CHINESE / 中国語

卵巢切除大鼠之鈦螺旋式植體周圍骨癒合的實驗研究：利用骨髓基質細胞移植促進骨癒合。

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摘要：
目的：本研究旨在利用將骨髓基質細胞 (BMSC) 移植到大鼠股骨評估植體周圍區域的骨質，這已經成為引致骨質疏鬆的模型。

資料與方法：將 SD 大鼠分成三組：第一組切除卵巢 (OVX 組)，第二組進行假手術，第三組將 BMSC 移植到 OVX 組 (OVXBMSC 組)。在 OVXBMSC 組中，將 1×10　BMSC移植到有植體的股骨內。取得各組的骨植體接觸 (BIC) 值以及各皮質骨和海綿骨的骨面積 (BA)。從寬度 500 μm 的植體測量骨質密度 (BD)。

結果：OVX-BMSC 組海綿骨的 BIC、BA 和 BD 比率明顯高於 OVX 組。

結論：BMSC 移植療法改善植體周圍海綿骨的局部骨癒合，還同時改善骨植體結合度。

關鍵字：骨質疏鬆、骨髓基質細胞、鈦植體、細胞移植、骨整合

KOREAN / 한국어

난소적출 랫드에서 티타늄 스크류 임플란트 주변 골치유 실험 연구: 골수기질세포이식에 의한 골치유 증강

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요약:
목적: 본 시험의 목적은 골다공증 유발 모델인 랫드 대퇴골의 골수기질세포(BMSCs)와 임플란트 주변 부위 골질을 평가하기 위함이다.

재료 및 방법: SD 랫드는 다음과 같은 3 군으로 나뉘었다:난소를 제거한 첫번째 군(OVX 군), 모의수술(sham surgery)를 시행한 두번째 군(SHAM 군), OVX군에 BMSC를 이식한 세번째 군(OVX-BMSC군). OVX-BMSC군에, 임플란트와 함께 1×10┌ BMSCs 를 대퇴골에 이식하였다. 각각의 뼈와 임플란트 간 접촉값(BIC), 그리고 각 골피질과 해면골의 골 부위(BA)를 얻었다. 임플란트로부터 500 μm 폭의 골밀도(BD)가 측정되었다.

결과: OVX-BMSCs 군에서 해면골의 경우 BIC, BA 및 BD의 각각의 비는 OVX군보다 OVX-BMSC군에서 유의하게 높았다.

결론: BMSC이식요법은 임플란트 주변 해면골의 국소 골 치유를 개선시켰으며 또한 임플란트와 골결합을 유의하게 개선시켰다.

키워드: 골다공증, 골수기질세포, 티타늄 임플란트, 세포이식, 골유합

INVERSE ASSOCIATION BETWEEN TOOTHBRUSHING AND UPPER AERODIGESTIVE TRACT CANCER RISK IN A JAPANESE POPULATION

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Accepted 22 September 2010
Published online 00 Month 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/hed.21649

Abstract: *Background.* Oral hygiene is attracting increasing attention as a potential risk factor for cancers. To investigate the association between toothbrushing frequency and upper aerodigestive tract (UADT) cancer, the authors conducted a large-scale case-control study.

Methods. A total of 856 UADT cancer case participants and 2696 age- and sex-matched control subjects without cancer were included. Edentulous or participants with unknown frequency of toothbrushing or number of remaining teeth were excluded. Associations were assessed by odds ratios and 95% confidence intervals in logistic regression models with adjustment for potential confounders.

Results. Compared with toothbrushing once per day, the adjusted odds ratio for brushing twice or more was 0.82 (95% confidence interval: 0.68, 0.99) whereas that for not brushing was 1.79 (0.79, 4.05). This association was observed especially in subjects who had a history of heavy smoking or drinking.

Conclusions. The authors suggest that toothbrushing could have a protective effect for UADT cancer. © 2011 Wiley Periodicals, Inc. *Head Neck* **00:** 000–000, 2011

Keywords: toothbrushing; oral hygiene; upper aerodigestive tract cancer; preventive care; case-control study

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This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan, for Cancer Research from the Ministry of Health, Labour and Welfare of Japan, and for the Third Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan. These grantors were not involved in the study design, subject enrollment, study analysis or interpretation, or submission of the manuscript for this study.

Worldwide, more than 1 million new cases and 680,000 deaths caused by upper aerodigestive tract (UADT) cancer are reported each year, with 560,000 cases and 300,000 deaths for head and neck cancer and 450,000 cases and 380,000 deaths for esophageal cancer. Collectively, these account for approximately 10% of the world's total new cancer cases.[1] Many studies have investigated the association between tobacco use and alcohol consumption and UADT cancer, and these are now considered the main risk factors for this cancer.[2–4] However, given that these cancers also occur among people who never smoke and never drink,[5] other risk factors are also likely to be present. To date, the potential importance of fruit and vegetable intake, frequent drinking of hot beverages, low body mass index (BMI), and socioeconomic status were also considered substantial risk factors for head and neck cancer and esophageal cancer, in addition to smoking and drinking.[6–8]

Recently, oral health status, such as periodontal disease and tooth loss, has attracted attention as a risk factor for cancer. Several epidemiologic studies have suggested that these diseases may play a role in head and neck, esophageal, lung, pancreatic, kidney, and hematopoietic cancers,[9–13] and among these, the association is most consistently observed with UADT cancer. Toothbrushing is a daily means of maintaining oral health and is thus closely related to oral health and hygiene.[14–16] If a bad oral environment is associated with a risk of cancer, it can easily be suggested that toothbrushing may reduce the risk. To our knowledge, there are 7 studies that showed odds ratios (OR)

with regard to the association between toothbrushing frequency and cancer: 2 studies in the oral region (Spain and India), 3 studies in the head and neck region (Brazil, Europe, and Italy), 1 study in the esophageal region (Iran), and 1 study in the UADT region (Latin America). In these studies, toothbrushing seemed to provide a protective effect against head and neck and esophageal cancer, although these studies have heterogeneity in race and ethnicity.[11,17–22] We conducted a case-control study to investigate the association between toothbrushing frequency and UADT cancer in a Japanese population.

MATERIALS AND METHODS

Study Population. The case participants were 959 patients with no history of cancer who were histologically diagnosed with UADT cancer (278 with oral cavity cancer, 75 with oropharyngeal cancer, 79 with hypopharyngeal cancer, 93 with laryngeal cancer, and 434 with esophageal cancer) between January 2001 and December 2005 at Aichi Cancer Center Hospital in Japan. All participants were recruited within the framework of the hospital-based Epidemiologic Research Program at the Aichi Cancer Center, as described elsewhere.[23–25] The study was approved by the Institutional Ethical Committee of Aichi Cancer Center Hospital.

UADT cancer was defined according to the following codes of the International Classification of Diseases and Related Health Problems (ICD10): oral cavity (C02.0-C02.3, C03, C04, C05.0, C06), oropharynx (C01, C02.4, C05.1-C05.2, C09, C10), hypopharynx (C12, C13), larynx (C32), and esophagus (C15). Malignant neoplasms of the lip (C00.0–C00.9), salivary glands (C07, C08), nasopharynx (C11), nasal (C30), and paranasal (C31) were excluded because these have quite distinct causes. The control participants were 2877 first-visit outpatients seen during the same period who were confirmed to have no cancer and no history of neoplasms. Noncancer status was confirmed by medical examination, including radiographic examination, with participants suspected of having UADT cancer first examined by physical or endoscopic inspection and subsequently with radiography if indicated. Control subjects were selected randomly and individually matched by age (±4 years) and sex (male; female), with a case-control ratio of 1:3. Patients whose brushing frequency data are unknown (25 cases and 55 control subjects) or edentulous (78 cases and 115 control subjects) were excluded from both case and control groups. A total of 3552 participants (856 cases and 2696 control subjects) were included.

Toothbrushing and Number of Remaining Tooth Information. Information on lifestyle factors, as well as other relevant factors, including brushing status and number of remaining teeth, was collected from first-visit outpatients aged 20 to 79 years by use of a self-administered questionnaire. Each participant was asked at the time of the first visit to our hospital about lifestyle factors with regard to environmental exposures before the symptoms that made them visit our hospital developed. Brushing status was determined by the question, "On which occasions do you brush your teeth?" Participants could choose multiple occasions from among the following: 1, at awakening; 2, after breakfast; 3, after lunch; 4, after snacks between meals; 5, after dinner; 6, before sleeping; and 7, none. Number of remaining teeth was determined by the question "How many of your own teeth do you have?," and responses were categorized into 4 groups: group 1, number of remaining teeth, >21; group 2, 9 to 20; group 3, 1 to 8; and group 4, 0. Responses were checked by trained interviewers.

