For reinforcement of the stump of the aorta in case of acute aortic dissection, gelatin–resorcin–formaldehyde glue or BioGlue surgical adhesive (CryoLife, Inc, Kennesaw, Ga) was usually applied to obliterate the false lumen. In addition to the use of chemical glue, all stumps were reinforced with Teflon felt strips. For total arch replacement, Teflon felt strips were placed on the outer side of the aortic stump and a graft 5 to 7 cm in length and 18 to 22 mm in diameter was inserted into the true lumen of the descending aorta as an elephant trunk. They were sutured and fixed with 5-0 polypropylene running suture, obliterating the false lumen in a sandwich-like fashion. The quadrifurcated graft was anastomosed to the stump of the descending aorta, where sandwich-like reinforcement was applied. For hemiarch replacement, Teflon felt strips were placed on the outer and inner sides of the aortic stump. The stump was thus reinforced and the false lumen obliterated in sandwich-like fashion with 5-0 polypropylene running suture.

Concomitant Procedures

Concomitant procedures included aortic valve resuspension in 28 patients, aortic valve replacement in 4, and coronary artery grafting in 25 patients.

Definitions

Hospital mortality was defined as death within the hospital. *Postoperative stroke* was defined as newly developing neurologic deficit, confirmed by computed tomography. *Transient neurologic dysfunction* was defined as postoperative confusion, agitation, delirium, or prolonged obtundation with negative brain computed tomographic findings and complete resolution before discharge. The neurologic diagnosis was made by neurologists.

Statistical Analysis

Values are presented as the mean ± standard deviation. Data were analyzed by the χ^2 test for categorical variables. Factors that tended to be of statistically significant risk by univariate testing ($P < .10$) were entered into a multivariate analysis. Stepwise logistic regression analysis of predictor variables for hospital mortality and stroke was performed with estimate odds ratios and 95% confidence intervals for each of the independent variables in the model. The Kaplan–Meier estimate was used to depict survival over time.

RESULTS

The average duration of circulatory arrest, myocardial ischemic time, and pump time was 57 ± 21 minutes, 123 ± 45 minutes, and 224 ± 80 minutes, respectively.

The total hospital mortality was 7.9% (9/114), 5.2% (4/77) for elective operations and 13.5% (5/37) for emergency operations ($P = .12$). All patients stayed in our hospital to the end. The average length of hospital stay of the patients was 36.8 ± 26.1 days (1–89 days, median 33.9 days). The 30-day mortality was 2.6% (3/114). The cause of death was sepsis in 3 patients, low output syndrome in 1, rupture of residual aneurysm in 1, respiratory failure in 2, and gastrointestinal tract problems in 2. The hospital mortality was 19.2% (5/26) until 2001, and it decreased to 4.5% (4/88) after 2002 ($P = .015$). Figure 2 shows the number of patients distributed by year and the number of deaths in each year. There were 19 patients aged 86 years and older. The hospital mortality of the 19 patients was 9.7%, and that of the rest of the younger patients was 7.2% ($P = .67$).

In total, 11 (9.6%) patients had a perioperative stroke, 6.5% (5/77) associated with elective operations and 16.2% (6/37) associated with emergency operations ($P = .12$). Eight (7.0%) patients had transient neurologic dysfunction. Other complications were respiratory failure in 17 (14.9%) patients, bleeding in 6 (5.3%), gastrointestinal tract problems in 3 (2.6%), and mediastinitis in 1 (8.8%).

Table 1 shows the results of univariate analysis of risk factors for hospital deaths, and Table 2 shows those of univariate analysis of risk factors for stroke. Tables 3 and 4 show the results of multivariate analysis of risk factors for hospital death and perioperative stroke.

TABLE 1. Univariate analysis of risk factors for hospital death

Variables	No.	*P* value
Operation		
Total arch replacement	7/74	.40
Hemiarch replacement	2/40	
Urgency		
Elective	3/76	.03
Emergency	6/38	
Concomitant operations		
Yes	2/41	.58
No	5/73	
Circulatory arrest		
>60 min	4/42	.62
≤60 min	5/72	
Cardiac ischemic time		
≤3 h	8/98	.79
>3 h	1/16	
Cardiopulmonary bypass		
≤4 h	4/79	.09
>4 h	5/35	
Postoperative stroke		
Yes	2/11	.18
No	7/103	
Hypertension		
Yes	9/99	.22
No	0/15	
Coronary artery disease		
Yes	4/35	.42
No	5/73	
Cerebral artery disease		
Yes	2/26	.96
No	6/81	
Diabetes		
Yes	3/12	.01
No	5/101	
Hyperuricemia		
Yes	0/3	.27
No	9/110	
Chronic obstructive pulmonary disease		
Yes	4/15	.0006
No	3/93	
Smoking		
Yes	4/36	.18
No	3/70	

TABLE 2. Univariate analysis of risk factors for stroke

Variables	No.	*P* value
Operation		
Total arch replacement	9/74	.21
Hemiarch replacement	2/40	
Urgency		
Elective	5/76	.12
Emergency	6/38	
Concomitant operations		
Yes	4/41	.98
No	7/73	
Circulatory arrest		
>60 min	5/42	.53
≤60 min	6/72	
Cardiopulmonary bypass		
≤4 h	5/35	.26
>4 h	6/79	
Cardiac ischemic time		
≤3 h	10/98	.62
>3 h	1/16	
Hypertension		
Yes	10/99	.67
No	1/15	
Coronary artery disease		
Yes	4/35	.59
No	6/73	
Cerebral artery disease		
Yes	3/26	.66
No	7/81	
Diabetes		
Yes	1/12	.86
No	10/101	
Hyperuricemia		
Yes	0/3	.56
No	11/110	
Chronic obstructive pulmonary disease		
Yes	2/15	.56
No	8/93	
Smoking		
Yes	5/36	.26
No	5/70	

The mean follow-up was 2.3 ± 2.0 years. Figure 3 shows the postoperative survival curve. The postoperative 1-year survival was 84.8%, the 3-year survival was 68.5%, and the 5-year survival was 58.1%.

DISCUSSION

Advances in perioperative care, anesthesia management, myocardial and brain protection during surgery, and sophisticated techniques have resulted in favorable results of cardiac surgery in octogenarians.[2,3] However, aortic arch surgery, which requires hypothermia and circulatory arrest, is still an invasive intervention. The number of octogenarians undergoing aortic surgery in our institute has been steadily increasing. The reasons are the increase in average life expectancy and the extension of the surgical indication. The surgical outcome of aortic arch surgery is improving recently,[4] and it is natural to extend the surgical indication to the patients of more advanced age with aortic disease.

Previous reports from our institute described that from 1991 to 1997 the postoperative mortality of septuagenarians and octogenarians after thoracic aortic surgery was 15.6%.[5] In this study, although the population was limited to octogenarians, the mortality rate was low; even more, it decreased to 4.5% after 2002.

The reason for the recent improvement of surgical results is not simple, but one of the major recent modifications of the surgical strategy is brain protection. For brain protection, RCP and ASCP were respectively used before 2000. Although deep hypothermia has usually been used for brain protection, the availability of ASCP has led to recent trends toward use of moderate hypothermia together with circulatory arrest.[6-10] We have used ASCP with circulatory arrest

TABLE 3. Multivariate analysis of risk factors for hospital death

	P value	Odds	95% CI
COPD	.013	8.48	1.6–46.0

CI, Confidence interval; *COPD*, chronic obstructive pulmonary disease.

TABLE 4. Multivariate analysis of risk factors for stroke

	P value	Odds	95% CI
Urgency	.029	4.62	1.2–18.2

CI, Confidence interval.

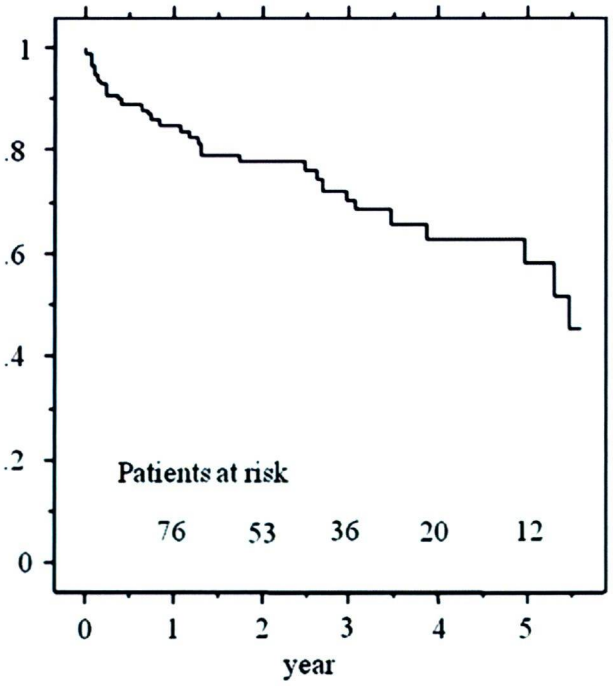

FIGURE 3. Postoperative survival curve.

as the standard technique since 2001, because ASCP yielded better results than RCP under circulatory arrest in our institute.[11] The system used for cerebral perfusion has gradually been modified, and right axillary cannulation is now a routine maneuver.[12,13] In addition, since 2002 we have gradually elevated the temperature of hypothermic circulatory arrest from 20°C to 28°C for aortic arch surgery, as noted above.[14]

The ratio of emergency operations in this study was more than 30%. Acute aortic dissection is not easy to prevent, but early recognition and treatment of *asymptomatic large aneurysms* in octogenarians might be possible. Our indication for elective aortic arch surgery in octogenarians is essentially the same as that for younger patients. When patients can still carry out their activities of daily living, even in the ninth decade of life, we believe the patient is in condition to survive an aortic arch operation.

