Achillion Pharmaceuticals, Avexa, Boehringer Ingelheim, Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline, Hoffmann LaRoche, Idenix, Janssen, Merck, Pfizer, Sangamo, Taimed, Tobira, and Vertex; and consulting fees as a member of advisory boards for Bristol-Myers Squibb, Gilead Sciences, Janssen, Merck, and Vertex. MS reports grant support to his Institution from Gilead Sciences, Merck, Janssen, BMS, and ViiV. AP has received research grant support and consulting fees as a member of advisory boards for GSK, BMS, ViiV, Gilead Sciences, Roche, Abbott, Janssen, and Merck. MT reports grant support to her Institution from Gilead Sciences, BMS, GeoVax, Kowa Research Institute, Merck, Pfizer, Tobira, ViiV, and Janssen. DP has received research grants and/or honoraria for advisories and/or conferences from Boehringer Ingelheim, GSK, ViiV, Pfizer, BMS, Abbott, Gilead, Janssen, and Merck. JMM reports grants and personal fees from Gilead Sciences, Merck, BMS, ViiV, Janssen, and Tobira. SO reports grants and personal fees from MSD KK, Janssen, ViiV, Torii, Gilead Sciences, Abbvie, and Daiichisankyo. EK has no conflicts of interest to disclose. BT reports grants and personal fees from Abbvie, BMS, Gilead, Merck, Janssen, and ViiV. JA-V has no conflicts of interest to disclose. GC reports grants and personal fees from Gilead, GSK, Pfizer, Jansse, Sangamo, ad Merck. JMC, AP, LZ, HC, HM, CC, AKC, MWF, SM are employees of Gilead Sciences.

Acknowledgments

We thank the patients, their partners, and families, and all principal investigators and their study staff for the GS-US-292-0104 and GS-US-292-0111 studies. We also thank Sandra Friborg, Kathleen Villamejor, Jay Huang, Susan Guo, Hui Wang, Michael Miller, Nicolas Margot, and the complete GS-US-292-0104 and GS-US-292-0111 study teams. Parts of this study were presented at the 22nd Conference on Retroviruses and Opportunistic Infections, Seattle, WA, USA; February 23–26, 2015.

References

1 DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. 2011; 1–166. http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf (accessed Feb 2, 2015).

2 Gupta SK. Tenofovir-associated Fanconi syndrome: review of the FDA adverse event reporting system. *AIDS Patient Care STDS* 2008; **22:** 99–103.

3 Mocroft A, Kirk O, Reiss P, et al, and the EuroSIDA Study Group. Estimated glomerular filtration rate, chronic kidney disease and antiretroviral drug use in HIV-positive patients. *AIDS* 2010; **24:** 1667–78.

4 Morlat P, Vivot A, Vandenhende MA, et al, and the Groupe D'epidémiologie Clinique du Sida en Aquitaine (Gecsa). Role of traditional risk factors and antiretroviral drugs in the incidence of chronic kidney disease, ANRS CO3 Aquitaine cohort, France, 2004–2012. *PLoS One* 2013; **8:** e66223.

5 McComsey GA, Kitch D, Daar ES, et al. Bone mineral density and fractures in antiretroviral-naive persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: Aids Clinical Trials Group A5224s, a substudy of ACTG A5202. *J Infect Dis* 2011; **203:** 1791–801.

6 Bedimo R, Maalouf NM, Zhang S, Drechsler H, Tebas P. Osteoporotic fracture risk associated with cumulative exposure to tenofovir and other antiretroviral agents. *AIDS* 2012; **26:** 825–31.

7 Van Rompay KK, Durand-Gasselin L, Brignolo LL, et al. Chronic administration of tenofovir to rhesus macaques from infancy through adulthood and pregnancy: summary of pharmacokinetics and biological and virological effects. *Antimicrob Agents Chemother* 2008; **52:** 3144–60.

8 Fux CA, Simcock M, Wolbers M, et al, and the Swiss HIV Cohort Study. Tenofovir use is associated with a reduction in calculated glomerular filtration rates in the Swiss HIV Cohort Study. *Antivir Ther* 2007; **12:** 1165–73.

