61.5% (Table 3), but CVDs were not analyzed deeply, presumably because of the scarcity of such cases in these studies.[1,4,5,7,8,12-15] To the authors' knowledge, the present study of 97 cases is the first to undertake detailed analysis of baseline CVDs in pregnancy-associated stroke.

This study has revealed 2 important findings: first, hemorrhagic stroke conceals baseline CVDs at high frequency, especially before the 32nd week of gestation, and most had not been diagnosed until the bleeding had occurred. It can also be said that CVD-unrelated hemorrhages caused by obstetric complication or unknown etiology occur significantly later than those related to CVDs. The cause of this phenomenon has not been proven, but it is likely that the remarkable physiological changes occurring in late gestation are related to the increase in CVD-unrelated hemorrhage. As to the absolute CVD detection rate, the authors must clearly acknowledge the possibility of inclusion bias: this being a survey of neurosurgical institutes, it is possible that hemorrhagic cases diagnosed as having CVDs in the previous hospital could have been transferred selectively. An examination of the patient transfer state, however, revealed that 55.0% of the pregnant patients with intracranial hemorrhage were directly admitted in the surveyed institutes and that 36.7% had been transferred from the obstetric institution immediately after the diagnosis of hemorrhage without advanced examination of cerebral vascular lesions. This indicates that a total of 91.7% of our cases were free from this bias. Another highly possible bias is that patients with mild hemorrhage were treated by obstetricians or neurologists without a neurosurgeon being consulted and were thus excluded from the present study. Although there has been no evidence that severe hemorrhages are likely to be accompanied with CVDs and mild ones are not, the authors must admit the limitation of the present study with regard to this point. Accordingly, it might be proper to discuss the significant difference in CVD detection rate between the period before and the period after the 32nd week, rather than to argue the absolute value itself. At any rate, it is essential that patients presenting with intracranial hemorrhage during pregnancy be carefully examined for underlying CVDs.

The second novel finding is that AVMs are the predominant bleeding source, being 1.8 times more frequent than cerebral aneurysms during pregnancy. In the general population, AVM rupture is approximately one tenth as frequent as aneurysmal rupture[16,17]; even when confined to young adults, it is still one third as frequent as bleeding from an aneurysm.[18] These findings strongly suggest that physiological changes during pregnancy have a significant impact on the vasculature of AVMs, and ruptures during pregnancy are by no means coincidental. A review of the literature by neurosurgeons once

counted the number of past cases of pregnancy-related hemorrhage from aneurysms and AVMs and described the predominance of aneurysms compared with AVMs (77% versus 23%, respectively),[19] but these data were compiled from different countries and times and included many old case reports before the CT era. Recently, several studies showed the predominance of vascular malformations as shown in Table 2.[1,4,5,8] The small number of cases, however, precluded a robust conclusion about their prevalence. The present survey has clearly disclosed the predominance of AVMs at least in the Japanese population. Because no study proves a higher prevalence of AVMs in Asians than in Caucasians, the authors believe that this predominance is also applicable to Western populations.

All the aneurysms in the present series were emergently clipped or embolized except for 1 case found to be dead on arrival. This strategy apparently follows the recent recommendation that ruptured aneurysms should be managed in the same way as in the nonpregnant population.[19-21] Management of ruptured AVMs during pregnancy, on the other hand, has not yet been discussed in depth. Unlike cerebral aneurysms, AVMs exhibit a wide diversity in their amenability to surgical resection, from small resectable lesions in a noneloquent cortex to huge, deep-seated ones that cannot be removed.[17,22] Consequently, various surgical approaches were applied in the present study, including emergent nidus resection, ventricular drainage, and hematoma removal leaving the nidus unresected. The authors believe that ruptured AVMs should also be managed in the same manner as they are in the nonpregnant population, even during gestation. The mode and timing of surgery should be determined according to the size and location of the nidus, anatomical pattern of drainage, and volume of the intracerebral hematoma.[17,22]

This survey detected 10 hemorrhages caused by moyamoya disease, which accounted for 10.3% of all cases. Recently, pregnancy-associated stroke in moyamoya disease was closely studied in Japan, and the significance of both ischemia and hemorrhage has been emphasized.[23] The authors believe that the findings regarding moyamoya disease are applicable at least to other Asian countries.