Evaluation of Lifestyle Factors. Information on smoking status was obtained in the 3 categories of nonsmoker, former smoker, and current smoker, with former smokers defined as those who had quit smoking at least 1 year before the survey. Cumulative smoking dose was evaluated as pack-years, the product of the number of packs consumed per day and years of smoking, and categorized into 4 groups: never smoked, 0 to <20 pack-years, ≥20 to <40 pack-years, and ≥40 pack-years. Lifetime alcohol consumption of various common beverages (Japanese sake, beer, shochu, whiskey, and wine) was determined in terms of the average number of drinks per day, which was then converted into a Japanese sake (rice wine) equivalent. We asked about the amount consumed in terms of one *go* (180 mL) of Japanese sake equivalent, which contains 23 g ethanol, namely 1 large bottle (633 mL) of beer, 2 shots (57 mL) of whiskey, or 2.5 glasses of wine (200 mL). One drink of "shochu" (distilled spirit), which contains 25% ethanol, was rated as 108 mL. Drinking status was classified into the 4 categories of never drank, moderate drinker (drinks less than 5 days per week), frequent moderate drinker (drinks 5 or more days per week at less than 2 go per day), and frequent heavy drinker (drinks 5 or more days per week at 2 or more go per day). Consumption of fruits and vegetables was determined by use of a food frequency questionnaire (FFQ). Briefly, the FFQ consisted of 43 single food items, with frequencies in 8 frequency categories. We estimated average daily intake by calculating as the sums of consumption of contributing single food items as estimated from the food composition table, food frequency, and portion size. Energy-adjusted intake of fruits and vegetables was calculated by the residual method.[26] The FFQ was validated with a 3-day weighted dietary record as standard, which showed that reproducibility and validity were acceptable.[27,28] Participants were divided into 3 groups on the basis of distribution among control subjects (tertile). With

regard to hot beverage intake, we defined those who consume coffee or green tea more than 3 times per day as frequent consumers. BMI was calculated from reported height and weight: BMI (kg/m^2). In the analysis, we dichotomized participants with a threshold of 25 kg/m^2. Participants were also asked about their occupation as a measure of socioeconomic status (SES) and were categorized into following 3 groups, white collar, blue collar, or other. Others included working at a part time job, homemaker, student, and people without an occupation.

Statistical Analyses. We assessed the effect of toothbrushing frequency on the risk of UADT cancer in terms of odds ratios (OR) and 95% confidence intervals (95% CI) as estimated by conditional and unconditional logistic regression models with adjustment for potential confounders. We defined those who brush their teeth once a day as a reference category because of the small number of those who do not brush their teeth. Potential confounders considered in the models were age, sex, smoking and drinking status, vegetable and fruit intake, frequent intake of hot beverages, BMI, occupation as SES, and number of remaining teeth. Unconditional logistic regression models were applied in stratified analyses for sex, age, amount of smoking and alcohol consumption, vegetable and fruit intake, frequent intake of hot beverages, BMI, occupation, and number of remaining teeth with adjustment of potential confounders other than stratifying variable. Missing values for covariates were treated as dummy variables in the models. All analyses were done with Stata SE version 10 (Stata Corporation, College Station, TX).

RESULTS

Characteristics of participants are shown in Table 1. As anticipated, the proportion of those who ever smoked and drank was higher in the case participants than in the control subjects. In a univariate analysis, smoking and drinking had a significant dose-dependent relationship with UADT cancer. Compared with control participants, case participants were less likely to eat vegetables and fruits, were more likely to be slender, and to work a blue collar job, and were less likely to have remaining teeth. These relationships were significantly observed in the trend test (Table 1).

Table 2 presents the association between frequency of toothbrushing and cancer stratified by subsite. In overall analysis, compared with brushing once a day, brushing 2 or more times showed an inverse association (OR: 0.82, 95% CI: 0.68, 0.99) in UADT cancer risk after adjustment for potential confounders, whereas no brushing showed a direct association (OR, 1.79; 95% CI, 0.79, 4.05), with the trend test demonstrating statistical significance ($p = .016$). In subsite analysis, in brushing once per day versus more than twice per day, this association was consistently observed in all sites with ORs lower than unity. In none versus once, a direct association was observed in head and neck, oral cavity, hypopharyngeal/laryngeal but not oropharyngeal and esophageal cancer. In total, only 27 participants did not brush their teeth. The trend test was only significant in head and neck and oral cavity cancer (Table 2).

Table 3 presents the stratified analysis by potential confounder to examine the consistency of the association between frequency of toothbrushing and UADT cancer. We also considered stratified analysis in subsite, but because of the low number of those who didn't brush their teeth, we only compared between once per day versus twice or more per day in Table 4. The stratifying variables included were sex, age, smoking and drinking status, frequency of drinking hot beverages, BMI, vegetable and fruit intake, occupation, and number of remaining teeth, which could be regarded as potential confounders for UADT cancer. In overall analysis, compared with brushing once a day, those who brush their teeth 2 or more times had ORs with point estimates lower than unity in all strata, and those not brushing their teeth had increased ORs in all strata except for those who drink frequently moderately and have more than 21 teeth (Table 3). This trend was similarly observed in oral cavity, oropharyngeal, hypopharyngeal/laryngeal, and esophageal cancer (Table 4). In UADT cancer, a significant association was observed among category of male, age less than 70, heavy smoker, heavy drinker, lowest vegetable intake, lowest fruit intake, hot drink intake less than 2 times, BMI less than 25, other occupation, and having more than 21 teeth (Table 3).

DISCUSSION

In this case-control study, we found that frequent toothbrushing could have a protective effect for UADT cancer regardless of other risk factors (Table 2). This association was consistently observed in stratified analyses by potential confounders (Table 3). Furthermore, OR was especially decreased among category of heavy smoking, heavy drinking, lowest vegetable intake, lowest fruit intake, BMI less than 25, and other occupation with significance, which may suggest that toothbrushing is more effective for people who have risk factors for UADT cancer. This relationship was almost similarly observed in oral cavity, oropharyngeal, hypopharyngeal/laryngeal, and esophageal cancers, respectively. However, because we could not have a large enough number of cases to analyze in subsite, a larger study is warranted (Table 4).

The main effect of toothbrushing is the control of dental plaque, which is biofilm consisting almost entirely of bacteria.[29] More frequent brushing results in a decrease in dental plaques[14–16] and further prevents periodontitis and gingivitis.[30,31] Given this,

Table 1. Participant characteristics.

	Cases		Control subjects				
	No.	%	No.	%	OR*	95% CI	*p* value for trend
Cancer site							
Upper aerodigestive tract cancer (overall)	856		2696				
Head and neck cancer	469						
Oral cavity cancer	261						
Oropharyngeal cancer	61						
Hypopharyngeal cancer	69						
Laryngeal cancer	78						
Esophageal cancer	387						
Sex							
Male	700	81.8	2178	80.8			
Female	156	18.2	518	19.2			
Age							
<40, y	41	4.8	119	4.4			
40–49, y	67	7.8	217	8.1			
50–59, y	272	31.8	840	31.2			
60–69, y	312	36.5	1042	38.7			
≥70, y	164	19.2	478	17.7			
Amount of smoking							<.001
Never	158	18.5	983	36.5	1.00	Reference	
0 to <20 pack-years	99	11.6	488	18.1	1.65	1.21, 2.26	
≥20 to ≤40 pack-years	240	28.0	547	20.3	4.11	3.11, 5.45	
≥40 pack-years	350	40.9	651	24.2	5.49	4.15, 7.25	
Alcohol consumption							
Never	150	17.5	870	32.3	1.00	Reference	<.001
Light	137	16.0	746	27.7	1.26	0.96, 1.65	
Frequently moderate	195	22.8	623	23.1	2.15	1.64, 2.80	
Frequently heavy	354	41.4	424	15.7	6.27	4.81, 8.18	
Unknown	20	2.4	33	1.2			
Vegetable intake (range: g/d)							<.001
Lowest tertile (0.83–73.7)	340	39.7	886	32.9	1.00	Reference	
Middle tertile (30.2–81.2)	277	32.4	887	32.9	0.80	0.66, 0.97	
Highest tertile (131–902	209	24.4	877	32.5	0.61	0.50, 0.75	
Unknown	30	3.5	46	1.7			
Fruit intake (range: g/d)							<.001
Lowest tertile (4.16–30.2)	388	45.4	889	33.0	1.00	Reference	
Middle tertile (73.7–131)	261	30.5	886	32.9	0.61	0.50, 0.75	
Highest tertile (81.2–506.2)	170	20.9	877	32.5	0.46	0.36, 0.57	
Unknown	27	3.2	44	1.6			
Hot beverage intake frequency							
<3 times/d	471	55.0	1553	57.6	1.00	Reference	
>3 times/d	352	41.1	1078	40.0	1.11	0.94, 1.30	
Unknown	33	3.9	65	2.4			
BMI, kg/m^2							
<25	725	84.7	2061	76.5	1.00	Reference	
≥25	126	14.7	617	22.9	0.55	0.45, 0.69	
Unknown	5	0.6	18	0.7			
Occupation							
White collar	317	37.0	728	27.0	1.00	Reference	
Blue collar	185	21.6	832	39.9	0.50	0.41, 0.62	
Other	347	40.5	1119	41.5	0.74	0.61, 0.92	
Unknown	7	0.8	17	0.6			
Teeth number							<.001
1–8	185	21.6	351	13.0	1.00	Reference	
9–20	293	34.2	832	30.9	0.63	0.50, 0.79	
≥21	378	44.2	1513	56.1	0.41	0.33, 0.52	

**ORs were calculated by conditional univariate logistic regression.*

several mechanisms can be considered behind the protective effect for UADT cancer. First, because toothbrushing can reduce the number of microorganisms by removing plaque, levels of inflammation might be reduced locally or in general, which is induced by production of bacteria or bacteria itself. Bacterial infection induces chronic inflammation, with increased concentrations of circulating inflammatory cytokines,[32,33] possibly leading to cancer.[34] Treatment of periodontal infection reduces these cytokines.[35] Second, because oral microorganisms can induce the production of carcinogenic byproducts, such as nitrosamines[36] and acetaldehyde,[37,38] it is possibly protected from these production if

Table 2. Associations between frequency of toothbrushing and upper aerodigestive tract cancer.