9 Goicoechea M, Liu S, Best B, et al, and the California Collaborative Treatment Group 578 Team. Greater tenofovir-associated renal function decline with protease inhibitor-based versus nonnucleoside reverse-transcriptase inhibitor-based therapy. *J Infect Dis* 2008; **197:** 102–08.

10 Kiser JJ, Carten ML, Aquilante CL, et al. The effect of lopinavir/ritonavir on the renal clearance of tenofovir in HIV-infected patients. *Clin Pharmacol Ther* 2008; **83:** 265–72.

11 Ruane PJ, DeJesus E, Berger D, et al. Antiviral activity, safety, and pharmacokinetics/pharmacodynamics of tenofovir alafenamide as 10-day monotherapy in HIV-1-positive adults. *J Acquir Immune Defic Syndr* 2013; **63:** 449–55.

12 Sax PE, Zolopa A, Brar I, et al. Tenofovir alafenamide vs. tenofovir disoproxil fumarate in single tablet regimens for initial HIV-1 therapy: a randomized phase 2 study. *J Acquir Immune Defic Syndr* 2014; **67:** 52–58.

13 Guidance for Industry: Human Immunodeficiency Virus-1 Infection: Developing Antiretroviral Dtrugs for Treatment. U.S.Department of Health and Human Services Food and Drug Administation, Center for Drug Evaluatin and Research, June 2013. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm355128.pdf (accessed Feb 2, 2015).

14 Koch GG, Carr GJ, Amara IA, et al. Categorical data analysis. In: Berry DA, ed. Statistical methodology in the pharmaceutical sciences. New York: Marcel Dekker, Inc. 1989: 41–21.

15 Wiens BL, Dmitrienko A. The fallback procedure for evaluating a single family of hypotheses. *J Biopharm Stat* 2005; **15:** 929–42.

16 Lewden C, Chene G, Morlat P, et al, and the Agence Nationale de Recherches sur le Sida et les Hepatites Virales (ANRS) CO8 APROCO-COPILOTE Study Group, and the Agence Nationale de Recherches sur le Sida et les Hepatites Virales (ANRS) CO3 AQUITAINE Study Group. HIV-infected adults with a CD4 cell count greater than 500 cells/mm3 on long-term combination antiretroviral therapy reach same mortality rates as the general population. *J Acquir Immune Defic Syndr* 2007; **46:** 72–77.

17 Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S, Tonelli M. Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients. *Clin Infect Dis* 2010; **51:** 496–505.

18 Scherzer R, Estrella M, Li Y, et al. Association of tenofovir exposure with kidney disease risk in HIV infection. *AIDS* 2012; **26:** 867–75.

19 Brown TT, Qaqish RB. Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: a meta-analytic review. *AIDS* 2006; **20:** 2165–74.

20 Grund B, Peng G, Gibert CL, et al, and the INSIGHT SMART Body Composition Substudy Group. Continuous antiretroviral therapy decreases bone mineral density. *AIDS* 2009; **23:** 1519–29.

21 Grant PM, Kitch D, McComsey GA, et al. Low baseline CD4+ count is associated with greater bone mineral density loss after antiretroviral therapy initiation. *Clin Infect Dis* 2013; **57:** 1483–88.

22 Stellbrink H-J, Orkin C, Arribas JR, et al, and the ASSERT Study Group. Comparison of changes in bone density and turnover with abacavir-lamivudine versus tenofovir-emtricitabine in HIV-infected adults: 48-week results from the ASSERT study. *Clin Infect Dis* 2010; **51:** 963–72.

23 Mateo L, Holgado S, Marinoso ML, et al. Hypophosphatemic osteomalacia induced by tenofovir in HIV-infected patients. *Clin Rheumatol* 2014; published online May 3. DOI:10.1007/s10067-014-2627-x.

24 Reynes J, Trinh R, Pulido F, et al. Lopinavir/ritonavir combined with raltegravir or tenofovir/emtricitabine in antiretroviral-naive subjects: 96-week results of the PROGRESS study. *AIDS Res Hum Retroviruses* 2013; **29:** 256–65.