Whether an emergency surgical procedure for acute aortic dissection should be done in octogenarians is controversial.[15–17] In this series, 28 patients were operated on to treat acute aortic dissection and the hospital mortality rate was 10.7%. Our indication for acute aortic dissection in octogenarians is also the same as for younger patients. Unless the patient has severe preoperative brain damage, we believe the patient has a good chance to survive.

Kirsch and his colleagues[18] reported that the actuarial survival was 79.2% at 1 year, 9% at 3 years, and 56.2% at 5 years after cardiac surgical procedures in octogenarians. Melby and his colleagues[3] reported the actuarial survival was 82% at 1 year, 70% at 3 years, and 56% at 5 years after aortic valve replacement in octogenarians. Aortic arch surgery is still one of the most invasive procedures in cardiovascular surgery. The survivals found in this study, however, are comparable with those mentioned above.

In conclusion, the results of conventional aortic arch surgery in octogenarians are improving. The operations were performed with an acceptable operative risk even under emergency situations, including acute aortic dissection. The option of conventional surgery for aortic arch diseases in octogenarians should not be abandoned only because of the high chronologic age of the patients.

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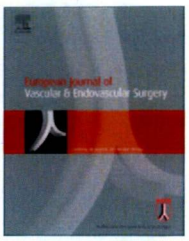

Spinal Cord Injury is Not Negligible after TEVAR for Lower Descending Aorta☆

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KEYWORDS
Thoracic aortic aneurysm;
Endovascular repair;
Stent graft;
Spinal cord injury;
Paraplegia

Abstract *Objectives:* To clarify the incidence of spinal cord injury (SCI) after thoracic endovascular aneurysm repair (TEVAR), we investigate the intercostal/lumbar arteries that supply the Adamkiewicz artery (ICA-AKA).
Patients: Among 81 patients subjected to TEVAR, we retrospectively reviewed the clinical records of 50 patients (range: 57–86 (median age: 77) years, 41 males) who underwent TEVAR for part of or the whole distal descending aorta (T7 to L2) after identification of ICA-AKA by magnetic resonance angiography (MRA) or computed tomography angiography (CTA).
Results: The 50 patients were classified into group A: 17 patients whose patent ICA-AKA was not covered, group B: 24 patients whose ICA-AKA was covered and group C: nine patients in whom no patent ICA-AKA was identified. Only three patients in group B suffered paraplegia and of them two recovered full ambulation. The estimated incidence of permanent and transient paraplegia was 3.7% in all TEVAR patients, 6.0% when part of or the entire distal aorta was covered and 12.5% when the patent ICA-AKA was covered. The length of aortic coverage in patients with paraplegia was >300 mm.
Conclusions: Paraplegia after TEVAR occurred in one of eight patients in whom the stent graft covered ICA-AKA. Long coverage of the aorta including the ICA-AKA was critical. To prevent this serious complication, identification of the ICA-AKA is crucial.

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The incidence of spinal cord injury (SCI) after thoracic endovascular aneurysm repair (TEVAR) has been reported to vary according to the demographics of the patients.[1–20] Whether the integrity of the Adamkiewicz artery (AKA) is essential for spinal cord function is still to be investigated.[21] However, after reattachment of the intercostal/lumbar arteries, which supply AKA (ICA-AKA), or of the adjacent intercostal/lumbar arteries during thoracoabdominal aortic replacement, motor-evoked potentials (MEPs) recover.[22] TEVAR has been reported to reduce SCI.[12] In principle, the longer the length of the aorta including both landing zones that is covered by TEVAR, the larger the number of ICAs that will be sacrificed and whose revascularisation will be impossible.[23]

To clarify the incidence and cause of SCI after TEVAR, we have investigated the patency of ICA-AKA in relation to other factors which may cause SCI.

Materials/Methods

Patient demographics

In the past 27 months, of 81 patients, we performed TEVAR with Gore TAG (W. L. Gore & Associates, Flagstaff, AZ, USA) in 47 patients, Talent thoracic stent graft (Medtronic, Inc., Santa Rosa, CA, USA) in five, both TAG and Talent in one and Matsui-Kitamura (MK) stent graft in 28 patients.[24] In this study, we included 50 patients who underwent TEVAR for part of or the whole distal descending aorta after ICA-AKA was identified by magnetic resonance angiography (MRA) or computed tomography angiography (CTA). The distal descending aorta was defined as the segment between T7 and L2.[25] Fifteen patients who underwent TEVAR above T6 and 16 patients who had not undergone MRA or CTA to identify ICA-AKA were not included in this investigation.

In general, the patients were senescent, debilitated and presented co-morbidities. (Table 1) Thirty-seven patients were ≥75 years old and the median age was 77. Thirty-seven patients were in ASA class 3 or 4, and 32 patients had a history of aortic surgery (48 surgeries in total).

Of the 18 patients who had undergone AAA repair, TEVAR had been indicated more than 1 year later in 11 patients, scheduled within 3 months in five and performed simultaneously in two. Emergency TEVAR was performed in three for haemoptysis, acute aneurysm dissection and persistent back pain. They were haemodynamically stable and could undergo CTA for ICA-AKA.

In all patients, another CTA was carried out to precisely measure the aneurysm and access. CTA also revealed the patency of the left subclavian (LSCA) and bilateral internal iliac arteries (IIA). Occlusion of left IIA (LIIA) was confirmed in three patients but LSCA and right IIA (RIIA) were patent in all the patients regardless of whether total arch replacement (TAR) or AAA repair was performed.

Identification of ICA-AKA

ICA-AKA was identified by MRA in 39 and by CTA in 11 patients.

The details of contrast MRA were previously reported by Yamada et al.[26] For the CTA, an Aquilion 16 multi-detector row CT scanner (Toshiba, Tokyo, Japan) was used. To detect AKA, the reconstruction field of view was set to the area around the aorta and spine. The images were processed in a workstation (Ziostation; Amin, Tokyo, Japan). Volume-rendered images of the entire aorta were routinely generated. Multiplanar reformation (MPR) images, including oblique coronal images with craniocaudal angulations and curved planar reformation images, were reconstructed to investigate the side and level of the origin of AKA.

Diagnostic criteria for the anterior spinal artery and AKA were as previously reported.[26] We preferred MRA as CTA is disadvantageous due to the influence of the spine and lack of accurate differentiation of the AKA from the anterior radicular vein.[22] However, the selection of MRA or CTA

Table 1 Patient demographics.

Number of Patients	50			
Age	57–86 [median 77] year-old			
Gender	41 male			
ASA class	Class 2: 13, Class 3: 19, Class 4: 18			
History of aortic surgery	Root to Ascending	3		
	Arch	21	Total arch replacement	20
			TEVAR after debranch	1
	Descending		Replacement	3
			TEVAR	1
	Thoraco-abdominal	2		
	AAA	18	Replacement	17
			EVAR	1
Aortic pathology	Degenerative aneurysm	39		
	Chronic dissection	3		
	Acute dissection on aneurysm	2		
	Penetrating atherosclerotic ulcer	3		
	Anastomotic false aneurysm	3		

Table 2 Distribution of ICA-AKA.

	Right	Left	(Occlusion at origin)
Th7	0	1	
Th8	1	6	(2)
Th9	0	18	(1)
Th10	1	10	
Th11	0	7	(1)
Th12	2	4	(2)
L1	0	1	
L2	0	0	
Total	4	47	(6)

ICA-AKA: intercostal/lumbar arteries which supplies Adamkiewicz artery.

depended on the availability of the equipment. CTA was used in all three emergency cases.

When AKA was not identified by MRA, it was diagnosed as 'absent' ($n = 3$). In 47 patients, 51 ICA-AKAs were identified (Table 2). In four patients, there were double ICA-AKAs. Occlusion of ICA-AKA at its origin was diagnosed in six patients, in all of them on the left side. When the ICA-AKA was occluded, blood supply from adjacent intercostal or lumbar arteries was suspected to be significant. However, we were unable to distinguish the critical collateral flow to AKA.

TEVAR

To create a landing zone, a carotid–subclavian bypass was performed in two and visceral vessel bypass was performed in one. In nine patients who had extensive/multiple aneurysm(s) from the aortic arch to the descending aorta, TAR was performed using elephant trunk (ET) implantation. Regarding patients who had a history of aortic surgery, an artificial graft was used to create a proximal landing zone in 19 and a distal landing zone in three.

In all patients, TEVAR was carried out under general anaesthesia. The access route for TEVAR was a native artery in 35, an iliac conduit in 13 and a graft limb or a side branch of AAA graft in two patients.

MEP monitoring and cerebrospinal fluid drainage

In all patients trans-cranial MEPs were monitored during TEVAR and a cerebrospinal fluid drainage (CSFD) tube was placed before TEVAR in 31 patients.