	Frequency of toothbrushing												
	None				Once a day				2 or more times a day				*p* value
	Case	Control	OR*	95% CI	Case	Control	OR*	95% CI	Case	Control	OR*	95% CI	for trend
Upper aerodigestive tract cancer	18	19	1.79	0.79, 4.05	425	990	1.00	Reference	413	1687	0.82	0.68, 0.99	.016
Head and neck cancer	14	9	2.86	1.07, 7.66	221	519	1.00	Reference	234	938	0.78	0.61, 1.01	.010
Oral cavity cancer	8	3	6.11	1.35, 27.6	110	245	1.00	Reference	143	503	0.81	0.57, 1.14	.046
Oropharyngeal cancer	1	1	0.63	0.03, 13.5	32	79	1.00	Reference	28	124	0.63	0.30, 1.34	.287
Hypopharyngeal/ Laryngeal cancer	5	5	1.35	0.22, 8.17	79	188	1.00	Reference	63	290	0.81	0.50, 1.29	.334
Esophageal cancer	4	10	0.67	0.12, 3.88	204	471	1.00	Reference	179	749	0.86	0.63, 1.16	.379

**ORs were calculated by unconditional logistic regression, and adjusted for age, sex, amount of smoking and alcohol consumption, intake of vegetables, fruits, and hot beverages, BMI, occupation, and number of remaining teeth.*

microorganisms are removed. Acetaldehyde, a metabolite of ethanol, has been implicated as an important risk factor for UADT cancer on the basis of molecular epidemiologic studies.[39–42] Poor dental status increases acetaldehyde production from ethanol.[37,38,43] Third, toothbrushing might directly wash out carcinogens in components of tobacco such as nitrosamines or acetaldehyde and alcohol itself. This

Table 3. Association between frequencies of toothbrushing and upper aerodigestive tract cancer stratified by potential confounder.

	Frequency of toothbrushing												
	None				Once a day				2 or more times a day				*p* value
Stratified by	Case	Control	OR*	95% CI	Case	Control	OR*	95% CI	Case	Control	OR*	95% CI	for trend
Sex													
Male	17	19	1.60	0.75, 3.43	376	898	1.00	Reference	307	1261	0.78	0.64, 0.94	.003
Female	1	0	NE	NE	49	92	1.00	Reference	106	426	0.75	0.40, 0.73	.167
Age													
<60, y	9	4	4.29	1.16, 15.9	184	432	1.00	Reference	187	740	0.82	0.62, 1.08	.039
60–69, y	5	8	1.06	0.28, 4.01	162	392	1.00	Reference	145	642	0.74	0.55, 0.99	.042
≥70, y	4	7	1.16	0.25, 5.46	79	166	1.00	Reference	81	305	0.79	0.2, 1.20	.251
Amount of smoking													
Never	1	2	2.37	0.17, 33.0	47	258	1.00	Reference	110	723	0.83	0.54, 1.27	.319
0 to <40 pack-years	7	9	1.15	0.36, 3.64	159	400	1.00	Reference	173	626	0.89	0.68, 1.18	.397
≥40 pack-years	16	16	1.97	0.67, 5.77	376	710	1.00	Reference	300	909	0.58	0.44, 0.78	<.001
Alcohol consumption													
Never	3	8	2.42	0.53, 11.0	50	286	1.00	Reference	97	576	0.87	0.56, 1.34	.301
Light, frequently moderate	3	9	0.44	0.11, 1.83	157	486	1.00	Reference	172	874	0.79	0.60, 1.03	.161
Frequently heavy	12	2	4.93	0.99, 24.5	207	203	1.00	Reference	135	219	0.69	0.51, 0.95	.003
Vegetable intake (range: g/d)													
Lowest tertile (0.83–73.7)	11	8	3.06	1.03, 9.14	196	405	1.00	Reference	133	473	0.67	0.50, 0.91	.001
Middle tertile (73.7–131)	4	4	2.21	0.50, 9.76	131	333	1.00	Reference	142	550	0.87	0.64, 1.18	.235
Highest tertile (131–902)	2	4	0.99	0.13, 7.29	81	235	1.00	Reference	126	638	0.89	0.62, 1.28	.522
Fruit intake (range: g/d)													
Lowest tertile (4.16–30.2)	8	7	2.52	0.81, 7.84	200	388	1.00	Reference	141	462	0.70	0.53, 0.94	.004
Middle tertile (30.2–81.2)	4	4	2.92	0.59, 14.5	92	305	1.00	Reference	119	543	0.90	0.64, 1.28	.343
Highest tertile (81.2–506.2)	1	2	1.37	0.06, 32.1	59	231	1.00	Reference	103	607	0.89	0.59, 1.33	.540
Hot beverage intake frequency													
<3 times/d	11	11	1.88	0.69, 5.12	234	578	1.00	Reference	226	964	0.81	0.64, 1.04	.041
>3 times/d	6	7	1.65	0.49, 5.59	172	386	1.00	Reference	174	685	0.79	0.59, 1.04	.056
BMI, kg/m^2													
<25	15	12	1.76	0.75, 4.10	362	707	1.00	Reference	348	1342	0.73	0.60, 0.89	.001
≥25	2	7	1.33	0.25, 7.02	61	274	1.00	Reference	63	336	0.94	0.61, 1.44	.698
Occupation													
White collar	2	0	NE	NE	83	305	1.00	Reference	100	527	0.88	0.61, 1.27	.290
Blue collar	7	8	1.73	0.53, 5.60	177	339	1.00	Reference	133	381	0.90	0.66, 1.22	.328
Other	9	11	1.19	0.42, 3.39	160	340	1.00	Reference	178	768	0.66	0.50, 0.88	.004
Number of teeth													
1–8	9	8	2.67	0.82, 4.66	101	158	1.00	Reference	75	185	0.80	0.52, 1.24	.102
9–20	7	5	3.28	0.86, 12.5	147	331	1.00	Reference	139	496	0.84	0.61, 1.14	.091
≥21	2	6	0.78	0.12, 5.15	177	501	1.00	Reference	199	1006	0.74	0.57, 0.96	.027

Abbreviation: NE, not estimated.
**ORs were calculated by unconditional logistic regression, and adjusted for age, sex, amount of smoking and alcohol consumption, intake of vegetables, fruits, and hot beverages, BMI, occupation, and number of remaining teeth.*

Table 4. Association between frequencies of toothbrushing and oral cavity, oropharyngeal, hypopharyngeal/laryngeal, and esophageal cancer, respectively, stratified by potential confounder.

	Frequency of toothbrushing					
	Once a day		2 or more times a day			
Stratefied by	Case	Control	Case	Control	OR*	95% CI
Oral cavity cancer						
Sex						
Male	89	198	75	276	0.80	0.53, 1.20
Female	21	47	68	227	0.75	0.37, 1.52
Age						
<60, y	61	143	86	293	0.86	0.55, 1.35
60–69, y	32	77	36	137	0.97	0.47, 2.02
≥70, y	17	25	21	73	0.61	0.23, 1.60
Amount of smoking						
Never	25	75	70	265	0.79	0.43, 1.46
0 to <40 pack-years	47	102	52	173	0.82	0.49, 1.38
≥40 pack-years	38	68	21	65	0.58	0.26, 1.28
Alcohol consumption						
Never	20	74	57	216	0.76	0.37,1.55
Light, frequently moderate	52	124	60	241	0.68	0.43, 1.10
Frequently heavy	31	45	22	41	1.02	0.40, 2.61
Vegetable intake (range: g/d)						
Lowest tertile (0.83–73.7)	53	108	56	129	0.96	0.55, 1.67
Middle tertile (73.7–131)	27	73	38	167	0.74	0.38, 1.45
Highest tertile (131–902)	22	62	45	196	0.85	0.42, 1.75
Fruit intake (range: g/d)						
Lowest tertile (4.16–30.2)	56	99	53	135	0.83	0.49, 1.42
Middle tertile (30.2–81.2)	22	72	40	148	0.85	0.42, 1.69
Highest tertile (81.2–506.2)	15	57	32	195	0.58	0.25, 1.36
Hot beverage intake frequency						
<3 times/d	64	152	81	284	0.96	0.61, 1.52
>3 times/d	40	90	58	206	0.74	0.42, 1.29
BMI, kg/m^2						
<25	88	168	88	412	0.77	0.52, 1.13
≥25	21	75	21	88	1.29	0.58, 2.86
Occupation						
White collar	19	78	33	146	1.33	0.62, 2.84
Blue collar	50	92	42	125	0.80	0.45, 1.41
Other	40	74	67	229	0.64	0.36, 1.13
Number of teeth						
1–8	20	29	24	42	0.79	0.30, 2.09
9–20	33	80	40	130	0.82	0.42, 1.61
≥21	57	136	79	331	0.77	0.49, 1.22
Oropharyngeal cancer						
Sex						
Male	89	198	75	276	0.80	0.53, 1.20
Female	21	47	68	227	0.75	0.37, 1.52
Age						
<60, y	61	143	86	293	0.86	0.55, 1.35
60–69, y	32	77	36	137	0.97	0.47, 2.02
≥70, y	17	25	21	73	0.61	0.23, 1.60
Amount of smoking						
Never	2	17	6	49	NE	NE
0 to <40 pack-years	10	33	14	62	1.05	0.36, 3.05
≥40 pack-years	16	27	6	26	0.39	0.10, 1.46
Alcohol consumption						
Never	5	25	6	32	20.7	1.15, 3.75
Light, frequently moderate	9	33	12	63	0.69	0.20, 2.37
Frequently heavy	16	21	10	28	0.50	0.15, 1.72
Vegetable intake (range: g/d)						
Lowest tertile (0.83–73.7)	12	39	9	36	0.69	0.18, 2.63
Middle tertile (73.7–131)	11	19	16	35	0.53	0.13, 2.23
Highest tertile (131–902)	9	19	3	51	NE	NE
Fruit intake (range: g/d)						
Lowest tertile (4.16–30.2)	15	37	10	24	1.49	0.47, 4.80
Middle tertile (30.2–81.2)	9	24	6	49	0.52	0.08, 3.19
Highest tertile (81.2–506.2)	2	12	10	39	2.59	0.03, 1.95

(Continued)

Table 4. (Continued).