25 Bedimo RJ, Drechsler H, Jain M, et al. The RADAR study: week 48 safety and efficacy of RAltegravir combined with boosted DARunavir compared to tenofovir/emtricitabine combined with boosted darunavir in antiretroviral-naive patients. Impact on bone health. *PLoS One* 2014; **9:** e106221. DOI:10.1371/journal.pone.0106221.

26 Shepherd JA, Lu Y. A generalized least significant change for individuals measured on different DXA systems. *J Clin Densitom* 2007; **10:** 249–58.

27 Tungsiripat M, Kitch D, Glesby MJ, et al. A pilot study to determine the impact on dyslipidemia of adding tenofovir to stable background antiretroviral therapy: ACTG 5206. *AIDS* 2010; **24:** 1781–84.

Clinical Infectious Diseases Advance Access published April 17, 2015

MAJOR ARTICLE

High-Dose Oral Amoxicillin Plus Probenecid Is Highly Effective for Syphilis in Patients With HIV Infection

Ryutaro Tanizaki,[1] Takeshi Nishijima,[1,2] Takahiro Aoki,[1] Katsuji Teruya,[1] Yoshimi Kikuchi,[1] Shinichi Oka,[1,2] and Hiroyuki Gatanaga[1,2]

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, and [2]Center for AIDS Research, Kumamoto University, Japan

Background. Intramuscular benzathine penicillin G (BPG) is widely used for the treatment of syphilis. However, BPG is not available in some countries. This study examined the effectiveness and safety of high-dose oral amoxicillin plus probenecid for the treatment of syphilis in patients with human immunodeficiency virus type 1 (HIV-1).

Methods. This retrospective observational study included 286 HIV-infected male patients with syphilis (median age, 36 years; median CD4 count, 389 cells/μL) who were treated with oral amoxicillin 3 g plus probenecid. Syphilis was diagnosed by both serum rapid plasma reagin (RPR) titers ≥8 and positive *Treponema pallidum* hemagglutination test. Patients with neurosyphilis diagnosed by cerebrospinal fluid examination were excluded. Successful treatment was defined as a at least 4-fold decrement in RPR titer.

Results. The overall treatment efficacy was 95.5% (95% confidence interval [CI], 92.4%–97.7%; 273/286 patients), and efficacy for primary, secondary, early latent, late latent, and unknown duration syphilis was 93.8% (95% CI, 68.1%–99.8%; 15/16), 97.3% (95% CI, 92.9%–99.2%; 142/146), 100% (95% CI, 90.5%–100%; 37/37), 85.7% (95% CI, 58.6%–96.4%; 18/21), and 92.4% (95% CI, 81.9%–97.3%; 61/66), respectively. Treatment duration was mostly 14–16 days (49.7%) or 28–30 days (34.3%), with efficacy of 94.4% (134/142) and 95.9% (94/98), respectively; 96.3% of successfully treated patients achieved a ≥4-fold decrement in RPR titer within 12 months. Adverse events were noted in 28 (9.8%) patients, and 25 of these (89.3%) were successfully treated. Only 6% of patients underwent lumbar puncture.

Conclusions. The combination of oral amoxicillin 3 g plus probenecid was highly effective and tolerable for the treatment of syphilis in patients with HIV-1 infection.

Keywords. syphilis; amoxicillin; HIV; MSM; treatment.

Syphilis is a common sexually transmitted infection caused by *Treponema pallidum*, and in recent years, an increase in the number of cases with syphilis has been reported among men who have sex with men (MSM), particularly among those with human immunodeficiency virus type 1 (HIV-1) infection in resource-rich settings [1, 2]. A recent study of syphilis [3] in HIV-1–infected men, comprised mostly of MSM, showed that syphilis is associated with an increase in HIV-1 RNA load and with a small decrement in CD4 cell count, suggesting that syphilis may increase a risk of HIV-1 transmission. Furthermore, progression to neurosyphilis may be faster in HIV-1–infected patients than patients without HIV-1 infection [4–6], and HIV-1 infection may exacerbate the clinical symptoms of neurosyphilis [7]. For the above-mentioned reasons, early diagnosis and treatment of syphilis are important in HIV-infected patients.