Immediately after the stent graft was placed, the mean blood pressure was raised above 80 mmHg and MEP was monitored every 5 min. When the amplitude of MEPs decreased under general anaesthesia, or when symptoms and signs of SCI were noted during the postoperative period, CSFD (<15 cmH_2O) was started with the infusion of methylprednisolone (30 mg kg^{-1} bolus and 5.4 mg kg^{-1} h^{-1} for 23 h followed by 2.7 mg kg^{-1} h^{-1} for 2 days) and naloxone (1200 μg day^{-1}). Intensive spinal care with CSFD, methylprednisolone and naloxone was continued for 72 h if the symptom did not resolve or was discontinued 24 h after full recovery.

CSFD was started only after paraplegia or a decrease of less than 25% of the amplitude of MEPs was noted. CSFD was not indicated as a prophylactic measure after TEVAR.

Measurement of the aortic length

The length of 'proximal uncovered aorta' (from LSCA to stent graft), 'aortic coverage' by stent graft and 'distal uncovered aorta' (from stent graft to coeliac axis (CA)) was measured on CTA using curved planar reformation images processed in a workstation (GE Advantage workstation 4.3).

After TAR with a multibranch graft, the length of aortic coverage was measured from the distal anastomosis. This site coincided with the origin of ET and was several centimetres distal to the branch graft of LSCA. When ET was installed, the proximal edge of the stent graft was positioned inside the multibranch graft and not only inside ET. After replacement of the descending or the thoracoabdominal aorta, the position of LSCA and/or CA served as the point of reference for the measurement.

Statistical analysis

Values are the mean ± SD. Data were analysed using the chi-square test for categorical variables, and continuous variables were examined using analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$.

Results

Mortality and morbidity

Initial success of TEVAR was achieved in all patients except for two patients with Type I endoleaks detected by CTA who were successfully treated by a repeat TEVAR. No operative (30 days) death was encountered. Injury and occlusion of access arteries occurred in one. Two patients were complicated with cerebral embolism due to the guidewire pull-through technique and atrial fibrillation.

The following three patients were complicated with paraplegia: Patient 1 was a 59-year-old man with a history of closure of ventricular septal defect, aortic valve replacement and repair of a Valsalva sinus aneurysm. He also suffered from liver cirrhosis. He developed aneurysmal dilatation of the whole thoracic aorta and underwent TAR with ET installation as the first-stage repair. MRA revealed the AKA arose from the left Th9-ICA. TEVAR with Gore TAG was performed 5 weeks later from ET (Z3) to T11. The iliac conduit was connected to the right common iliac artery but the haemostasis was time consuming because of obvious coagulopathy due to liver cirrhosis. Paraplegia was confirmed 24 h after TEVAR after the patient suffered much pain. Despite treatment for SCI, the patient could not ambulate. Retroperitoneal haematoma had to be removed twice. He eventually died from methicillin-resistant *Staphylococcus aureus* (MRSA) mediastinitis 4 months after TEVAR. The length of aortic coverage from the origin of ET to the distal flair was 325 mm.

Patient 2 was an 81-year-old man with ascending, arch and descending aorta aneurysms. MRA revealed the AKA branching from the left Th9-ICA. Four weeks after TAR with ET installation, TEVAR with TAG was performed from ET (Z3) to T12 (Fig. 1). The iliac conduit was required and

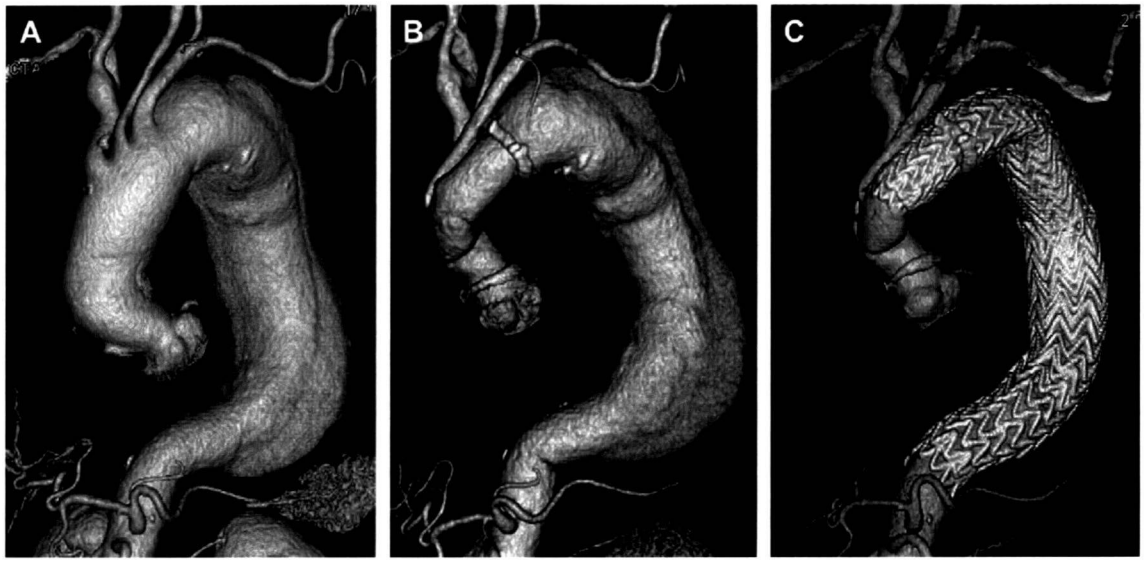

Figure 1 Sequence of CTA in Patient 2. Panel A: Preoperative, Panel B: After total arch replacement, Panel C: After TEVAR.

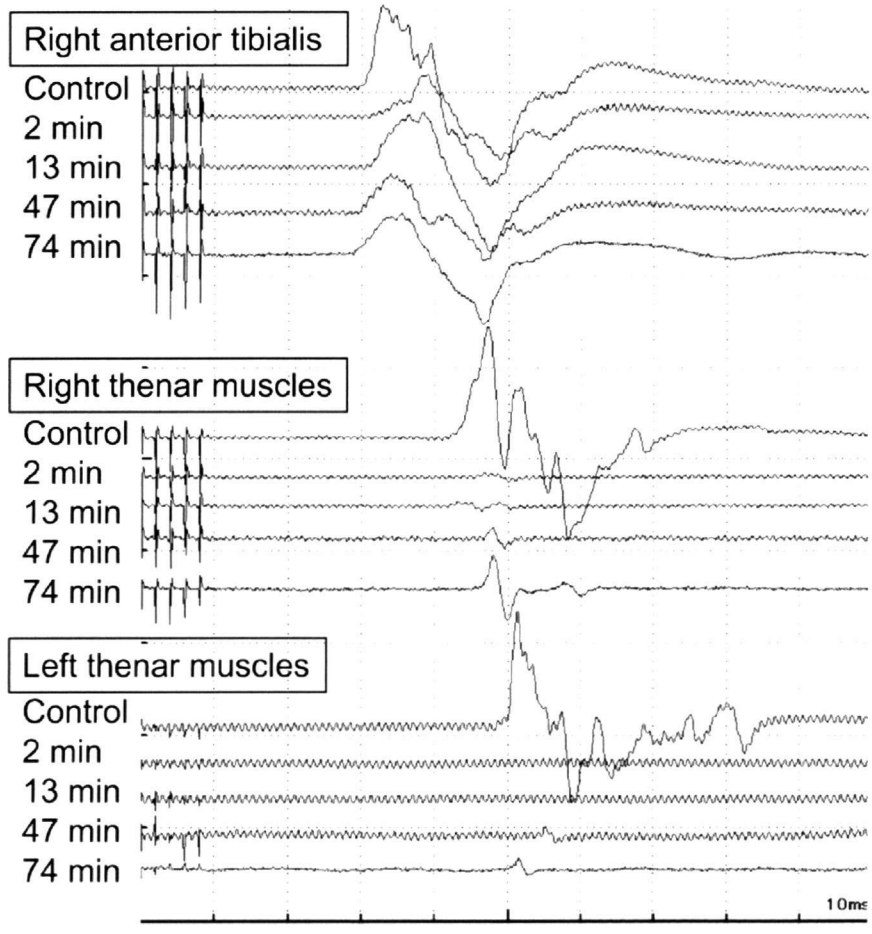

Figure 2 Sequence of MEPs in Patient 3. MEPs of the right anterior tibialis and both thenar muscles before TEVAR over Th9-ICA (control) and 2, 13, 47, and 74 minutes after TEVAR.

haemostasis took a long time due to co-existing consumption coagulopathy caused by aortic lesions. Six hours after TEVAR, the patient suddenly complained of back pain and paraplegia was confirmed. One hour after intensive spinal treatment, he could move his legs and on the next morning he could walk. The retroperitoneal, femoral and brachial haematomas were removed twice. The length of aortic coverage was 302 mm.

Patient 3 was a 78-year-old woman who had undergone TEVAR (Z3 to T7) for a proximal descending aortic aneurysm 6 months earlier. MRA revealed the ICA-AKA branching from the left Th9-ICA. Due to the rapid growth of the distal descending aortic aneurysm, TEVAR was performed again from the previous stent graft to L1. The CA was closed to create a distal landing zone. Immediately after the deployment over the Th9-ICA, the MEPs of both thenar muscles diminished and the amplitude of the MEPs of the right anterior tibialis decreased about 50% from the control amplitude (Fig. 2). Despite treatment for SCI, ankle dorsiflexion was slight when she awoke from anaesthesia. Intensive spinal care was continued and she gradually gained muscle strength within 3 h after TEVAR. On the following morning she could ambulate. The length of aortic coverage after the first TEVAR was 157 mm and was extended to 308 mm by the second TEVAR.