A poor prognosis was identified in 36.1% of all the cases, with mortality reaching 10.3% despite aggressive treatment. This raises the question of whether it is possible to avoid these tragedies. Pre-existing CVDs, as described earlier, play a significant role in pregnancy-associated hemorrhagic stroke, and most remain undiagnosed until stroke onset. Certainly, nonpreventable strokes can occur in the absence of CVDs. Some obstetric complications might also be unavoidable. Clearly, however, one key to prevent a tragic hemorrhage is to detect the underlying CVDs before gestation. A routine brain checkup with MR angiography before pregnancy might reveal these lesions, but implementing such a strategy is not realistic from the viewpoints of medical economics, social and ethical issues surrounding marriage, and morbidity resulting from therapeutic intervention for CVDs that have remained asymptomatic. The familial occurrence of cerebral aneurysms, however, is well recognized, although most AVMs are sporadic.[24,25] The incidence of familial intracranial aneurysms (at least 2 affected first-degree relatives in the same family) among the patients of subarachnoid hemorrhage is 6%-10%,[26-28] and the relative risk for cerebral aneurysms among first-degree relatives in familial intracranial aneurysms families has been reported to be 4.2.[24] Moyamoya disease is also known to have genetic components,[29] and a lot of highly aggregated families with moyamoya disease has been reported.[30] Therefore, it should not be unreasonable to consider a medical checkup with brain MR angiography, at least for women anticipating pregnancy who have dense familial history. Although much discussion is needed, a poor maternal prognosis demands that we continue to address ways to prevent tragic pregnancy-associated strokes.

Conclusions

A nationwide survey revealed that underlying CVDs play an important role in hemorrhagic stroke associated with pregnancy, among which AVM is the predominant bleeding source. Careful examination for vascular lesions is, therefore, essential when dealing with intracranial hemorrhage, especially before the 32nd week of gestation. As maternal prognosis after hemorrhagic stroke has been proved to be poor, a greater effort should be made to prevent tragic pregnancy-associated stroke.

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Annual report of Subcommittee for Examination of Causes of Maternal Death and their Prevention in Perinatology Committee, Japan Society of Obstetrics and Gynecology, 2013

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Introduction

Hemorrhage in the third stage of labor is the most frequent cause of maternal death. A national survey conducted by the subcommittee last year revealed the following bleeding-related factors during the third stage of labor: (i) atonic bleeding; (ii) abnormal placental adherence; (iii) abnormal placental adherence plus atonic bleeding; and (iv) placental abruption. In short, atonic bleeding is the most important factor associated with massive bleeding during the third stage of labor. In addition to this, the following two studies have been conducted this year:

Study 1

A secondary investigation to clarify the pathology of frequently occurring atonic bleeding, involving the same patients as those studied last year.

Study 2

To examine the relationship between the type of amniotic fluid embolism and autopsy findings, in order to clarify the pathology of amniotic fluid embolism and improve the survival rate.

Discussion

In study 1, the results demonstrated that the fibrinogen level decreases earlier than the platelet count and antithrombin III (AT III) activity when atonic bleeding occurs; however, the fibrinogen level was measured immediately after occurrence in only 33% of all patients. Considering that the fibrinogen level was not correlated with the platelet count or AT III activity, it may be important to measure fibrinogen levels in early stages, in order to determine the pathological condition and severity of atonic bleeding. While myometrial fatigue due to prolonged labor and weak pains generally regarded as the main cause of atonic bleeding, in this study, its occurrence was not associated with prolonged labor, weak pains or the use uterotonic agents. On the other hand, with an increase in the volume of bleeding and obstetrical disseminated intravascular coagulation (DIC) scores, packed red blood cells and fresh frozen plasma (FFP) were administrated. As the fibrinogen level decreases early in atonic bleeding, the early administration of FFP may be important as an initial approach to treat the disease.