One-shot intramuscular benzathine penicillin G (BPG) injection is widely used for the treatment of primary, secondary, and early latent syphilis based on its high efficacy and convenience [8–10]; however, intramuscular injection is painful and, for the treatment of late latent syphilis and syphilis of unknown duration,

Received 9 January 2015; accepted 21 March 2015.

Correspondence: Takeshi Nishijima, MD, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku, Tokyo 162-0052, Japan (tnishiji@acc.ncgm.go.jp).

Clinical Infectious Diseases®

DOI: 10.1093/cid/civ270

3 injections at 1-week intervals (3 hospital visits in total) is required. BPG is not available in some countries, including Japan [11, 12], and oral amoxicillin plus probenecid has been used as an alternative for BPG in the treatment of syphilis.

However, there is no evidence available on the efficacy and safety of oral amoxicillin plus probenecid for the treatment of syphilis; the only available evidence is from pharmacokinetic studies published in the 1970s–1980s [13, 14].

We investigated the efficacy and safety of high-dose oral amoxicillin (3 g) plus probenecid for the treatment of syphilis (excluding neurosyphilis) in patients with HIV-1 infection in an observational setting.

METHODS

Patients and Study Design

We conducted a retrospective cohort study of HIV-1–infected patients with syphilis to investigate the efficacy and safety of oral amoxicillin plus probenecid for the treatment of syphilis at the AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, Japan. The enrollment criteria were HIV-1–infected patients who were diagnosed with syphilis and started treatment with 3 g oral amoxicillin plus probenecid at our center between January 2000 and June 2014. We included all patients treated with the combination of 3 g amoxicillin and probenecid, irrespective of the dose of the latter. The diagnosis of syphilis was based on both serum rapid plasma regain (RPR) titers ≥8 and positive *T. pallidum* hemagglutination (TPHA) result [15]. The following exclusion criteria were applied: (1) lack of follow-up tests, (2) patients with neurosyphilis diagnosed based on the findings of cerebrospinal fluid (CSF) [16], or ocular or auditory syphilis, (3) patients who started treatment with antibiotics other than 3 g amoxicillin plus probenecid, (4) patients with clinical symptoms compatible with primary or secondary syphilis but RPR titers <8, (5) patients suspected of reinfection after initiation of syphilis treatment (≥4-fold rise in RPR titer after 4-fold decrement with or without symptoms) [15, 17].

Data Collection

Data on the following parameters were collected at the time of treatment of syphilis: stage of syphilis infection, age, sex, race, route of HIV-1 infection, antiretroviral therapy (ART) use, history of syphilis treatment, status of hepatitis B and C infection, RPR titer, CD4 count, and HIV-1 RNA load. The stage of syphilis was classified into early syphilis (including primary, secondary, and early latent syphilis) and late syphilis (including late latent syphilis and syphilis of unknown duration) [8, 9]. Early latent syphilis was defined as asymptomatic syphilis that was confirmed to be infected within a year from the day of diagnosis, and late latent syphilis was defined as asymptomatic syphilis confirmed to be infected more than a year before diagnosis [8, 9]. Syphilis with unknown duration was defined as asymptomatic syphilis that could not be classified into either early latent or late latent syphilis [8, 9]. The methods of amoxicillin and probenecid administration, treatment duration, treatment efficacy, adverse events during treatment, and changes to doxycycline from amoxicillin were also collected from the medical records. Among the adverse events, the presence of fever and/or acute exacerbation of maculopapular skin rash within 24 hours of administration of amoxicillin was defined as Jarisch-Herxheimer reaction for syphilis and was not regarded as drug-related adverse events [18].