Incidence of paraplegia

The 50 patients were classified into group A: 17 patients whose patent ICA-AKA was not covered by TEVAR, group B: 24 patients whose ICA-AKA was covered by TEVAR and group C: nine patients in whom no patent ICA-AKA was identified. Group C included six patients whose ICA-AKA occluded at its origin and three patients whose ICA-AKA was absent.

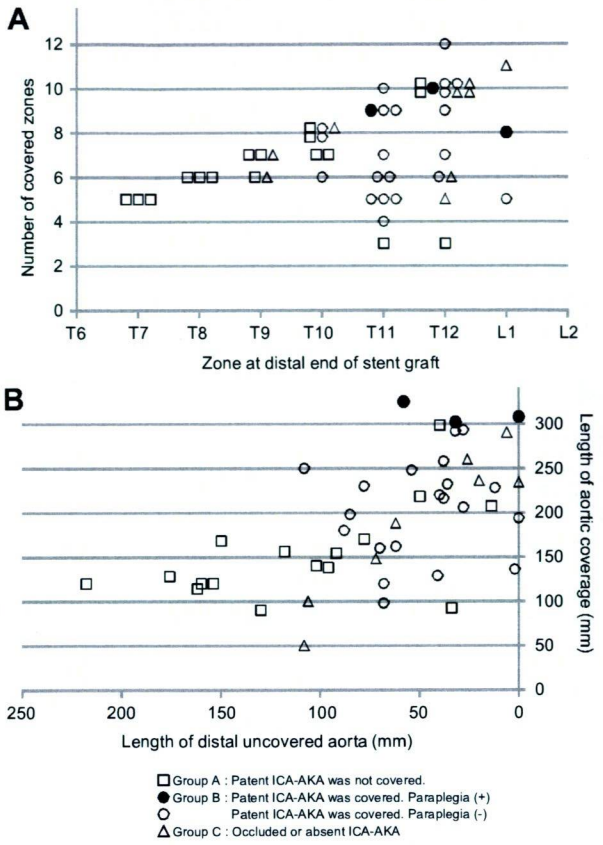

Figure 3 Distribution of patients with paraplegia in accordance with covered aorta and distal uncovered aorta expressed as number of aortic zones (Panel A) and measured length (Panel B).

Table 3 Comparison of patients with and without paraplegia.

	Paraplegia $N = 3$	No paraplegia $N = 47$	p
Age (year-old)	72.7 ± 11.9	76.1 ± 6.1	.3736
Male gender	2 (67%)	39 (83%)	.5094
ASA classification	3.7 ± 0.6	3.1 ± 0.8	.2026
Renal dysfunction	1 (33%)	18 (38%)	.3362
History of aortic repair (descending, thoracoabdominal, abdominal)	1 (33%)	19 (40%)	.8060
LSCA patency	3 (100%)	47 (100%)	–
RIIA patency	3 (100%)	47 (100%)	–
LIIA patency	3	44	.5359
Op time (minutes)	252 ± 117	141 ± 76	.0200
Blood loss (ml)	557 ± 274	363 ± 423	.4482
Use of an iliac conduit	2 (67%)	15 (32%)	.2335
Zones of aortic coverage	9 ± 1	7.2 ± 2.2	.1691
Proximal uncovered aorta (mm)	0	36 ± 49	.2191
Aortic coverage (mm)	312 ± 12	179 ± 64	.0009
Distal uncovered aorta (mm)	30 ± 29	72 ± 52	.1839
Hypotension	2 (67%)	3 (6%)	.0116

LSCA: Left subclavian artery, R(L)IIA: Right internal iliac artery.

Table 4 Reported incidence of spinal cord injury (SCI).

	Patients	Location	N	SCI	
Criado, 2002	TEVAR	Arch-descending	47	0	(0.0%)
Bergeron, 2003	TEVAR	Descending	38	0	(0.0%)
Czerny, 2004	TEVAR	Descending	54	0	(0.0%)
Orend, 2003	TEVAR	Various	74	2	(2.7%)
Mitchell, 1999	TEVAR	n/d	103	3	(2.9%)
Makaroun, 2005	TAG phase II	Various	142	4	(3.0%)
Ellozy, 2003	TEVAR	Descending	84	3	(3.6%)
Morales, 2007	TEVAR	n/d	186	7	(3.8%)
Bell, 2003	TEVAR	Various	67	3	(4.0%)
Greenberg, 2008	TEVAR	TAAA	352	15	(4.3%)
Gravereaux, 2001	TEVAR	Descending	53	3	(5.7%)
Greenberg, 2005	TXI & TXII	Various	100	6	(6.0%)
Sandroussi, 2007	TEVAR	n/d	65	4	(6.2%)
Cheung, 2005	TEVAR	Various	75	5	(6.5%)
Amabile, 2008	TEVAR	Descending	67	5	(7.5%)
Feezor, 2008	TEVAR	n/d	326	33	(10.0%)

The three patients who developed paraplegia were in group B, that is, ICA-AKA was covered by TEVAR. The estimated incidence of permanent and transient paraplegia was 3.7% in all patients subjected to TEVAR (81 patients), 4.5% in patients in whom part or the entire distal aorta was covered, regardless of ICA-AKA identification by MRA (66 patients) and 6.0% in those whose ICA-AKA was identified (50 patients, groups A, B and C). The incidence increased to 12.5% only when the patent ICA-AKA was covered by TEVAR (24 patients, group B).

Comparison of patients with and without paraplegia after TEVAR showed that the operation time and the length of aortic coverage were significantly longer in those with paraplegia (Table 3). Episodes of hypotension below 80 mmHg for more than 10 min during and after surgery were more frequent in patients with paraplegia. No difference was found in the patency rate of LSCA[27] and IIA. Other risk factors previously reported[28] such as the abdominal aortic surgery and the renal dysfunction showed no difference.

Fig. 3 shows the occurrence of paraplegia in relation to aortic coverage and distal aortic uncoverage length. When these were divided into zones, the stent grafts in the three patients with paraplegia were placed at T11 or distal to it and covered more than eight zones. Fourteen patients without paraplegia had the same range of intervened zones (Fig. 3A). In the three patients with paraplegia, the length of aortic coverage was more than 300 mm. The length of distal uncovered aorta was within 60 mm. Four other patients whose length of aortic coverage was between 270 mm and 300 mm and the length of distal uncovered aorta was less than 60 mm did not experience paraplegia (Fig. 3B).

Discussion

We have reported the low risk of paraplegia for patients subjected to descending and thoraco-abdominal aorta open repair by combined use of AKA identification by MRA and MEP measurement.[22] The risk of paraplegia has been considered to be lower after TEVAR than after open repair, but the incidence of SCI in the previous reports varied (Table 4). This variation might be due to differences in case mix as the area subjected to TEVAR was not the same and was not specified in some of the reports.[23]

The theoretical advantages of TEVAR concerning protection of the spinal cord are the maintenance of distal perfusion, stable haemodynamics and no reperfusion of the spinal cord.[12] However, additional ICAs are sacrificed for the landing zones and revascularisation of the ICAs is impossible. Paraplegia occurs after TEVAR when the arteries that supply the spinal cord are sacrificed, as well as after a period of hypotension or as a result of emboli from aortic atheromatous lesions.[29]

We encountered paraplegia in three patients whose patent ICA-AKA was covered and the length of aortic coverage was more than 300 mm. The rate of permanent and transient paraplegia was 12.5% (1/8), when the patent ICA-AKA was covered by TEVAR. This result was in agreement with that of a previous study showing that SCI occurred in 9.1% of the patients with occlusion of the ICA-AKA.[13] The authors did not encounter paraplegia in patients whose ICA-AKA was patent. This fact is also relevant to our result as none of our patients, whose ICA-AKA was already occluded at its origin before TEVAR or was absent, experienced paraplegia.

The spinal cord blood supply depends on many interchangeable collateral arteries that supply the anterior spinal cord artery, rather than a single dominant AKA.[21] However, the importance of the patency of the ICA-AKA during TEVAR[13] and the restoration of blood flow in the spinal cord after revascularisation of ICA-AKA at the aortic replacement have been reported.[22] Patency of ICA-AKA is sufficient to prevent paraplegia and occlusion of the patent ICA-AKA is critical. To preserve the patency of ICA-AKA, this should be identified preoperatively to allow the creation of an adequate landing zone.[12]

The rate of paraplegia, 12.5%, means that 87.5% (7/8) patients did not develop paraplegia after the coverage of patent ICA-AKA. Between three patients who suffered paraplegia and 21 patients who did not in group B, the threshold of the length of aortic coverage was 300 mm. The length of aortic coverage has been described as a risk for SCI in previous reports.[13,30] Amabile et al. reported that

205 mm was the critical length of aortic coverage for SCI.[18] Feezor et al. described that both the extent (>200 mm) and distal location of aortic coverage (20 mm from CA) were associated with an increased risk for SCI.[16]

We tried to locate the critical segment for paraplegia by dividing the aorta into zones but the length measured by CTA demonstrated the critical length of aortic coverage more clearly. This particular threshold, 300 mm, might vary in the future as experience accumulates and the index is modified, for instance, according to height. Nevertheless, it can be emphasised that long aortic coverage is another important risk factor for paraplegia. Long coverage of the aorta including the patent ICA-AKA is critical.