In study 2, amniotic fluid embolism was classified into two types: that involving cardiopulmonary collapse; and that following DIC. Pathologically, the former type is conventional, in which fetal and amniotic fluid

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components are observed in pulmonary blood vessels. The pathological characteristics of the latter type include uterine atony, and the presence of fetal and amniotic fluid components in uterine blood vessels. In this type, fetal and amniotic fluid components are occasionally absent in the lungs. Among cases of clinical amniotic fluid embolism without fetal and amniotic fluid components in the lungs (or pulmonary examination findings are unavailable in life-saving settings), those involving uterine atony in the presence of fetal and amniotic fluid components in uterine blood vessels may be called uterus-type amniotic fluid embolism.

Disclosure

The authors have no conflict of interest to declare.

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

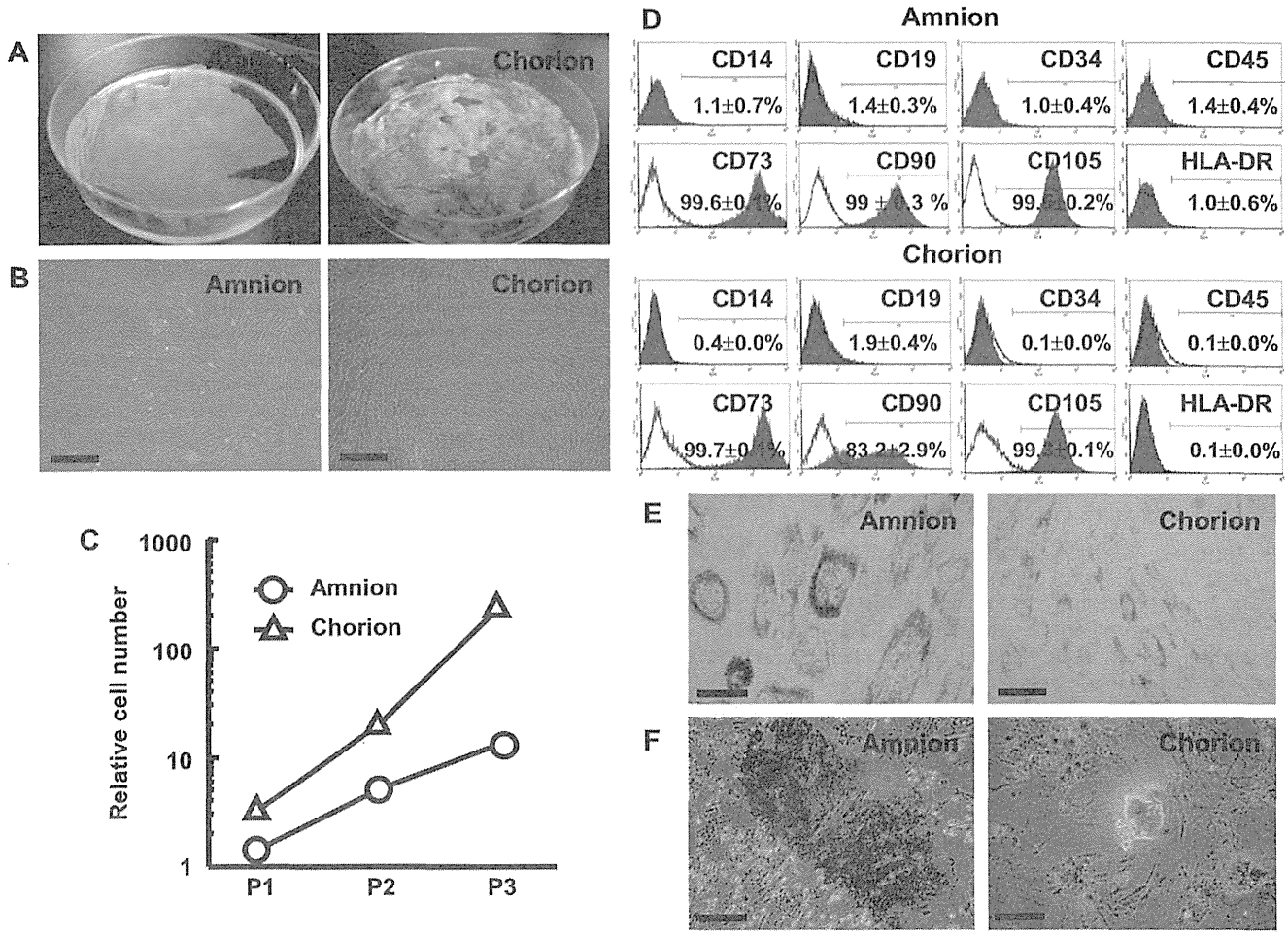

Figure 1. Characterization of human amnion- and chorion-derived MSCs. (A) Representative photographs of human amnion and chorion. (B) Photographs of cultured MSCs obtained from human amnion and chorion at passage 3. Scale bars = 500 μm. (C) Relative cell number of amnion- and chorion-derived MSCs at 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-