Successful treatment of syphilis was defined as at least 4-fold decrement in RPR titer within 24 months after initiation of treatment. Follow-up serum RPR titer was examined at the discretion of the attending physician. Because at our clinic, written informed consent was obtained from each patient to store serum samples at the first and subsequent visits [19], the RPR data based on stored samples were also used to supplement the RPR data from daily clinical practice order to determine treatment response and the syphilis staging. RPR Test "Sankoh" (EIDIA Co, Ltd, Tokyo, Japan) was used for the measurement of RPR titer both in clinical practice and with stored samples.

Patients visited our clinic at least once every 3 months for monitoring and prescription, as the prescription period under the Japanese healthcare system is limited to 3 months [20]. The study was approved by the Human Research Ethics Committee of NCGM, and was conducted according to the principles expressed in the Declaration of Helsinki.

Statistical Analysis

The study patients were classified according to the results of the combination treatment into the success group (patients with successful treatment of syphilis) and failure group (failure of treatment). The baseline characteristics were compared between the 2 groups using the Student t test or χ^2 test (Fisher exact test when appropriate) for continuous or categorical variables, respectively. To estimate risk factors for treatment failure, univariate logistic regression model was constructed. Because the number of patients in the failure group was small (n = 13) in this study, the multivariate analysis was not performed. Statistical significance was defined as 2-sided P values <.05. We used odds ratios (ORs) with 95% confidence intervals (CIs) for logistic regression analysis. All statistical analyses were performed with Stata 11 (Stata Corp, College Station, Texas).

RESULTS

During the study period, 403 HIV-1–infected patients were diagnosed with syphilis. One hundred seventeen patients were excluded from the study based on the inclusion and exclusion criteria set for this study (Figure 1), and data of the remaining

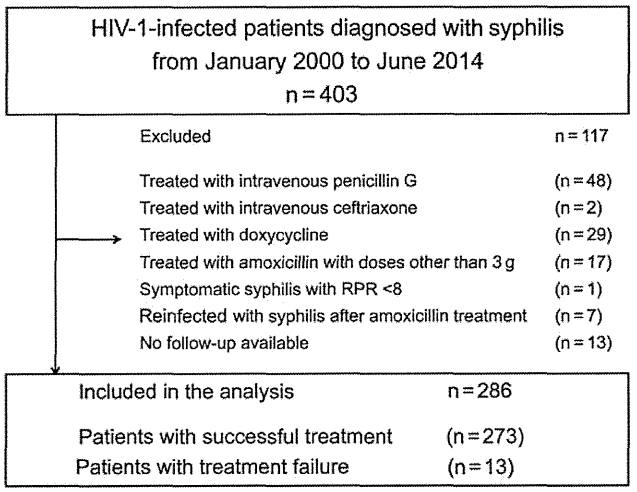

Figure 1. Patient enrollment process. Abbreviations: HIV-1, human immunodeficiency virus type 1; RPR, rapid plasma reagin.

286 patients were analyzed. All study patients were men with a median age of 36 years (interquartile range [IQR], 30–42 years). They were mostly Asians who were infected with HIV-1 through homosexual contact. The median CD4 count was 389 cells/μL (IQR, 276–538 cells/μL), ART had been started in 156 (54.5%) patients, and 170 patients (59.4%) had a history of syphilis treatment. Primary syphilis, secondary syphilis, early latent syphilis, late latent syphilis, and syphilis with unknown duration were diagnosed in 16 (5.6%), 146 (51.0%), 37 (12.9%), 21 (7.3%), and 66 (23.1%) patients, respectively. Furthermore, 199 (69.6%) patients were categorized with early syphilis, which included primary, secondary, and early latent syphilis, and 87 (30.4%) with late syphilis, which included late latent syphilis and syphilis of unknown duration.