We found intra-operative coagulopathy related to prolonged operation time and postoperative retroperitoneal bleeding in patient 1 and patient 2. Hypotension associated with retroperitoneal bleeding contributes to SCI.[6] Consumption coagulopathy is another risk which is heightened by long coverage of the aorta.

Paraplegia occurred when the stent graft covered the zones at T11 or was placed less than 60 mm from CA. It can be concluded that the zones above T10 or a distance of more than 60 mm from CA are safe. However, the length of distal uncovered aorta would only express the probability of the occlusion of the ICA-AKA according to its distribution. The length of distal uncovered aorta might be less significant than the closure of the ICA-AKA or the length of aortic coverage.

Similarly, high percentages of paraplegia, 12.5% and 14.3%, after TEVAR were reported in patients with prior AAA repair.[12,20] In our series, a history of abdominal, thoraco-abdominal, or descending aneurysm repair was not a significant risk for paraplegia. However, AAA repair sacrifices several pairs of lumbar arteries that significantly contribute to spinal cord perfusion and/or IIA, which are the possible sources of direct or collateral blood flow to spinal arteries. Indeed, previous AAA repair was described as a risk factor in various other reports.[2,6,12,27,31]

Limitations of this study include the retrospective review of prospectively collected data, the retrospective measurement of aortic length and the small number of patients. Further accumulation of patients treated by TEVAR after identification of ICA-AKA is crucial for more precise diagnosis of the risk for paraplegia after TEVAR.

Conclusions

Paraplegia after TEVAR occurred in 1 of 8 (12.5%) patients in whom the stent graft covered the distal descending aorta below Th7. Long (>300 mm) coverage of the aorta including the ICA-AKA is a critical risk factor for SCI and paraplegia. To prevent this serious complication, it is imperative to identify the ICA-AKA before performing TEVAR.

Conflict of Interest/Funding

None.

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Methods Article

A Suspension Induction for Myocardial Differentiation of Rat Mesenchymal Stem Cells on Various Extracellular Matrix Proteins

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AU 1 ▶ The microenvironment of bone marrow-derived mesenchymal stem cells (MSCs) strictly regulates their differentiation. In this study, we have developed a new suspension induction method for myocardial differentiation of bone marrow-derived rat MSCs (rMSCs) *in vitro* on various extracellular matrix (ECM) proteins. Myocardial differentiation of rMSCs was induced with a conventional monolayer method and our suspension method. In our suspension induction, a cell suspension was treated with the medium in the presence of an inducer, incubated for 2 h under a suspension conditions, and moved to a monolayer culture on gelatin-coated, collagen type I-coated, fibronectin-coated, or polystyrene dishes until the total induction time was 24 h. We evaluated the myocardial differentiation by counting the number of colonies of beating cells, performing immunohistochemical staining, and measuring the expression of cardiac-specific gene mRNA using real-time quantitative polymerase chain reaction. We found that rMSCs induced with the conventional monolayer method did not differentiate efficiently, whereas beating cell colonies were found on ECM-coated dishes of suspension-induced cells, after 3 weeks of culture, especially on gelatin-coated dishes. The beating cells were positively stained with anti-troponin T-C antibody and expressed specific cardiac markers. In conclusion, these results demonstrated that the suspension induction followed by subsequent culture on gelatin ECM substrates is a promising method for differentiating rMSCs into cardiomyocytes *in vitro*.

Introduction

ISCHEMIC HEART DISEASE is the primary cause of death throughout the world.[1] Adult cardiac muscle, unlike skeletal muscle, lacks the ability to regenerate after ischemic injury. The only eventual therapy is cardiac transplantation. However, this option is limited by a lack of donor organs.

An implantable left ventricular assist device has been proposed as a bridge to transplant for many patients who are on a waiting list for donor organs.[2] Left ventricular assist device can improve organ perfusion, reduce wall stress, and improve functional capacity and quality of life, but it is not an option for the majority of people with heart failure.[3,4] Thus, the ultimate goal is to repair the injured myocardium by cell transplantation.

Some fundamental studies and clinical trials suggest that cell-based therapies can improve cardiac function.[5–8] The isolation of cardiomyocytes from a patient's heart is unrealistic at present. In general, three types of potential cell sources have been proposed, but the search for these sources and types of cells are still under investigation.[9] One potential source is allogeneic cells, including human embryonic stem cells or fetal allogeneic cardiomyocytes, but there remain ethical issues in their use. Another option is transgenic sources. Genetically engineered animal cardiomyocytes have been studied in an attempt to reduce the rejection reaction *in vivo*, which is still a long-term problem in recipients.[9]

To deal with this problem, autograft bone marrow-derived mesenchymal stem cells (MSCs) are foreseen to be the most promising candidate for transplantation, because they are easy to obtain and less immunogenic than other stem cells. The differentiation of MSCs into cardiomyocytes *in vivo* has been observed, but it occurs at an extremely low rate and its efficiency is under debate.[7,10]

The production of autologous beating cardiomyocytes is thus an attractive goal for cell-based therapy. For this purpose, it is preferable to differentiate MSCs into cardiomyocytes

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in vitro before transplantation, and it is crucial to understand how best to achieve this.

Based on traditional isolation of MSCs and monolayer culture, Wakitani *et al.* reported that rat MSCs (rMSCs) were differentiated to myogenic cells after 24 h of exposure to DNA-demethylating agent 5-azacytidine,[10] and Makino *et al.* reported that the repeated treatment of murine MSCs with 5-azacytidine differentiated the cells into cardiomyocytes with high cardiac marker expression *in vitro*.[11] These findings are in contrast with a report that functional cardiac cells and gene expression were not obtained after treatment with 5-azacytidine.[12] Xu *et al.* also reported cardiac marker expression in 5-azacytidine-treated MSCs, but they did not observe any beating cells.[13] The differences in these observations might be related to the efficiency of the inducer and the timing of induction.

Clemmons *et al.* reported that fibroblasts in the suspension did not undergo DNA synthesis and division.[14] Griffin and Houstan reported that cells in monolayer cultures are in a static environment and have a relatively small surface area for diffusion, in contrast to suspension cultures in which the entire surface area is exposed to the drug.[15] In addition, in suspensions, efflux transporters are not retained because of the loss of cell polarity and redistribution of canalicular membranes[16]; therefore, the compound remains in the cell. Hence, we assumed that by treating the cells with the inducer in suspension culture, the treated cells were more likely to proceed toward the differentiation phase instead of the division phase.

Langer and Vacanti reported that three important components of tissue-engineered constructs were the cell source, soluble chemical factor, and extracellular matrix (ECM).[17] ECM proteins and the cooperation between signaling pathways triggered by soluble factors such as growth and differentiation factors were found to determine cell proliferation and cell differentiation.[18] In our previous study, ECM components were seen to affect the beating behavior of primary neonatal cardiomyocytes and cardiac differentiated P19.CL6 cells in which enhanced beating behavior and cardiac differentiation on gelatin-coated dishes were observed.[19]

The aim of our studies was to produce spontaneously beating cardiac cells from rMSCs by our new induction method on different substrates. Optimal substrates for stem cell attachment, proliferation, and differentiation have been reported for various types of stem cells.[20] In this study, treated rMSCs were cultured on gelatin-coated, fibronectin-coated, collagen type I-coated, and polystyrene dishes. We treated rMSCs using a newly established suspension method, and the differentiation tendency was compared with those treated by the conventional monolayer method.

Materials and Methods

Bone marrow cell preparation

Femora and tibiae of 4-week-old, male Sprague Dawley rats with average body weight of 80 g were collected and adherent soft tissues were removed. Institutional guidelines for the care and use of laboratory animals were observed. The rMSCs were obtained from collected femora and tibiae by flushing the marrow cavities. Isolated cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM-HG; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (lot no. 7297H, MP Biomedicals, Eschwege, Germany), 5% heat-inactivated horse serum (lot no. 076K8430; Sigma-Aldrich, St. Louis, MO), and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Wako, Osaka, Japan).

The cells were seeded on 10 mm fibronectin-coated dishes (BD Falcon, BD BioCoat, BD Biosciences, Bedford, MA) and incubated in a 5% carbon dioxide (CO_2)/air atmosphere at 37 C. At 24 h after plating, nonadherent cells were removed, and the medium was changed every 3 days until the adherent cells reached 80% confluence. The cells in one dish were harvested with 0.25 mg/mL trypsin (Lonza, Walkersville, MD), washed with phosphate-buffered saline (PBS), and seeded onto three new dishes.

Isolation of neonatal heart

Cardiomyocytes were isolated from neonatal (2-day-old) Sprague Dawley rat hearts by the collagenase digestion method with modifications.[21,22] Institutional guidelines for the care and use of laboratory animals were followed. The hearts were removed and carefully minced with a scalpel blade into fragments and rinsed several times with Hanks' balanced salt solution (Sigma-Aldrich) to remove blood and cellular debris. The minced hearts were gently stirred in 50 mL collagenase solution (0.15 M NaCl, 5.63 mM KCl, 0.02 M HEPES, 0.02 M $NaHCO_3$, 3.74 mM $CaCl_2 \cdot 2H_2O$, and 6.5×10^4 U collagenase [lot no. 06032W; Wako]) at 37 C for 30 min. The resulting cell suspension was filtered through a 40 μm pore-sized nylon cell strainer (BD Falcon, BD BioCoat, BD Bioscience) and centrifuged at 78 g for 3 min.