Stratefied by	Frequency of toothbrushing					
	Once a day		2 or more times a day			
	Case	Control	Case	Control	OR*	95% CI
Hot beverage intake frequency						
<3 times/d	18	46	17	66	0.84	0.32, 2.20
>3 times/d	14	32	11	54	0.80	0.24, 2.62
BMI, kg/m^2						
<25	29	55	22	99	0.58	0.26, 1.25
≥25	3	23	6	25	NE	NE
Occupation						
White collar	8	25	11	46	1.34	0.37
Blue collar	12	31	13	33	1.05	0.22, 5.02
Other	11	23	4	43	0.51	0.06, 4.37
Number of teeth						
1–8	8	12	1	12	NE	NE
9–20	11	27	12	35	0.46	0.11, 1.96
≥21	13	40	15	77	0.67	0.23, 1.98
Hypopharyngeal/Laryngeal cancer						
Sex						
Male	72	182	58	254	0.81	0.51, 1.28
Female	7	6	5	36	1.14	0.02, 84.3
Age						
<60, y	26	73	22	103	0.89	0.39, 2.02
60–69, y	39	69	25	123	0.46	0.23, 0.93
≥70, y	14	46	16	64	2.11	0.64, 7.01
Amount of smoking						
Never	4	43	7	94	0.70	0.13, 3.82
0 to <40 pack-years	23	73	27	122	1.25	0.55, 2.83
≥40 pack-years	52	72	29	74	0.47	0.24, 0.91
Alcohol consumption						
Never	17	57	12	93	1.11	0.37, 3.36
Light, frequently moderate	25	82	27	149	1.06	0.50, 2.24
Frequently heavy	37	46	22	47	0.53	0.24, 1.21
Vegetable intake (range: g/d)						
Lowest tertile (0.83–73.7)	36	79	14	95	0.46	0.20, 1.04
Middle tertile (73.7–131)	24	62	24	97	0.87	0.38, 1.99
Highest tertile (131–902)	15	44	20	94	1.13	0.42, 3.04
Fruit intake (range: g/d)						
Lowest tertile (4.16–30.2)	37	73	17	78	0.61	0.27, 1.39
Middle tertile (30.2–81.2)	14	53	18	106	0.76	0.29, 1.98
Highest tertile (81.2–506.2)	11	52	19	94	1.77	0.67, 4.69
Hot beverage intake frequency						
<3 times/d	41	108	33	167	0.74	0.41, 1.36
>3 times/d	33	74	26	117	0.73	0.33, 1.60
BMI, kg/m^2						
<25	69	138	51	221	0.71	0.43, 1.17
≥25	10	47	11	66	1.49	0.41, 5.36
Occupation						
White collar	11	58	20	94	2.64	0.88, 7.90
Blue collar	33	63	17	58	0.89	0.38, 2.09
Other	34	66	26	137	0.43	0.21, 0.89
Number of teeth						
1–8	26	31	14	37	0.72	0.22, 2.36
9–20	29	73	22	97	0.66	0.30, 1.46
≥21	24	84	27	156	0.88	0.42, 1.87
Esophageal cancer						
Sex						
Male	185	441	151	615	0.80	0.60, 1.07
Female	19	30	28	134	1.04	0.30, 3.65
Age						
<60, y	80	176	65	286	0.78	0.47, 1.30
60–69, y	84	218	73	328	0.87	0.57, 1.33
≥70, y	40	77	41	135	0.93	0.49, 1.77
Amount of smoking						
Never	16	119	27	302	0.73	0.33, 1.63
0 to <40 pack-years	75	188	78	279	0.84	0.53, 1.32
≥40 pack-years	113	164	74	168	0.70	0.46, 1.07

(Continued)

Table 4. (Continued).

Stratefied by	Frequency of toothbrushing					
	Once a day		2 or more times a day			
	Case	Control	Case	Control	OR*	95% CI
Alcohol consumption						
Never	8	128	22	226	1.34	0.48, 3.73
Light, frequently moderate	71	242	73	411	0.77	0.51, 1.16
Frequently heavy	123	91	81	102	0.68	0.44, 1.07
Vegetable intake (range: g/d)						
Lowest tertile (0.83–73.7)	95	178	54	210	0.59	0.36, 0.96
Middle tertile (73.7–131)	69	175	64	244	0.95	0.59, 1.54
Highest tertile (131–902)	35	108	58	286	1.11	0.61, 2.03
Fruit intake (range: g/d)						
Lowest tertile (4.16–30.2)	92	178	61	223	0.65	0.41, 1.05
Middle tertile (30.2–81.2)	47	152	55	232	1.22	0.70, 2.13
Highest tertile (81.2–506.2)	31	108	42	268	0.89	0.48, 1.65
Hot beverage intake frequency						
<3 times/d	111	269	95	435	0.76	0.52, 1.11
>3 times/d	85	186	79	299	1.06	0.68, 1.65
BMI, kg/m^2						
<25	176	342	154	592	0.81	0.59, 1.11
≥25	27	126	24	154	0.66	0.31, 1.41
Occupation						
White collar	45	144	36	239	0.72	0.39, 1.31
Blue collar	82	151	61	161	0.85	0.51, 1.41
Other	75	172	81	344	0.84	0.54, 2.26
Number of teeth						
1–8	47	84	36	88	1.14	0.57, 2.26
9–20	74	150	65	226	0.90	0.55, 1.46
≥21	83	237	78	435	0.69	0.45, 1.06

Abbreviation: NE, not estimated.
**ORs were calculated by unconditional logistic regression, and adjusted for age, sex, amount of smoking and alcohol consumption, intake of vegetables, fruits, and hot beverages, BMI, occupation, and number of remaining teeth. A group of those who brush their teeth once a day was used as the reference group.*

possibility may be partly supported by the stronger association in our heavy drinking and heavy smoking groups (Table 3). These potential mechanisms require clarification in biological and epidemiologic studies.

Our study had several methodologic strengths. First, it was conducted in a single region in central Japan. Second, potential confounding by age, sex, smoking and drinking status, vegetable and fruit intake, hot beverage intake, and BMI are considered as risk factors for UADT cancer and were adjusted for by matching or statistical adjustment in the analyses. Third, the size of the study was relatively large compared with others for UADT cancer conducted in Asian populations.

Several potential limitations of our study also warrant mention. First, measurement of toothbrushing might be affected by the status of cases at recruitment. Therefore, to avoid this, we asked about the self-reported frequency of toothbrushing when the participants were healthy or before the current symptoms developed. Furthermore the way of toothbrushing or attention for oral hygiene might be affected by innate respect for healthy or knowledge about oral hygiene. Second, the control participants were selected from among patients without cancer at our hospital. However, because cases and control subjects were selected from the same hospital and almost all patients lived in the Tokai area of central Japan, the internal validity of this case-control study is likely to be acceptable.[23] In addition, to dilute any bias that might have resulted from the inclusion of a specific diagnostic group that is related to the exposure, we did not set eligibility criteria for control diseases. Third, because the lifestyle factors considered as potential confounders were based on self-report, it is difficult to rule out potential inaccuracy. If present, however, any such misclassification would likely have been nondifferential and would likely have underestimated the causal association. Fourth, recall bias is 1 potential threat in this study. Patients with cancer might answer more badly for health-related questions, and this might bring overestimation. Finally, residual confounding by unmeasured factors such as infection with human papillomavirus (HPV) or *Helicobacter pylori* or SES cannot be ruled out. Because we had only occupational data for SES status, it is difficult to rule out potential residual confounding by educational status or income status. HPV and *H. pylori* are considered to have association with cancer, especially for HPV with oropharyngeal cancer[44,45] and *H. pylori* with esophageal cancer.[46–48] HPV is believed to

be a cause of head and neck cancer, especially for oropharyngeal cancer. In our research, we did subsite analysis, and the result was almost consistent in any sites, and only 61 cases were included as oropharyngeal cancer, then the effect might be small in our data. Recently, cumulative evidence has indicated that *H. pylori* infection plays a protective role in the development of reflux esophagitis and esophageal adenocarcinoma. In contrast, the relationship between *H. pylori* infection and esophageal squamous cell carcinoma is still inconclusive. Our data on esophageal cancer consisted mainly of squamous cell carcinoma (only 5% was adenocarcinoma), and the effect for esophageal adenocarcinoma might be small.