Treatment with 3 g oral amoxicillin and probenecid decreased RPR titer by 4-fold and was thus regarded successful in 273 (95.5% [95% CI, 92.4%–97.7%]) patients (success

Table 1. Baseline Characteristics of the Study Patients

Characteristic	All Patients (N = 286)	Patients With Successful Treatment (n = 273)	Patients With Treatment Failure (n = 13)	*P* Value
Age, y[a]	36 (30–42)	36 (30–42)	30 (24–37)	.003
Male sex	286 (100)	273 (100)	13 (100)	
Asian race	281 (98.3)	268 (98.2)	13 (100)	
Route of HIV-1 transmission				
Homosexual	274 (95.8)	262 (96.0)	11 (84.6)	
Heterosexual	7 (2.4)	6 (2.2)	1 (7.7)	
Injection drug user	3 (1.0)	2 (0.7)	1 (7.7)	
Unknown	2 (0.7)	2 (0.7)	0 (0)	
ART use	156 (54.5)	150 (54.9)	6 (46.2)	.579
History of syphilis treatment	170 (59.4)	163 (59.7)	7 (53.8)	.775
Stage of syphilis				
Early syphilis	199 (69.6)	194 (71.1)	5 (38.5)	.025[b]
Primary	16 (5.6)	15 (5.5)	1 (7.7)	
Secondary	146 (51.0)	142 (52.0)	4 (30.8)	
Early latent	37 (12.9)	37 (13.6)	0 (0)	
Late syphilis	87 (30.4)	79 (28.9)	8 (61.5)	
Late latent	21 (7.3)	18 (6.6)	3 (23.1)	
Unknown duration	66 (23.1)	61 (22.3)	5 (38.5)	
Baseline RPR titer[a]	96 (32–128)	128 (32–128)	64 (32–128)	.510
CD4 count, cells/μL[a]	389 (276–538)	393 (285–542)	286 (180–369)	.003
HIV-1 load, $\log_{10}$ copies/mL[a]	2.06 (1.70–4.49)	1.91 (1.70–4.45)	4.26 (3.04–4.70)	.048
Hepatitis	35 (12.2)	35 (12.8)	0 (0)	
Positive HBsAg	30 (10.5)	30 (11.0)	0 (0)	
Positive HCV antibody	5 (1.7)	5 (1.8)	0 (0)	
Lumbar puncture performed	17 (5.9)	12 (4.4)	5 (38.5)	<.001

Data are presented as No. (%) unless otherwise specified.

Abbreviations: ART, antiretroviral therapy; HBsAg, hepatitis B surface antigen; HCV, hepatitis C; HIV-1, human immunodeficiency virus type 1; RPR, rapid plasma regain.

[a] Median (interquartile range).

[b] By 2 × 2 table for early syphilis vs late syphilis and by Fisher exact test for successful treatment vs treatment failure.

group). Among 13 patients with treatment failure (failure group), none showed any evidence of clinical failure. The baseline HIV-1 load was lower in the success group than the failure group, whereas the baseline CD4 count was higher in the success group. Lumbar puncture for the examination of CSF was performed before treatment initiation in only 17 (5.9%) patients, including 13 patients with late syphilis, and neurosyphilis was ruled out in all 17 patients based on negative TPHA in the CSF [16]. The treatment efficacy of primary, secondary, early latent, late latent, and unknown duration syphilis was 93.8% (95% CI, 68.1%–99.8%; 15/16 patients), 97.3% (95% CI, 92.9%–99.2%; 142/146), 100% (95% CI, 90.5%–100%; 37/37), 85.7% (95% CI, 58.6%–96.4%; 18/21), and 92.4% (95% CI, 81.9%–97.3%; 61/66), respectively (Table 1). Also, the treatment efficacy for early syphilis (including primary, secondary, and early latent syphilis) and late syphilis (including late latent syphilis and syphilis of unknown duration) was 97.5% (95% CI, 94.1%–99.2%) and 90.8% (95% CI, 82.6%–96.4%), respectively. Treatment duration was mostly 14–16 days (49.7%) or 28–30 days (34.3%), with 94.4% (134/142) and 95.9% (94/98) efficacy, respectively. Among patients with early syphilis, treatment efficacy did not differ between the 2-week treatment and 4-week treatment (105 of 107 [98.1%] vs 109 of 113 [96.5%]; $P = .49$ by Fisher exact test). However, among patients with late syphilis, treatment efficacy tended to be lower among patients treated for 2 weeks than those treated for 4 weeks, although the difference was not statistically significant (29 of 35 [82.9%] vs 34 of 36 [94.4%]; $P = .15$). The frequency of oral amoxicillin administration was mainly 3 times a day (80.8%) with 96.1% (222/231) efficacy. The dosage of probenecid was 0.75 g/day in the majority of patients (60.1%), 1.0 g/day (23.4%), and 1.5 g/day (13.3%), with success rate of 96.5% (166/172), 92.5% (62/67), and 94.7% (36/38), respectively (Table 2). For patients with successful treatment, the median time for the documentation of ≥4-fold decrement in RPR titer after treatment was 4 months (IQR, 3–6 months), and 96.3% of the success group achieved the ≥4-fold decrease in RPR titers within 12 months (Figure 2). The median number of follow-up RPRs for the success group and the failure group was 1 (IQR, 1–2) and 4 (IQR, 2–6), respectively, and follow-up RPRs were more frequently measured for the failure group than for the success group ($P < .001$).