Isolated cardiomyocytes were cultured in minimum essential medium alpha (Gibco) supplemented with 10% (v/v) fetal bovine serum (lot no. 7297H; MP Biomedicals) and 100 IU/L penicillin-streptomycin (Wako) on 60 mm gelatin-coated dishes (Iwaki; Asahi Glass, Tokyo, Japan). Three days after isolation the mRNA levels of the cardiac marker genes were evaluated.

Cardiomyocyte differentiation

Monolayer induction. The rMSCs at fourth passage were seeded on 60 mm gelatin-coated dishes (Iwaki; Asahi Glass), fibronectin-coated dishes (BD Falcon, BD BioCoat, BD Biosciences), collagen type I-coated dishes, and noncoated polystyrene dishes (Iwaki; Asahi Glass) at a density of 1.0×10^5 cells/dish. The cells were cultured at 37 C in humidified air with 5% CO_2, reaching 80% confluence within 3 days. Afterward the cells were exposed to the inducers, 10 μM 5-azacytidine (Nacalai Tesque, Kyoto, Japan), 300 μM L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako), and 0.025 μg/mL human basic fibroblast growth factor (Sigma-Aldrich)-containing DMEM-HG for 24 h. Then, the inducers were washed away and cells were cultured for 5 weeks with DMEM-HG without inducers to develop the beating cells. The medium was changed every 3 days. The cell morphologies were observed every day using Nikon Eclipse TE 300 (Nikon, Tokyo, Japan) light microscope. An image was taken after 3 weeks of cultivation using Image Pro 4.5 software (Media Cybernetics, Silver Spring, MD).

Suspension induction. The suspension of 1.0×10^5 rMSCs was treated with and without 10 μM 5-azacytidine (Nacalai

Tesque), 300 μM L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako), and 0.025 μg/mL human basic fibroblast growth factor (Sigma-Aldrich)-containing DMEM-HG in a floating condition in a centrifuge tube (Iwaki; Asahi Glass) for 2 h at 37 C in humidified air with 5% CO_2. The treated cells were cultured on 60 mm gelatin-coated dishes (Iwaki, Asahi Glass), fibronectin-coated dishes (BD Falcon, BD BioCoat, BD Biosciences), collagen type I-coated dishes, and noncoated polystyrene dishes (Iwaki; Asahi Glass) in the presence of inducers until the total induction time was 24 h, then with DMEM-HG without inducers for 5 weeks. The medium was changed every 3 days. The cell morphologies were examined every day using a Nikon Eclipse TE 300 (Nikon) light microscope. Images were taken after 3 weeks of cultivation using Image Pro 4.5 software (Media Cybernetics).

The experiments were repeated to determine the expression of troponin C type-2 after suspension induction.

Total RNA isolation and reverse transcription

Total cellular RNAs from both noninduced and induced rMSCs with monolayer induction and suspension induction were extracted by QuickGene RNA cultured cell kit S (Fujifilm Life Science, Tokyo, Japan) after 1, 2, and 3 weeks of culture. In another experiment, total cellular RNAs from induced rMSCs with suspension induction were extracted after 1, 2, 3, 4, and 5 weeks. The cellular RNAs from neonatal cardiomyocytes were also extracted with the same protocol after 3 days of culture as a positive control for real-time quantitative polymerase chain reaction (PCR). Total cellular RNAs were calculated as follows: [RNA] = A_{260} (nm) × Dilution × 40 ng/mL. The RNAs from beating and nonbeating colonies were extracted separately.

First-strand cDNAs were synthesized using a mixture of oligo$(dT)_{18}$ primer. Total cellular RNAs (200 ng) were incubated with 2.5 μM oligo$(dT)_{18}$ primer at 70 C for 10 min to denature RNA secondary structure and then incubated at 4 C to let the primer anneal to the RNA. A given amount of 5× RT buffer (Toyobo, Osaka, Japan) and 2.5 mM dNTP mixture (Takara Bio, Shiga, Japan) (4 μL) were added and incubated at 37 C for 5 min. Reverse transcriptase (100 units; Toyobo) was added into the mixture and the RT reaction was extended at 37 C for 1 h. Then the reaction was heated at 94 C for 5 min to inactivate the enzyme and cooled at 4 C for 15 min. RNase (DNase-free, 0.5 μg; Roche Diagnostics GmbH, Mannheim, Germany) was added into the mixture and incubated at 37 C to remove the template RNA. To confirm that the beating cells were cardiomyocytes, an immunochemical study was conducted, in which the expressions of cardiac-specific markers (TNNC 1, TNNT 2, TNNI 3, GATA 4, and MEF2D) were measured.

Real-time quantitative PCR

Real-time quantitative PCR was conducted with SYBR Green. Primers for PCR analysis of troponin T type-2 (cardiac) (TNNT 2), troponin C type-1 (slow) (TNNC 1), troponin I type-3 (cardiac) (TNNI 3), GATA4, MEF2D, and troponin C type-2 (fast, TNNC 2) were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Warrington, UK). Primer sequences are shown in Table 1. The reaction mixtures contained 23.74 μL distilled water, 25 μL SYBR Green Real-Time PCR master mix (Toyobo), 100 nM of each

TABLE 1. POLYMERASE CHAIN REACTION PRIMERS USED IN THIS STUDY

Genes	Sense	Position (bp)	Antisense	Position (bp)	Accession no
TNNT 2	5′-GAAACAGGATCAACGACAACCA-3′	827–848	5′-CCCCCGGTGACTTTCC-3′	873–890	NM_012676
TNNC 1	5′-GATCTCTTCGCCATGTTTCAACA-3′	295–316	5′-TGCCTCTGCAGCATCATCTTT-3′	352–370	NM_001034105
TNNI 3	5′-CCAGGAATCTCCAATCCCATT-3′	n5–85	5′-CCCCATCGCCTGCTCTCA-3′	114–130	NM_017144
TNNC 2	5′-AGATCGAATCCCTGATGAAGGA-3′	392–413	5′-CATCTTCAGAAACTCGTCCAAGTC-3′	442–465	NM_001037351
GATA 4	5′-CAGTTCTGCACACCTGTATTCCA-3′	452–474	5′-GCTCCCCTTTATTTCCAACTCA-3′	515–536	NM_144730
MEF2D	5′-GCGGCTGGATACTTGGACATT-3′	1251–1271	5′-CGCTGAGATTGTCAACTCTTCATC-3′	2009–2032	NM_030860
GAPDH	5′-CTACCCCCAATGTATCCGTTGT-3′	742–763	5′-TACCCCAGGCATGCCCTTTAGT-3′	842–862	AB017801

* nt : Nucleotide

Troponin C type 1 (slow), Troponin T Type 2 Cardiac, Troponin I type 3 (cardiac), GATA Binding Protein 4, and Myocyte enhancer factor 2D.

primer, and 0.26 μL cDNA. The thermal profile for PCR was 50°C for 2 min, followed by 95°C for 10 min, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. We also performed a negative control PCR reaction using 0.26 μL distilled water to ensure the absence of template contamination in PCR reagents. The cycle number at which the reaction crossed an arbitrarily placed threshold (Cτ) was determined for each gene. The average Cτ values of triplicate measurements were used for all subsequent calculations on the basis of the delta Ct method (ΔCτ). The amount of mRNA levels was determined by $2^{\Delta C\tau}$. To correct any variation in mRNA content, the quantities of the genes of interest were normalized by the quantity of glyceraldehyde-3-phosphate dehydrogenase and expressed as relative values of mRNA.

Immunostaining analysis

To confirm the protein expression in addition to the mRNA expression, cells generated by the monolayer method and beating cells generated by suspension induction were stained with anti-troponin T-C antibody. After 4 weeks of culture, the cells were fixed with 10% formalin in PBS and washed with PBS three times. Next, the cells were incubated for 5 min in 0.1% hydrogen peroxide in PBS to quench endogenous peroxide activity and washed in PBS twice for 5 min each. Then the cells were incubated with 10% block-ACE (Dainippon Sumitomo Pharma, Osaka, Japan) in PBS for 20 min to suppress nonspecific binding of IgG. After three cycles of washing with PBS for 5 min each, the cells were incubated with 2.5 μL/mL primary antibody (troponin T-C(C-19), sc-8121; Santa Cruz Biotechnology, CA) for 60 min in PBS with 1.5% block-ACE, washed three times in PBS for 5 min, and incubated with 2 μL/mL secondary antibody (donkey anti-goat IgG-FITC, sc-8121; Cosmo Bio, Tokyo, Japan) for 45 min in PBS with 1.5% block-ACE. The cells were washed with PBS four times and mounted with aqueous mounting medium.

Stained cells were observed using Nikon Eclipse TE 300 (Nikon) fluorescence microscope. An image was taken using Image Pro 4.5 software (Media Cybernetics) with the following parameters: for bright file, an exposure time of 20 ms and gain of 7; for fluorescence, an exposure time of 2 s and gain of 7.

Statistical analysis

All data are presented as means ± standard deviations. Statistical analysis was performed using Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

Results

rMSCs form myotubes

After 3 weeks of cultivation, the shape of the cells induced with suspension induction was very different from that with monolayer induction, as shown in Figure 1. The shape of the suspension-induced cells appeared to be myotubular and seemed to correlate closely to beating colony formation. The phenotypic difference in these shapes was confirmed by measuring TNNT 2, TNNC 1, and TNNC 2 expression.