In conclusion, our case-control study demonstrated that frequent toothbrushing has a potential protective effect for UADT cancer risk, especially in having bad habits such as heavy smoking and heavy drinking, and less frequent toothbrushing might be indicated as a risk factor. These findings suggest the potential importance of toothbrushing as a potential candidate for intervention. Further investigation in various ethnicities is warranted.

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Acceleration of Wound Healing with Stem Cell–Derived Growth Factors

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Purpose: *Recently, it has been revealed that bone marrow–derived mesenchymal stem cells (MSCs) accelerate the healing of skin wounds. Although the proliferative capacity of MSCs decreases with age, MSCs secrete many growth factors. The present study examined the effect of mesenchymal stem cell–conditioned medium (MSC-CM) on wound healing.* ***Materials and Methods:*** *The wound-healing process was observed macroscopically and histologically using an excisional wound-splinting mouse model, and the expression level of hyaluronic acid related to the wound healing process was observed to evaluate the wound-healing effects of MSC, MSC-CM, and control (phosphate-buffered saline).* ***Results:*** *The MSC and MSC-CM treatments accelerated wound healing versus the control group. At 7 days after administration, epithelialization was accelerated, thick connective tissue had formed in the skin defect area, and the wound area was reduced in the MSC and MSC-CM groups versus the control group. At 14 days, infiltration of inflammatory cells was decreased versus 7 days, and the wounds were closed in the MSC and MSC-CM groups, while a portion of epithelium was observed in the control group. At 7 and 14 days, the MSC and MSC-CM groups expressed significantly higher levels of hyaluronic acid versus the control group (P < .05). The expression level of hyaluronic acid was lower at 14 days than at 7 days in all three groups.* ***Conclusions:*** *Both the MSC and MSC-CM groups accelerated wound healing versus the control group to a similar degree. Accordingly, it is suggested that the MSC-CM contains growth factor derived from stem cells, is able to accelerate wound healing as well as stem cell transplantation, and may become a new therapeutic method for wound healing in the future.* ORAL CRANIOFAC TISSUE ENG 2011;1:181–187

Key words: *cell therapy, human mesenchymal stromal cell, hyaluronic acid, stem cell–derived growth factor, wound healing*

An intractable chronic wound exerts considerable stress on patients, and treatment of such wounds is very difficult.[1,2] Surgery and medical treatments have been performed to treat such wounds. However, there are many problems, including failure of wound healing and scarring, with these treatments. In addition, wound closure and treatment effectiveness cannot be guaranteed in many cases.[3] Therefore, effective treatment measures must be established. Many therapeutic approaches, including ointments, artificial skin, and substitute skin, have been developed. Furthermore, cell therapy, which is a low-aggression method to promote scar-free wound healing, has been explored. Many reports have demonstrated that the differentiation and paracrine effects of mesenchymal stem cells (MSCs) accelerate wound healing.[4–6] MSCs are referred to as stromal progenitor, self-renewing, and expandable stem cells and are able to differentiate into osteoblasts, adipocytes, chondrocytes, and other cell types.[7] However, bone marrow aspiration is invasive,

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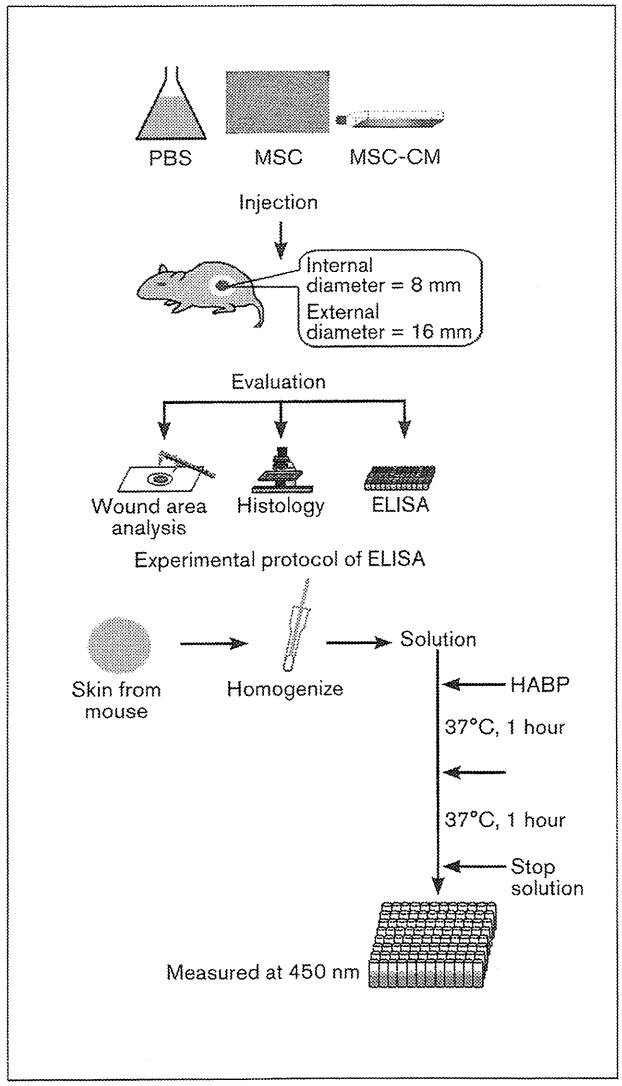

Fig 1 Scheme of the experiment.

and the quantity, proliferative capacity, and capability of differentiation of MSCs decrease with age.[8]

In general, the process of wound healing consists of inflammation, granulation, reepithelialization, and remodeling. The process also depends on various interactions between cells and extracellular matrix (ECM). Hyaluronic acid (HA), one of the components of ECM, is not solely a component of different matrices but is also involved in dynamic cellular processes in wound healing.[9–11] Therefore, it is important to observe the behavior of HA during the wound-healing process.

The present study is based on the assumption that the wound-healing accelerating effect of MSC is caused by growth factors secreted by MSCs, not by the function of the stem cells per se. The authors therefore examined the effect of MSC-conditioned medium (MSC-CM) in wound healing by using a wound-healing skin defect model and compared the effects of MSC-CM treatment with those of MSC and control (phosphate-buffered saline [PBS]) treatments. Furthermore, the amount of HA, which plays an important biologic role in skin wound healing, was measured in each group.

MATERIALS AND METHODS

Animals

Seven-week-old KSN/Slc nude mice (Chubu Kagaku Shizai) were used for the study resulting in 10 sites. Animal experimentation was conducted according to the Guidelines for Proper Conduct of Animal Experiments of the Nagoya University School of Medicine.

MSC Culture and Preparation of MSC-CM

MSCs were purchased from LONZA. Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing a low concentration of glucose supplemented with 10% fetal bovine serum, 100 IU penicillin G, 100 μg streptomycin, and 0.25 μg amphotericin B (Sigma) at 37°C in 95% humidified air and 5% carbon dioxide.[12,13]

MSC-CM was prepared by culturing MSCs (5 × 106) using serum-free DMEM/F12 medium (Invitrogen-Gibco BRL) for 72 hours. This was centrifuged at 300×g for 5 minutes, filtrated with a 0.22-μm syringe filter, and then used for the experiment.[14]

Wound-Healing Model, Cell Transplantation, and Administration of Test Materials

The experimental protocol is shown in Fig 1. The animals were randomly divided into three groups: control group, which would receive an injection of PBS; the MSC transplantation group; and the MSC-CM administration group. An excisional wound-splinting model that was established in a previous report was used.[15] In brief, two 8-mm full-thickness excisional skin wounds were formed on both sides of the midline on the back of a mouse. Doughnut-shaped silicone splints were placed on each wound so that the wound was at the center of the splint. Instant adhesive (Krazy Glue) was used to glue the splints to the skin, and the splint was then sutured to secure it.

For animals in the MSC transplantation group, PBS containing 4 × 106 cells (80 μL) was injected into four spots around each wound, PBS containing 1 × 106 cells (20 μL) was placed on the wound, and Tegaderm (3M) was placed on the wound. For the MSC-CM and control groups, 20 μL of MSC-CM or PBS (control) was injected in four spots around the wound and placed on the wound in the same manner as the MSC transplantation group.

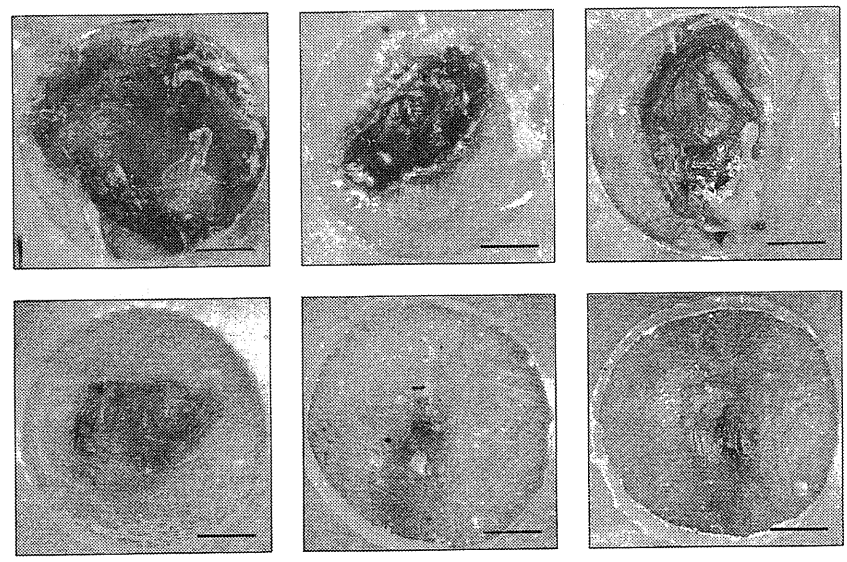

Fig 2 Macroscopic findings at 7 and 14 days after injection. *(Upper row)* 7 days after administration; *(bottom row)* 14 days after administration. *(Left column)* Control group; *(middle column)* MSC group; *(right column)* MSC-CM group. Bar = 2 mm.