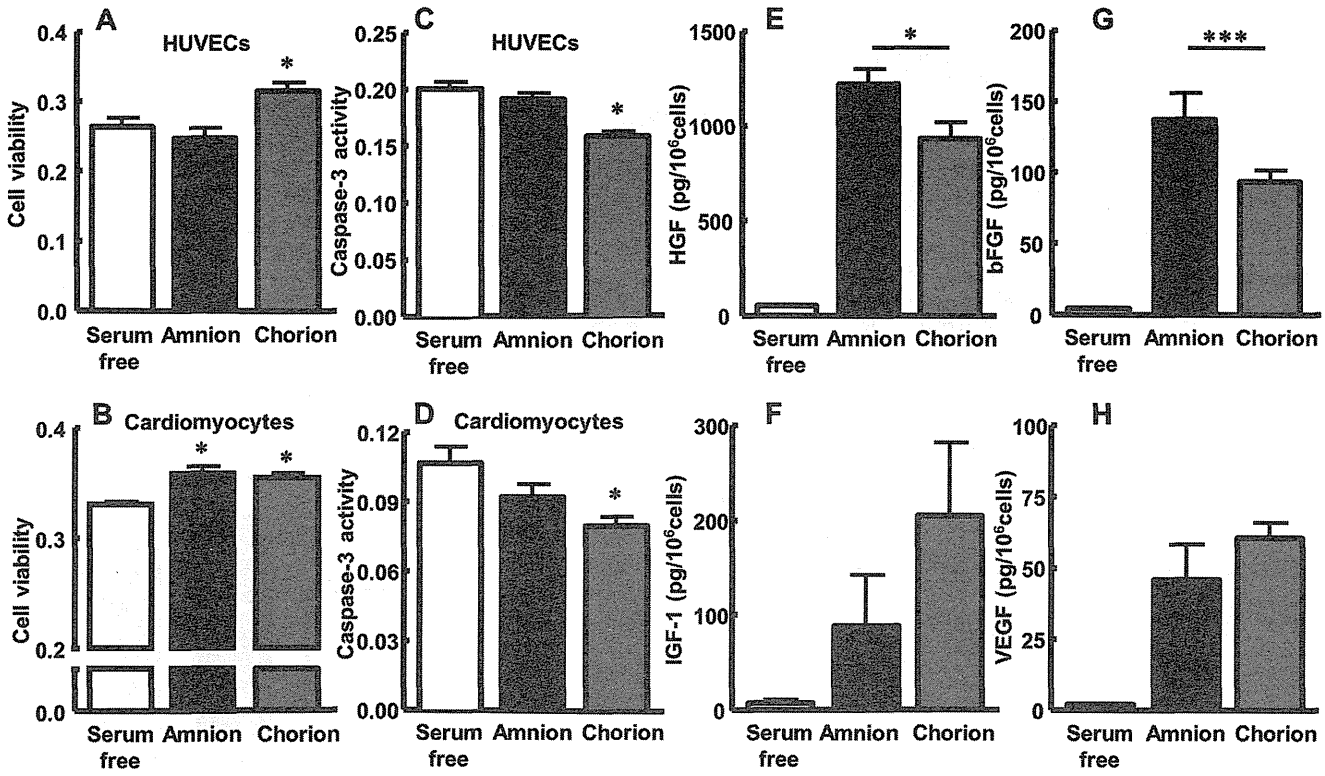

Figure 2. Growth factor secretion and the cytoprotective effect of amnion and chorion MSCs. (A–D) Cytoprotective effect of FM MSC-derived conditioned medium was analyzed by the MTS assay (A, B) and caspase-3 activity (C, D) in HUVECs (A, C) and cardiomyocytes (B, D). Values are mean ± SEM. *p<0.05 vs. 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+,