Adverse events related to treatment of syphilis were recorded in 28 (9.8%) patients, with skin rash being the most common symptom (n = 21), followed by fever (n = 9), diarrhea (n = 2),

Table 2. Frequency of Amoxicillin Administration, Probenecid Dosing, Treatment Duration, and Variables Related to Adverse Events

Variable	All Patients (N = 286)	Early Syphilis (n = 199)	Late Syphilis (n = 87)	Successfully Treated Patients (n = 273)	Patients With Treatment Failure (n = 13)	*P* Value*
Frequency of amoxicillin administration						
4 times daily	44 (15.4)	26 (13.7)	18 (20.7)	40 (14.7)	4 (30.8)	
3 times daily	231 (80.8)	165 (82.9)	66 (75.9)	222 (81.3)	9 (69.2)	.241[a]
Twice daily	11 (3.8)	8 (4.0)	3 (3.5)	11 (4.0)	0 (0)	
Dose of probenecid, g, median (IQR)	0.75 (0.75–1.0)	0.75 (0.75–1.0)	0.75 (0.75–1.0)	0.75 (0.75–1.0)	1.0 (0.75–1.0)	.213
1.5 g/d	38 (13.3)	26 (13.7)	12 (13.8)	36 (13.2)	2 (15.4)	
1.0 g/d	67 (23.4)	47 (23.6)	20 (23.0)	62 (22.7)	5 (38.5)	
0.75 g/d	172 (60.1)	119 (59.8)	53 (60.9)	166 (60.8)	6 (46.2)	.693[b]
0.5 g/d	8 (2.8)	6 (3.0)	2 (2.3)	8 (2.9)	0 (0)	
0.25 g/d	1 (0.3)	1 (0.5)	0 (0)	1 (0.4)	0 (0)	
Treatment duration, d, median (IQR)	14 (14–28)	14 (14–28)	21 (14–28)	14 (14–28)	14 (14–28)	.362
<14 d	18 (6.3)	12 (6.0)	6 (6.9)	17 (6.2)	1 (7.7)	
14–16 d	142 (49.7)	107 (53.8)	35 (40.2)	134 (49.1)	8 (61.5)	
17–27 d	7 (2.4)	3 (1.5)	4 (4.6)	7 (2.6)	0 (0)	.770[c]
28–30 d	98 (34.3)	62 (31.2)	36 (41.4)	94 (34.4)	4 (30.8)	
>30 d	21 (7.3)	15 (7.5)	6 (6.9)	21 (7.7)	0 (0)	
Adverse events	28 (9.8)	15 (7.5)	13 (14.9)	25 (9.2)	3 (23.1)	.123
Switched to doxycycline	21 (7.3)	13 (6.5)	10 (11.5)	19 (7.0)	2 (15.4)	.246

Data are presented as No. (%) unless otherwise specified.

Abbreviation: IQR, interquartile range.

[a] By use of 3 × 2 table, Fisher exact test.

[b] By use of 5 × 2 table, Fisher exact test.

[c] By use of 5 × 2 table, Fisher exact test.

* For comparison of patients with successful treatment and those with treatment failure.