Assessment of Wound Area

Digital photographs of the wounds were taken on the day of administration and on 3, 5, 7, 10, and 14 days after administration. The area of the wound was measured using an image analysis program (Scion Image, Scion). The percentage of the area of wound was evaluated with the formula (area of actual wound/area of original wound) × 100.

Mice were sacrificed at 7 and 14 days after administration of test materials. Samples were prepared for histologic and immunologic analyses and evaluated using the image analysis program.

Histologic Examination and Immunofluorescent Staining

Extracted tissue was fixed with 4% paraformaldehyde and embedded into OCT compound (Tissue-Tek, Miles). The samples were sliced into 5-μm sections and stained with hematoxylin-eosin. The expression of HA was confirmed with immunostaining (Rockland Immunochemicals) as follows. First, biotinylated HA binding protein (Seikagaku) was diluted 1:100 with PBS and incubated on slides with the samples at room temperature for 2 hours. Then, the slides were rinsed with PBS and incubated with fluorescein isothiocyanate–conjugated streptavidin (Beckman Coulter) diluted 1:200 with PBS at room temperature for 15 minutes. The slides were sealed with a mounting medium containing 4,6-diamidino-2-phenylindole-2-HCl (DAPI) (Vector Laboratories). To reduce intrinsic fluorescence in the tissue, the slide was incubated with 1% bovine serum albumin for 1 hour and then incubated with a streptavidin-biotin blocking kit (Vector Laboratories) at room temperature for 20 minutes to block endogenous biotin. Then, the tissue and immunofluorescence were observed with a fluorescence microscope (Microscope BX51; Olympus).

Enzyme-Linked Immunosorbent Assay of HA

An enzyme-linked immunosorbent assay (ELISA) HA quantitative test kit (Seikagaku) was used according to the provided protocol to determine the amount of HA in samples collected at 7 and 14 days after administration (Fig 1). The HA test kit is an enzyme-binding protein assay kit using a capture molecule known as HA binding protein (HABP). The samples are first reacted with biotinylated HABP solution and then with horseradish peroxidase–labeled streptavidin solution as the secondary reaction. The reaction is stopped with stop solution, and the amount of HA is colorimetrically measured at 450 nm. HA levels of all samples were calculated with reference to readings of blanks and a reference solution supplied with the kit.

Statistical Analysis

Differences between the groups were evaluated with the Tukey-Kramer test using one-way analysis of variance. A value of $P < .05$ was considered to indicate statistical significance.

RESULTS

Macroscopic Observation of Wound Healing

The wounds on the mice were observed macroscopically at 3, 5, 7, 10, and 14 days after surgery, and tissues were collected at days 7 and 14. At 7 days, healing of the wound was incomplete in all groups (Fig 2). The surface of the wound had not epithelialized macroscopically, especially in the control group. Exudate was

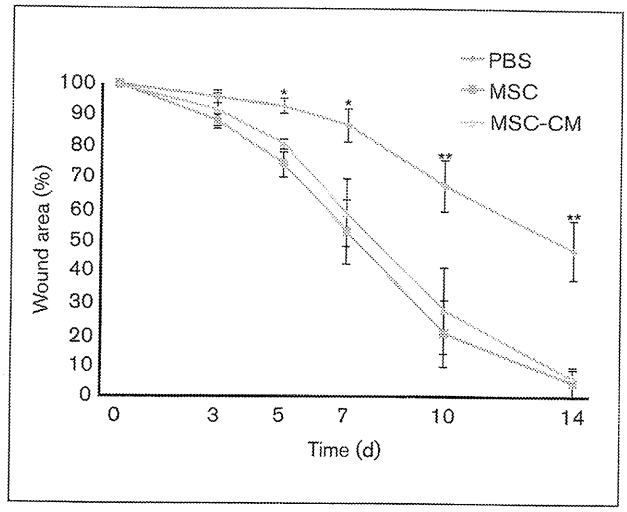

Fig 3 Time-dependent changes in the wound area determined with image analysis software (area of actual wound/area of original wound × 100). MSC-CM versus control: $^{*}P < .05$; $^{**}P < .01$.

observed in all groups. At 14 days, epithelialization appeared to be nearly complete in the MSC and MSC-CM groups, but the wounds were not completely closed in the control group (Fig 2). Changes in the wound area percentages at 7 and 14 days, as calculated with digital image analysis software, were as follows: 88.48% ± 3.49% and 42.64% ± 5.36% in the control group, 46.41% ± 5.49% and 4.90% ± 2.36% in the MSC group, and 47.77% ± 4.86% and 5.74% ± 2.85% in the MSC-CM group, respectively (Fig 3).

An acceleration of wound healing was observed in the MSC and MSC-CM groups as compared to the control group. At 5 days and later, there was a statistically significant difference in the wound area between the MSC and MSC-CM groups and the control group ($P < .05$, Fig 3); however, there were no significant differences between the MSC and MSC-CM groups.

Histopathologic Observations

At 7 days after surgery, inflammatory cells were observed in all groups. The epidermis of the wound was affected, especially in the control group. In the MSC and MSC-CM groups, epithelialization was advanced, and thick granulation tissue was observed (Fig 4).

At 14 days after wound creation and administration of the test materials, the number of inflammatory cells was decreased compared to 7 days. Reepithelialization was observed in the MSC and MSC-CM groups. Fluorescence microscopy showed decreases in HA production in all groups as compared to 7 days (Fig 5).

HA Production

The mean amounts of HA at 7 and 14 days were 1,512.0 ± 84.10 ng/mg and 683.7 ± 56.4 ng/mg in the control group, 2,450.3 ± 225.7 ng/mg and 1,690.2 ± 170.2 ng/mg in the MSC group, and 2,360.6 ± 230.0 ng/mg and 1,570.1 ± 142.5 ng/mg in the MSC-CM group, respectively. HA production in the MSC and MSC-CM groups at 7 and 14 days was significantly increased versus the control group. There were no statistically significant differences between the MSC and MSC-CM groups (Figs 6 and 7).

DISCUSSION

It is difficult to heal chronic wounds, and little improvement has been seen in the prevalence of complications and prevention of lesions in the past decade.[16] Even the most effective therapy for chronic wound repair has a healing rate of only 50%. In the present study, the area of the wound had shrunk by about 50% in the control group after 14 days. Therefore, an innovative therapy to accelerate wound healing and regeneration is needed. A major goal in wound healing is to discover methods to induce repair of the damaged site on the skin as completely as possible.[17] The present study revealed that MSC and MSC-CM can accelerate granulation and reepithelialization of wounds and demonstrated that these phenomena have a relationship with ECM, including HA.

The effect of this therapy on wound healing was examined using an excisional wound-splinting mouse model that prevents natural repair as a result of contracture of the skin; this allowed observation of granulation tissue and reepithelialization. The results indicated that closure of wounds in the control group was delayed significantly in comparison to the MSC and MSC-CM groups. In the MSC and MSC-CM groups, there were significant differences at 6 days and later as compared to the control group. To the best of the authors' knowledge, there are few reports