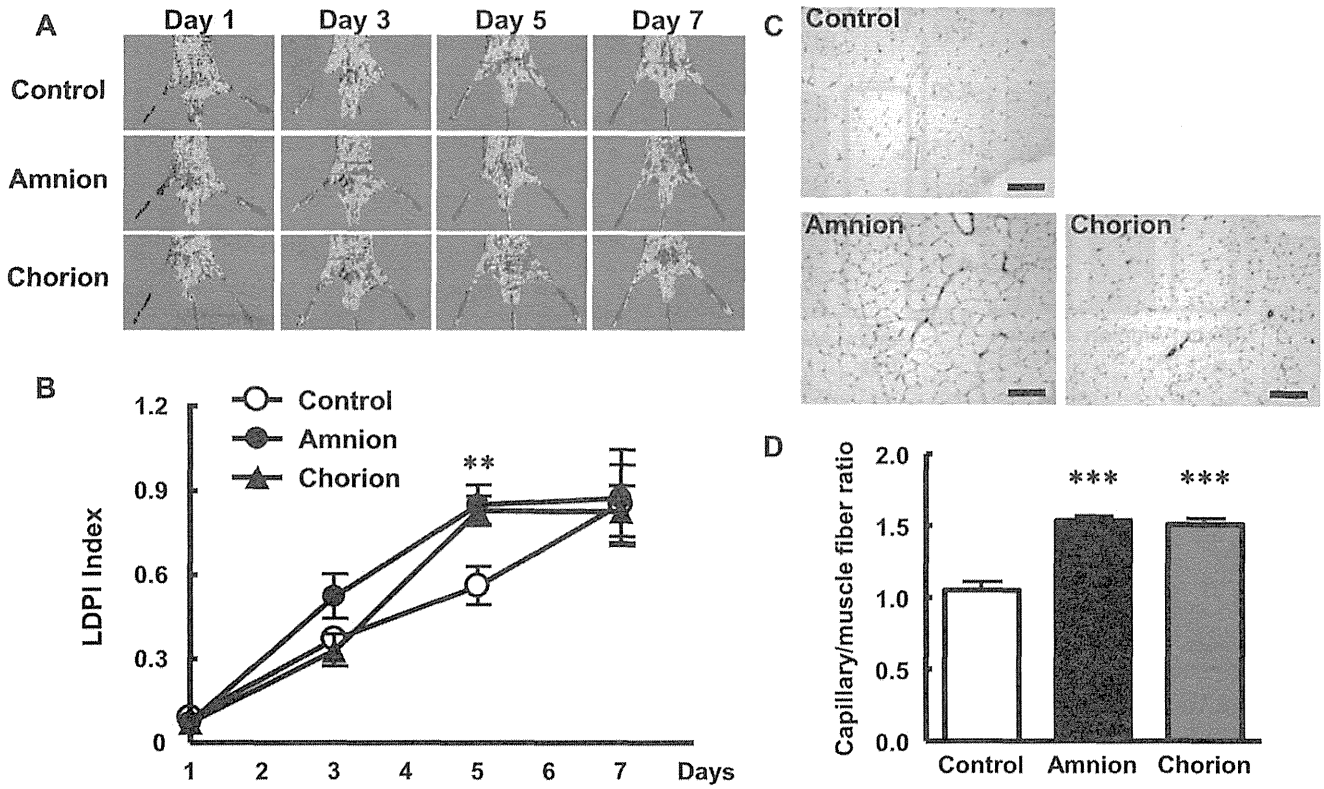

Figure 3. Angiogenic potential of amnion and chorion MSCs against hindlimb ischemia. (A) Representative images of serial hindlimb blood perfusion. Blood perfusion of ischemic hindlimb increased in the amnion and chorion MSC groups at day 5. (B) Quantitative analysis of hindlimb blood perfusion with the 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-