Expression of cardiomyocyte-associated genes in monolayer and suspension induction

Troponin T type-2 (cardiac, TNNT 2) and troponin C type-1 (slow, TNNC 1) are known to be markers of cardiomyocytes,[23,24] and troponin C type-2 (fast, TNNC 2) is reported to be expressed at the early stage of the cardiac development.[23] In this study, the expression of TNNT 2 was higher in the suspension induction than in the monolayer induction, as shown in Figure 2A. On the other hand, TNNC 1 expression was generally lower in the suspension induction than in the monolayer induction (Fig. 2B). However, the gene expression of TNNC 2 was detected only in the suspension induction and not in the monolayer induction (Fig. 2C). These results indicate that the suppression of TNNC 1 may have been affected by the expression of TNNC 2 and signify the initial stage of cardiac differentiation, as suggested by Stoutamyer and Dhoot.[23] In addition, the gene expression of TNNT 2 and TNNC 2 were detected only in rMSCs treated with inducers and not in the rMSCs treated without inducers (Supplemental Fig. S1 available online at www.liebertonline.com). The expression level of TNNC 1 was generally higher in the treated rMSCs with inducers than in the treated rMSCs without inducers.

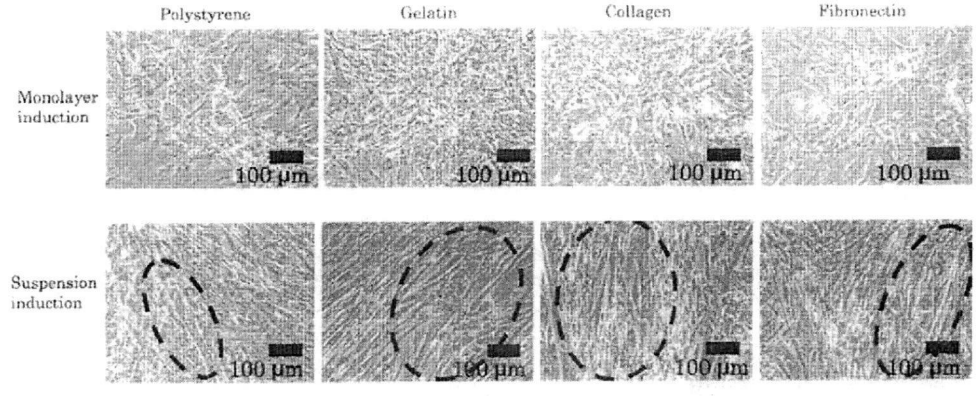

FIG. 1. Microscopic image of rMSCs after monolayer or suspension induction and 3 weeks of culture on several types of dishes. Dashed regions represent the regions of cells with myotube-like shape. rMSC, rat mesenchymal stem cell.

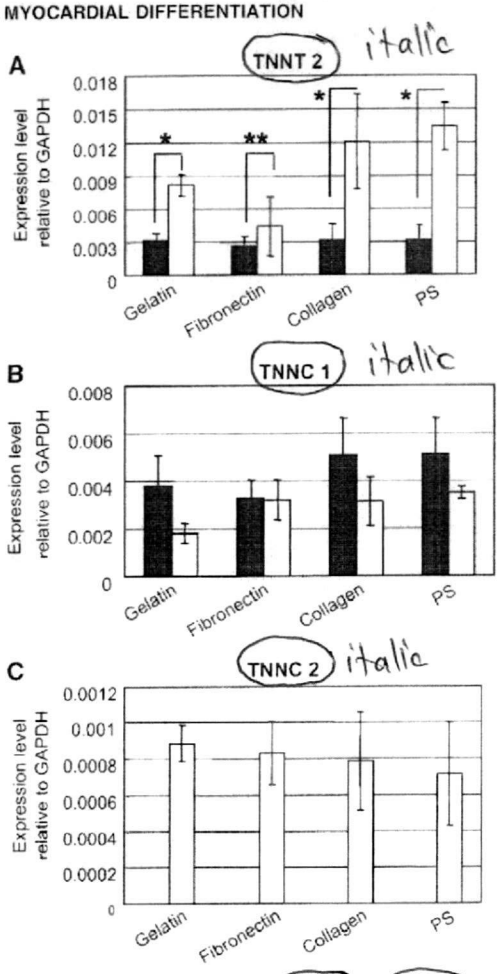

FIG. 2. Expression levels of (A) TNNT 2, (B) TNNC 1, and (C) TNNC 2 in cells after monolayer (■) or suspension induction (□) and culture on different extracellular matrix proteins or uncoated polystyrene dishes ($n = 3$; bars represent ± standard deviation; $^*p < 0.01$, $^{**}p < 0.16$).

In other experiments, the expression of TNN2 was detected after 2 weeks of differentiation and decreased by culture time as shown in Figure 3. This observation is possibly related to the cardiomyocyte differentiation. Besides, this result shows similarity with that during quail heart development *in ovo*.[23]

Myotube-like cells on ECM substrates show spontaneous contraction

In general, about 3 weeks are needed to observe spontaneous beating of the cells without the addition of any chemical reagent, such as acetylcholine.[10,25] Once the beatings are detected, it takes about another 1 week to enter the synchronous stage.

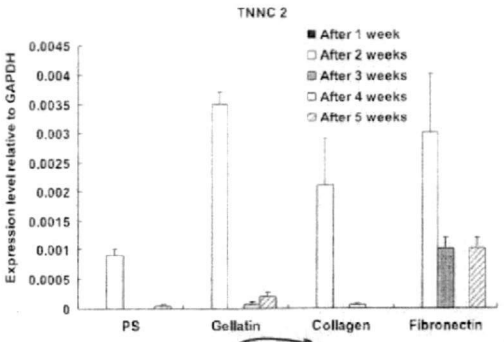

FIG. 3. The expression of TNNC 2 decreased with culture period. Data are means ± standard deviation; $n = 3$ for each sample.

Interestingly, the beating cells and colonies were detected only after they were induced with suspension induction on ECM protein-coated dishes, but not in monolayer induction. We carried out these induction experiments 14 times and found a beating colony only once in monolayer induction on gelatin-coated dishes. As five dishes were used for each experiment, the average number of beating colonies in one dish was calculated as 0.75 ± 1.5 (Table 2). However, the real probability of beating colony appearance was much lower than this value. A large number of beating colonies (4.5 ± 0.6) with sizes ranging from 400 to 500 μm were found in the five gelatin-coated dishes, and 1.3 ± 1.5 beating colonies with a similar size were found in the five fibronectin-coated and collagen type-I-coated dishes. No beating cells were detected in noncoated polystyrene dishes in either form of induction. Table 2 summarizes the colonies of beating cells. Supplemental Video S1 (available online at www.liebertonline.com) shows the beating colonies after 4 weeks of culture on gelatin-coated dishes.

In some cases, monolayer-treated and suspension-treated rMSCs were detached from the dishes after 3 weeks of culture. The nondetached cells proliferated and only suspension-treated rMSCs became beating cells after 6 weeks of culture (data not shown).

Immunostaining

Immunofluorescence examination clearly showed that when the dishes were stained with anti-troponin T-C

TABLE 2. AVERAGE NUMBER OF BEATING COLONIES FOUND IN A DISH (n = 5)

	Average number of beating colonies per dish	
Dish type	*Monolayer*	*Suspension*
Gelatin	0.75 ± 1.5	4.5 ± 0.6
Fibronectin	0	1.3 ± 1.5
Collagen type I	0	1.3 ± 1.5
Polystyrene	0	0

Five 60 mm culture dishes were used for calculation of average beating colony number.

antibody, the beating areas were positively stained as shown in Figure 4, but cells treated by monolayer induction remained at the background level of staining.

Expression of cardiomyocyte-associated genes in beating and nonbeating cells

To define the phenotype of the beating cells, the expression levels of cardiac-specific genes (TNNC 1, TNNT 2, TNNC 2, TNNI 3, GATA 4, and MEF2D) were evaluated. Expression levels of given genes were assessed using RNAs from neonatal hearts as positive qualitative controls. The expression levels of (TNNC 1, TNNT 2, and MEF2D) were higher in beating cells than in nonbeating cells, as shown in Figure 5A and B. The expressions of (TNNI 3 and GATA 4) were detected in only one of four isolated colonies of beating cells and were not detected in any nonbeating colonies. The high expression of (TNNC2) in Figure 5A is possibly because either skeletal muscle cells or initial cardiomyocytes are also present in the beating colonies.

In neonatal cardiomyocytes (3 days of cultivation), (TNNC 1, TNNT 2, TNNI 3, and GATA 4) were expressed (Fig. 5A, B). However, (TNNC 2) was not expressed. These data suggested that 25% of the beating cells were cardiomyocytes and 75% were late-maturing cardiomyocytes.

Discussion

MSCs derived from bone marrow are useful cells because they can be isolated from patients and can differentiate into many types of cells. The production of autologous beating cardiomyocytes is an attractive goal for cell-based therapy. However, in previous studies, differentiation into cardiomyocytes occurred at extremely low rates.[7,10] Therefore, it is essential to establish a new, more effective system for differentiating MSCs into beating cardiomyocytes *in vitro* before being transplanted into patients. Other reports have demonstrated that rat and mouse bone marrow cells can differentiate into cardiomyocytes *in vitro*.[10,11] On the other hand, Liu *et al.* reported that 5-azacytidine could not expand rMSCs or induce their differentiation into cardiomyocytes.[12] In our experience from 14 experiments in suspension and monolayer induction, beating cells were obtained in only 7 experiments with suspension induction and in 1 with monolayer induction. These results suggested that beating cardiomyocytes were not easily obtained after exposure to 5-azacytidine. In this regard, the induction method and the substrate are important to obtain beating cardiomyocytes.