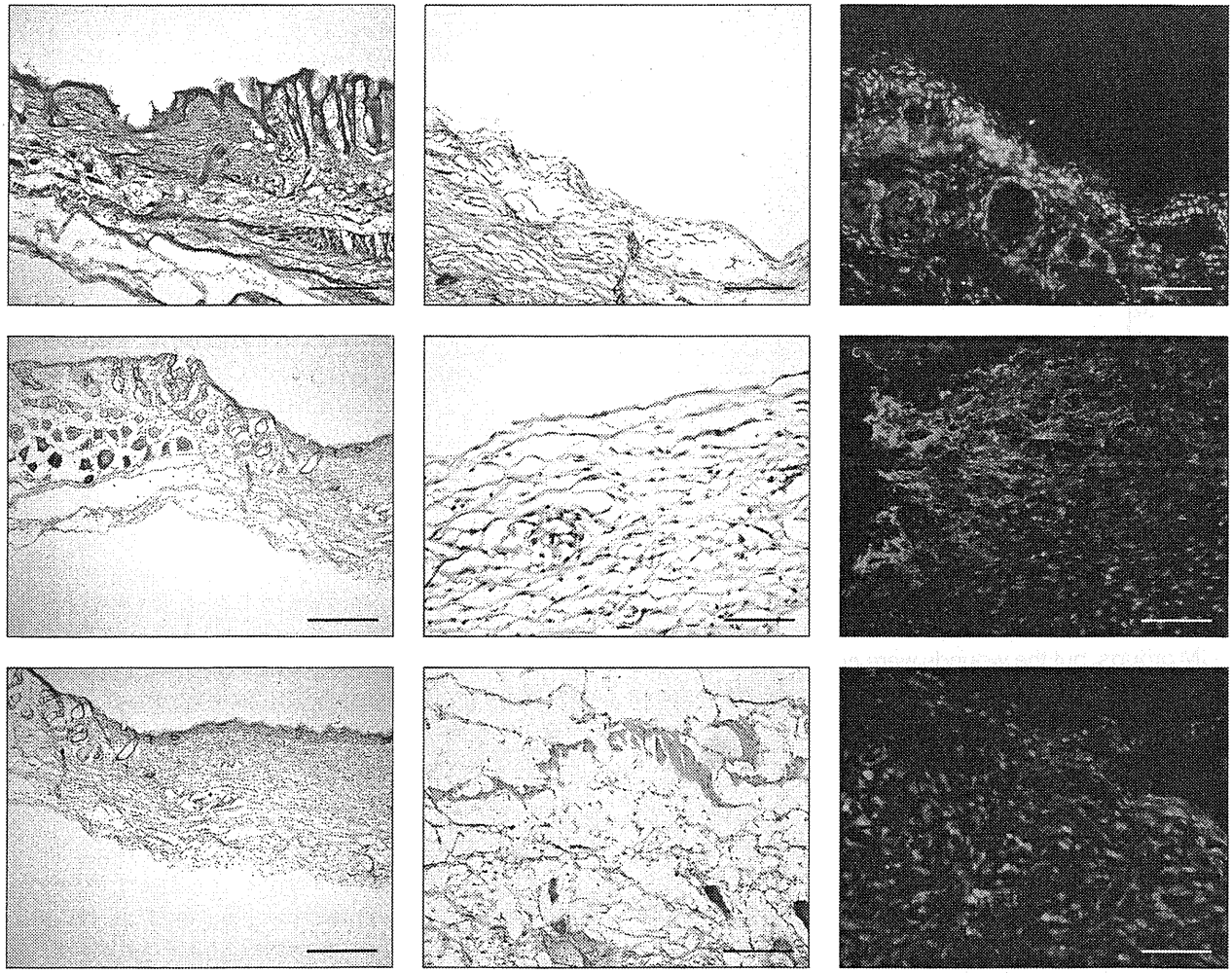

Fig 4 Histologic findings at 7 days after administration. Left and middle columns: hematoxylin-eosin staining; right column: immunostaining with HA. Blue, red, and green signals represent nuclei stained with DAPI, transplanted MSC, and HA, respectively. Top row: control group; middle row: MSC group; bottom row: MSC-CM group. Bar = 3 mm for left column; bar = 25 μm for center and right columns.

of culture supernatants of MSCs promoting wound healing, although many studies have demonstrated that MSC has a wound-healing effect. Ueda and Nishino[14] showed that CM of primary tooth stem cells was effective in improving ultraviolet-irradiated mouse skin tissue. This is the first experiment to show that CM can accelerate wound healing, even in a skin defect. It was already known that MSC-CM has effects on proliferation and migration of epithelium in vitro. Therefore, it was hypothesized that administration of MSC-CM to a wound might influence the epidermis and epidermal appendages, and thus regeneration of the epidermis. In a previous study, most MSCs remained at 7 days after surgery, and the number of MSCs was lower by 14 days.[18] The findings of this study correspond to these results. The amount of HA was reduced at 14 days as compared to that seen at 7 days. The mechanism that contributes to these phenomena is still not entirely clear. In the process of wound healing, cytokine and ECM molecules play favorable roles in epithelialization. Accordingly, the amount of HA was decreased because epithelialization of the skin was accelerated by the injected MSC and MSC-CM, and HA completed its role in this process. It was reported in a recent study that a peak of HA expression appeared at 7 days.[19] This study also confirmed expression of HA. HA plays an important role in the proliferation and migration of keratinocytes.[9–11] These results suggest that HA is important for the reepithelialization process and also helps reduce deposits of collagen and scars.[20]

Treatment using MSCs is effective; however, their numbers, proliferative capacity, and differentiation capability decrease with aging.[8] However, MSC-CM

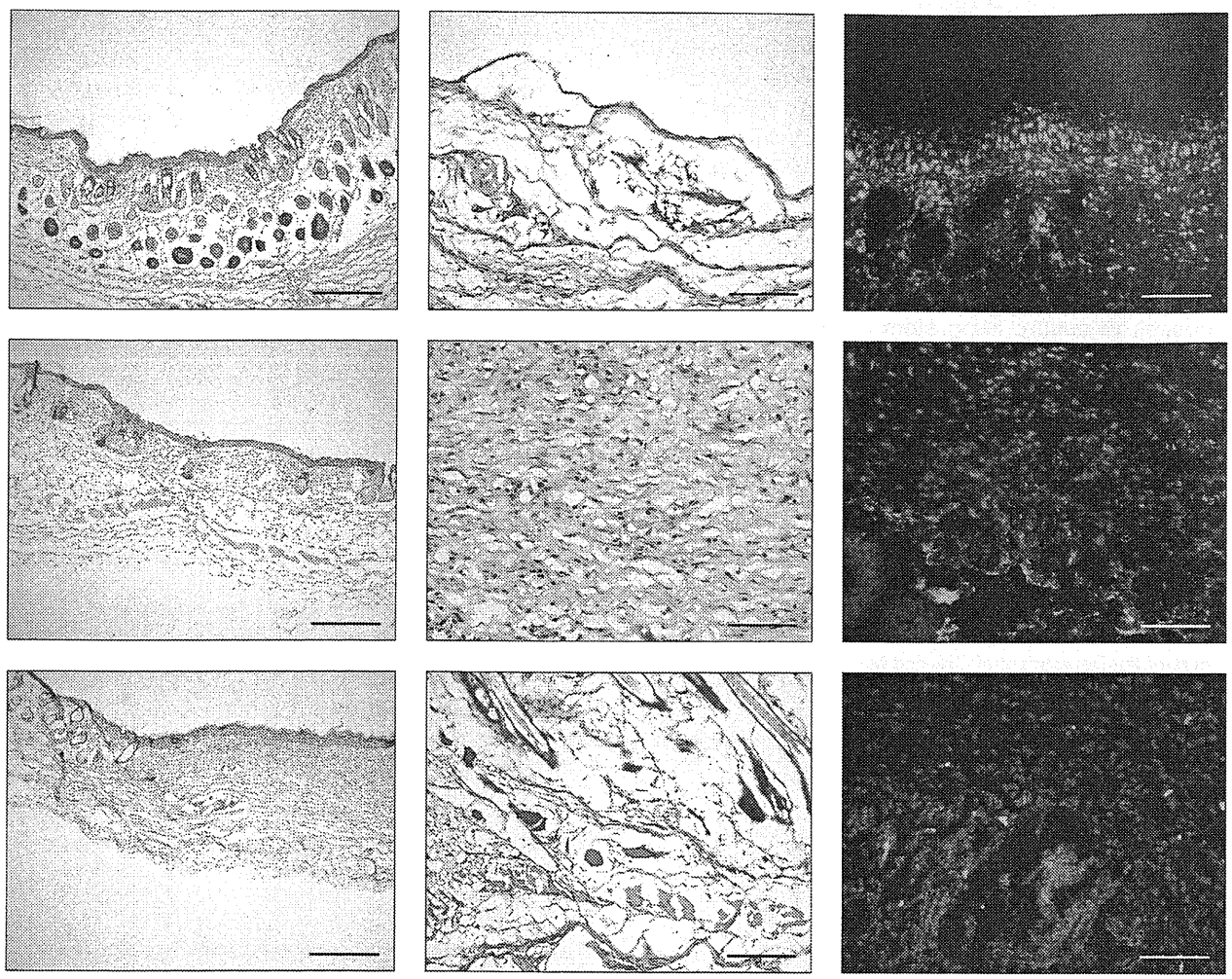

Fig 5 Histologic findings at 14 days after administration. Left and middle columns: hematoxylin-eosin staining; right column: immunostaining with HA. Blue, red, and green signals represent nuclei stained with DAPI, transplanted MSC, and HA, respectively. Top row: control group; middle row: MSC group; bottom row: MSC-CM group. Bar = 3 mm for left column; bar = 25 μm for center and right columns.

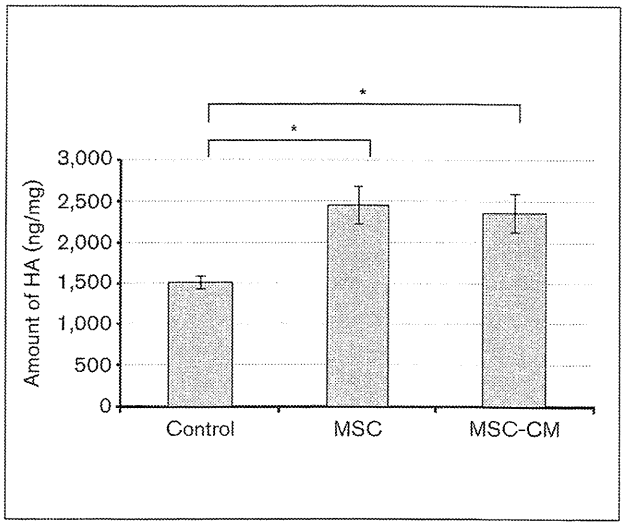

Fig 6 HA production at 7 days after administration, as determined with ELISA. *$P < .05$.