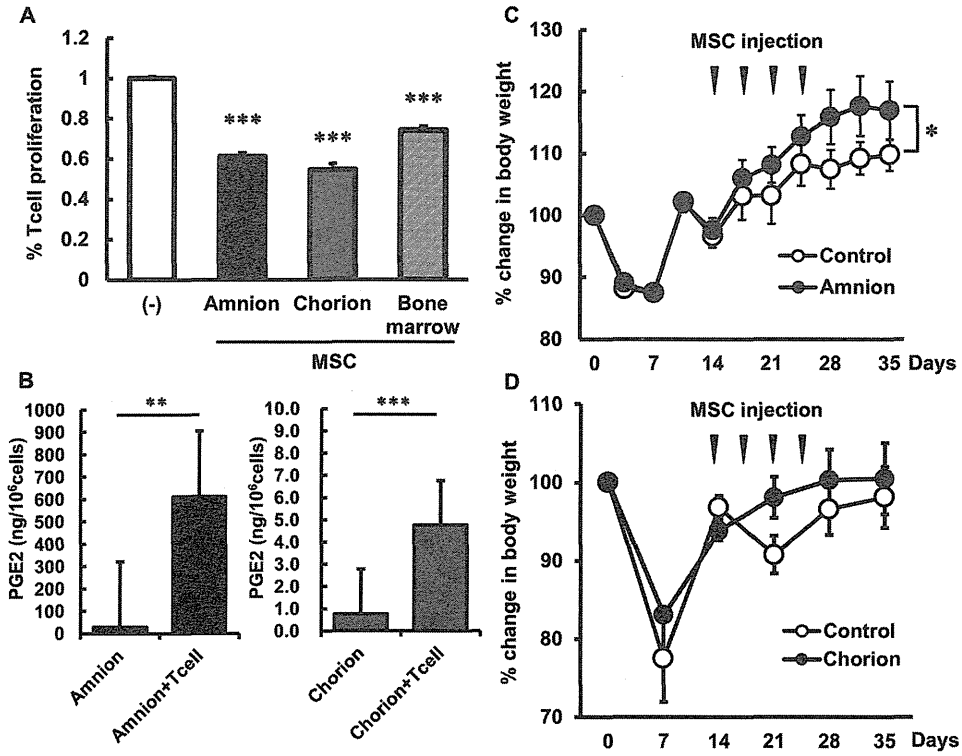

Figure 4. Immunosuppressive property of amnion and chorion MSCs. (A) Inhibition of human CD4+ T cell proliferation upon co-culture with human amnion, chorion, and bone marrow MSCs. (B) The concentration of PGE2 in FM-MSC-conditioned medium was measured by ELISA. Amnion MSCs secreted a significant amount 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