Some reports have described manipulating microenvironmental factors, such as cell dimensions, controlled delivery of soluble factors, chemical cues, mechanical cues, and culture substrates, for the controlled differentiation of stem cells.[26,27] Cells in monolayer culture are in a static environment and have a relatively small surface area for diffusion in contrast to cells in suspension culture.[15] In this study, more than 90% of cells induced with suspension method were adhered onto the dishes at 24 h after the cells were inoculated into monolayer cultures (Supplemental Fig. S2 available online at www.liebertonline.com). After 3 weeks in culture, the cells induced by the suspension method were shaped like myotubes on all types of dishes, as shown in Figure 1, and had begun to form colonies. These differentiated myotube shapes were similar to those described by Wakitani *et al.* and Makino *et al.*

In this study, the expression of (TNNT 2) was higher in suspension induction than in monolayer induction, as shown in Figure 2A. On the other hand, (TNNC 1) expression was generally lower in suspension induction than in monolayer induction (Fig. 2B). However, the gene expression of (TNNC 2) was detected only with the suspension induction and not with the monolayer induction (Fig. 2C). These results indicated that differentiation efficiency was affected by suspension induction. The enhancement mechanism is unclear but may be related to the proliferation activity, which had not yet started in the suspension condition.

The expression levels of cardiac-specific genes (TNNT 2, TNNC 1, and MEF2D) of the beating cells were higher than those of nonbeating cells and almost the same level as those of neonatal cardiomyocytes. The beating of neonatal cardi-

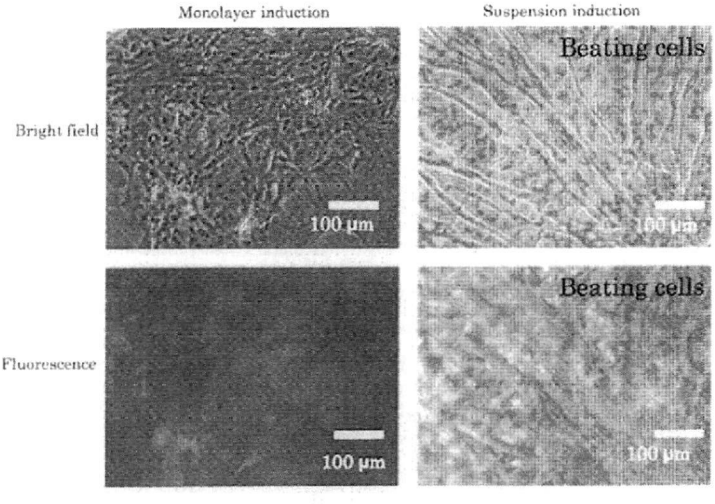

FIG. 4. rMSCs induced by monolayer or suspension method were stained with anti-troponin T-C antibody.

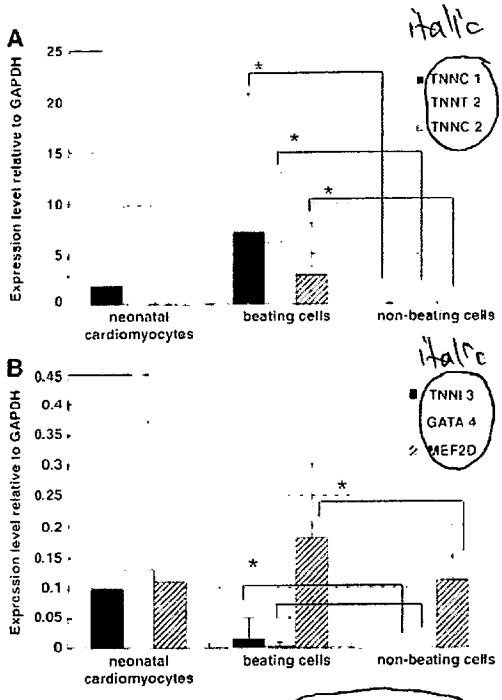

FIG. 5. (A) Expression levels of TNNC 1, TNNT 2, and TNNC 2, (B) TNNI 3, GATA 4, and MEF2D in the four colony types of beating and nonbeating cells after suspension induction. Cardiomyocytes isolated from rat neonatal heart were also evaluated after 3 days of cultivation as a qualitative positive control. Data are means ± standard deviation; $n = 4$ for each sample; $^*p < 0.2$.

omyocytes weakens with culture time. In these experiments, the gene expression of neonatal cardiomyocytes was measured at 3 days after cultivation, to use the well-beating cardiomyocytes as control cells.

Troponin, a calcium-receptive protein complex involved in the contraction of striated muscles, consists of three components: a calcium-binding component (troponin C), a component that inhibits contractile interaction (troponin I), and a tropomyosin-binding component (troponin T).[28] These contractile proteins are expressed in the developing mammalian heart.[24] Therefore, cardiac troponin T (TNNT2), slow troponin C (TNNC1), and cardiac troponin I (TNNI 3) are known to be markers of cardiomyocytes.[23,24] Myocyte enhancer factor (MEF2) genes are recognized to be expressed in differentiated skeletal and cardiac muscle.[29] MEF2D and GATA-4 are regulatory factors for cardiac troponin I.[30] Arceci *et al.* and Edmondson *et al.* reported that the GATA-4 gene was expressed in the early stage[31] and MEF2D in the middle stage of heart development.[32] Fukuda also reported that the MEF2D gene was expressed in cardiomyogenic cells after differentiation.[33] Consequently, in this study, we measured the expression of TNNT2, TNNC1, TNNC2, TNNI3, GATA-4, and MEF2D to examine the differentiation of rMSCs to cardiomyocytes.

Akavia *et al.* reported that GATA 4 and TNNI 3 were expressed only in cardiac muscle cells and not skeletal muscle cells.[34] However, the expression levels were decreased by culture time and were almost undetectable after 10 days of culture for TNNI 3. Referring to this report, we successfully differentiated the MSCs into beating cardiomyocytes by suspension induction. We concluded that expression of GATA 4 and TNNI 3 was not detected in the other three beating colonies because of the long cultivation period. TNNC 2 was also expressed in beating cells. The high expression of TNNC2 shown in Figure 5A, is possibly because either skeletal muscle cells or initial cardiomyocytes were also present in the beating colonies. As shown in Figure 3, TNNC 2 expression was decreased by the culture period. This observation was similar to that of Stoutamyer and Dhoot, who detected TNNC 2 on day 3 in quail hearts *in vivo* but could not detect it by day 17 using RT-PCR.[35]

Interestingly, our results indicated that the spontaneously beating cells were detected only on ECM protein-coated dishes and were induced with suspension induction. The beating cells or colonies were not detected in noncoated polystyrene dishes. These results indicated that the ECM substrate strongly affected cellular differentiation. The number of beating cell colonies on gelatin-coated dishes was much higher than on other substrate-coated dishes. This was similar to the result in our previous study, in which the beat and the beating period of isolated neonatal rat cardiomyocytes on gelatin-coated dishes were stronger and longer than on fibronectin- or collagen-coated dishes or on noncoated polystyrene dishes.[19] This phenomenon may be related to the cell biological activity and the physical properties of the substrate. It has been reported that during cell culture, large amounts of fibronectin are produced, which associates with collagen in a way that promotes fibrillogenesis.[35] It was also reported that fibronectin binds to gelatin more strongly than to collagen.[35,36] Therefore, fibril might be produced at higher levels on gelatin-coated dishes than on fibronectin- or collagen-coated dishes.

A very large difference was observed in the number of beating colonies on gelatin-coated and collagen type I-coated dishes. The dynamic storage modulus of gelatin is higher than that of collagen type I.[37] The high dynamic storage modulus of gelatin, as well as substantial movement and stretching, may have allowed easier contraction of differentiated rMSCs, which might have contributed to the large difference in the number of beating colonies. It was also reported that the elasticity of the substrate affects the differentiation of naive MSCs and mouse myoblast C2C12 cells into myogenic cells.[38,39]

In this study, cardiac gene expression analysis and immunostaining were performed to verify whether the beating cells derived from treated MSCs were cardiomyocytes or not. The beating cells and colonies were detected at 3 weeks after treatment ended and became synchronous after 1 week. Therefore, the immunostaining was done at 4 weeks after treatment ended. The results of immunofluorescence and cardiac gene expression demonstrated that the beating cells expressed cardiomyocyte-like phenotypes.

Conclusion

In conclusion, this study demonstrated that suspension induction is a promising method for differentiating rMSCs

into cardiomyocytes *in vitro*. In addition, the ECM protein is seen to affect the differentiation of rMSCs into beating cells. These findings indicate a bright future for the production of autologous beating cardiomyocytes for cell-based therapy and myocardial patch scaffold. In this study, a gelatin-based niche was found to be preferable for cardiac differentiation and beating function. Nevertheless, further study is required to characterize the full mechanism of the suspension method for the differentiation of MSCs into cardiomyocytes as well as the effect of differentiated MSCs after transplantation *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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