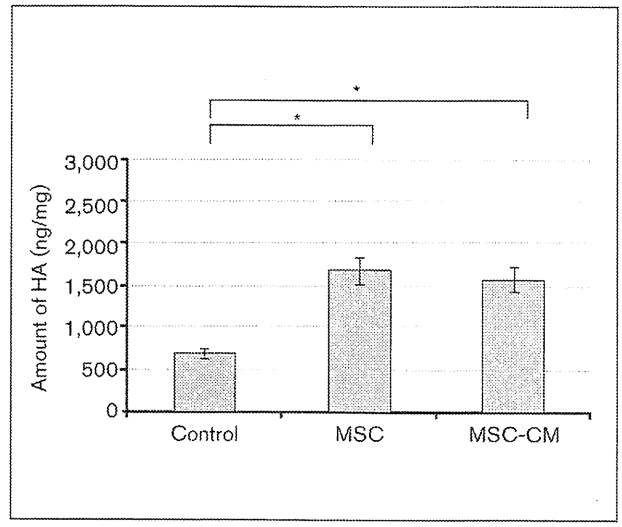

Fig 7 HA production at 14 days after administration, as determined with ELISA. *$P < .05$.

is obtained from an incubation process of the cells, and it is often discarded. This experiment showed that MSC and MSC-CM groups accelerated wound healing versus an untreated control group. The MSC-CM group showed wound healing comparable to that of the MSC group. There are the following advantages with the use of MSC-CM as compared with MSCs: *(1)* The risk of tumorigenesis is reduced because no stem cells are transplanted, and *(2)* no large-scale facilities, management expenses, or complicated processes to culture living stem cells are required. For example, freeze-dried CM can be prepared as a drug formulation. The authors consider that MSC-CM can be effective as a new treatment for wound-healing therapy for the skin as an alternative to stem cell transplantation.

ACKNOWLEDGMENTS

The authors wish to thank Naomi Nishio, Ken-ichi Isobe, and members of the Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, for their generous assistance and contributions to this study.

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Annals of Biomedical Engineering, Vol. 39, No. 7, July 2011 (© 2011) pp. 1872–1881
DOI: 10.1007/s10439-011-0327-0

Osteogenic Induction of Bone Marrow-Derived Stromal Cells on Simvastatin-Releasing, Biodegradable, Nano- to Microscale Fiber Scaffolds

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(Received 28 October 2010; accepted 23 February 2011; published online 18 May 2011)

Associate Editor Smadar Cohen oversaw the review of this article.

Abstract—Tissue engineering is an effective approach for the treatment of bone defects. Statins have been demonstrated to promote osteoblastic differentiation of bone marrow-derived stromal cells (BMSCs). Electrospun biodegradable fibers have also shown applicability to drug delivery in the form of bone tissue engineered scaffolds with nano- to microscale topography and high porosity similar to the natural extracellular matrix (ECM). The aim of this study was to investigate the feasibility of a simvastatin-releasing, biodegradable, nano- to microscale fiber scaffold (SRBFS) for bone tissue engineering with BMSCs. Simvastatin was released from SRBFS slowly. BMSCs were observed to spread actively and rigidly adhere to SRBFS. BMSCs on SRBFS showed an increase in alkaline phosphatase activity 2 weeks after cell culture. Furthermore, osteoclastogenesis was suppressed by SRBFS *in vitro*. The new bone formation and mineralization in the SRBFS group were significantly better than in the biodegradable fiber scaffold (BFS) without simvastatin 12 weeks after implantation of the cell-scaffold construct into an ectopic site on the murine back. These results suggest that SRBFS promoted osteoblastic differentiation of BMSCs *in vitro* and *in vivo*, and demonstrate feasibility as a bone engineering scaffold.

Keywords—Bone tissue engineering, Statin, Osteogenic differentiation, Electrospun biodegradable fiber scaffold, BMSCs.

INTRODUCTION

For reconstruction of a bone defect, the efficacy of various tissue engineering approaches using many kinds of scaffolds, bioactive substances, and cells has been demonstrated.[24] Although various cell sources have been introduced for application to bone tissue engineering, these cells commonly have been required for osteoinductive steps in the cell culture process before transplantation. For example, bone morphogenetic proteins (BMPs) are probably the most important osteoinductive factor in bone metabolism.[15] However, for broader clinical application, recombinant BMPs still pose problems in terms of safety and cost.[8,20]

For these reasons, this study focused on "statins" as osteoinductive factors that have been widely and safely used for the treatment of hyperlipidemia for more than two decades. Some studies have reported anabolic effects of statins on bone formation.[11] Also, statins are small, stable molecules that are not susceptible to proteolytic degradation and are stably produced, making them more cost effective than recombinant BMPs to produce.[11] Statins reportedly promoted osteogenic differentiation of bone marrow-derived stromal cells (BMSCs) and stimulated expression of BMP-2,[22] but suppressed osteoclastogenesis through blocking of the RANKL-induced NF-κB activation pathway.[2]

Meanwhile, our previous studies reported a sustained drug delivery system (DDS) using electrospinning biodegradable fiber with various types of drugs.[12,13,31] Sustained drug release can be adjusted by

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modification in the polymer degradation and can contribute to minimizing systemic influences and adverse side effects, while maximizing the local effects. Furthermore, the electrospinning process provides engineering scaffolds with nano- to microscale topography and high porosity similar to the natural extracellular matrix (ECM).[21] The large area-to-volume ratio of the fibers combined with their porous structure are beneficial for cell adhesion, proliferation,[27] migration,[32] and differentiation.[3] The high porosity of nano- to microscale fiber scaffolds assures more structural space for cell adaptation and promotes efficient exchange of nutrient and metabolic waste.

Therefore, we hypothesized that statin with electrospun biodegradable nano- to microscale fiber is a satisfactory combination for bone tissue engineering scaffold. A simvastatin-releasing, biodegradable, nano- to microscale fiber scaffold (SRBFS) was fabricated using a biodegradable polymer with simvastatin as the osteoinductive factor by an electrospinning procedure. The current study characterizes the complexes of SRBFS and BMSCs which have been major cell sources for bone regeneration, *in vitro* and *in vivo*, using an ectopic bone formation model in mice.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the "Guidelines and Regulations for Use and Care of Animals in Nagoya University" and approved by the "Animal Experiment Advisory Committee of the Nagoya University School of Medicine." This study used mice (C57/BL/6J, 4 weeks old, male) purchased from the Chubu Kagaku Shizai Corporation (Nagoya, Japan).

Isolation and Expansion of BMSCs

Mouse BMSCs were obtained by established techniques.[10] BMSCs were seeded in a culture medium containing α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and were subcultured by 4–6 passages and used for experiments. Osteogenic factors were not added in the culture medium. Characterization of BMSCs was determined by flow cytometry. Cells were labeled with the antibodies CD11b and Sca-1 (eBioscience, CA, USA). Cells used for this study were more than 70% positive for Sca-1 and less than 10% positive for CD11b (data not shown).

Fabrication of Simvastatin-Releasing, Biodegradable, Nano- to Microscale Fiber Scaffold (SRBFS)

SRBFS and BFS fabrication methods were the same as previously described.[12] In brief, simvastatin (Toronto Research Chemicals, Inc., North York, ON, Canada) (MW = 418.57) was mixed with biodegradable polymer to fabricate SRBFS, and not mixed to fabricate BFS. Poly(L-lactide-*co*-glycolide) (IV = 1.15 dL/g), which consists of 50% of each polymer, was prepared for the biodegradable polymer. To fabricate the nano- to microscale fiber, an electrospinning procedure was used. The polymer solution was added using a 1-mL syringe with a right angle-shaped metal capillary attached to it. The circular orifice of the capillary had an inner diameter of 1.2 mm. A flat counter electrode was located 35 cm from the capillary tip. Pressure was applied to the solution in the syringe, gradually forcing the piston to maintain a steady flow of solution from the capillary outlet. The flow rate of the polymer solution was 0.3 mL/min. The applied voltage ranged from 10 to 15 kV. The fibers released in the atmosphere were electrostatically removed and trapped with a rod-shaped collector located 20 cm from the capillary tip. The SRBFS shows a "cotton wool"-like formation (Fig. 1a). The fibers ranged from 500 nm to 10 μm in diameter (Figs. 1c, 1d). This configuration is flexible, making it easy to handle any bone defect. The simvastatin content in the SRBFS was 0.025 wt%.

Test of Simvastatin Release from SRBFS

The release test was carried out according to a previous report.[26] Five mg of SRBFS was placed in 1 mL phosphate-buffered saline (PBS) at 37 °C, and the PBS was exchanged at 1, 2, 3, 5, 7, 14, and 28 days, respectively. The PBS supernatant was freeze-dried, and dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Wako, Osaka, Japan). After centrifugation (8000 rpm, 5 min, 4 °C), the supernatant absorbance was measured at a wavelength of 248 nm while the simvastatin concentration was determined from the standard curve prepared with the HFP containing various amounts of simvastatin.

Cell Seeding on Scaffold

Sterilized scaffolds (0.2 cm^3 in volume, 5 mg in mass) were incubated in the culture medium for 12 h prior to cell seeding in a 96-well plate. Approximately 5×10^5 cells were suspended in 30 μL of culture medium and slowly injected into and around the nanofibrous scaffolds in a 96-well plate. Cell-scaffold constructs were subsequently cultured in α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with