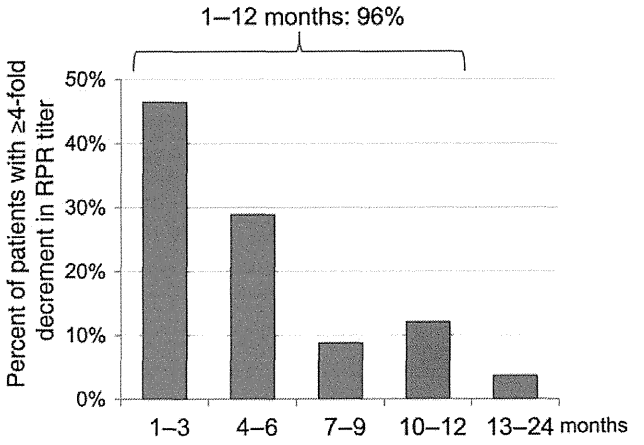

Figure 2. Time until 4-fold decrement in rapid plasma reagin (RPR) titer after initiation of syphilis treatment for successfully treated patients. The median time until ≥4-fold decrement in RPR titer was 4 months (interquartile range, 3–6 months). The ≥4-fold decrement in RPR titer within 12 months was accomplished in 96.3% of the patients. The number and proportion of patients with ≥4-fold decrement in RPR titer at 1–3, 4–6, 7–9, 10–12, and 13–24 months after treatment initiation were 127 (46.5%), 79 (28.9%), 24 (8.8%), 33 (12.1%), and 10 (3.7%), respectively.

and elevated liver enzymes (n = 1). Furthermore, 5 patients presented with both rash and fever. Analysis of the medical records showed that treatment with amoxicillin was changed to doxycycline in 21 (75%) patients due to the side effects. Despite the adverse events, treatment was considered successful in 25 of the 28 (89.3%) patients. Among the 7 patients who showed adverse events but did not change amoxicillin to doxycycline, treatment was successful in 6, although amoxicillin was administered for the median of only 10 days (IQR, 10–18 days). None of the patients discontinued amoxicillin due to Jarisch-Herxheimer reaction.

Logistic regression analysis was performed to identify the risk factors associated with treatment failure. Univariate analysis demonstrated that late syphilis (OR, 3.9 [95% CI, 1.25–12.4]; $P = .019$) and high HIV-1 load (per 1 $\log_{10}$ copies/mL: OR, 1.5 [95% CI, 1.03–2.26]; $P = .033$) before treatment were associated with treatment failure (Table 3). On the other hand, older age was associated with successful treatment (per 1 year: OR, 0.9 [95% CI, .84–.99]; $P = .025$), and higher CD4 count was also marginally associated (per 1 cell/μL: OR, 1.0 [95% CI, .99–1.00]; $P = .053$).

DISCUSSION

The present study investigated the treatment efficacy of 3 g oral amoxicillin plus probenecid for early and late syphilis among HIV-1–infected patients in an observational setting. The results showed that 95.5% of the study patients were successfully

Table 3. Results of Univariate Analysis to Estimate Risk Factors for Treatment Failure With Oral Amoxicillin Plus Probenecid for Syphilis

Variable	OR (95% CI)	*P* Value
Age (per 1 y)	0.9 (.84–.99)	.025
Late syphilis vs early syphilis	3.9 (1.25–12.4)	.019
HIV-1 load (per 1 $\log_{10}$ copies/mL)	1.5 (1.03–2.26)	.033
CD4 count (per 1 cell/μL)	1.0 (.99–1.00)	.053
ART use	0.7 (.23–2.15)	.536
History of syphilis treatment	0.8 (.26–2.41)	.675
Adverse events	3.0 (.77–11.5)	.114

Abbreviations: ART, antiretroviral therapy; CI, confidence interval; HIV-1, human immunodeficiency virus type 1; OR, odds ratio.

treated based on 4-fold decrement in RPR titer. Treatment efficacy was 97.5% in patients with early syphilis (including primary, secondary, and early latent syphilis) and 90.8% in patients with late syphilis (including late latent syphilis and syphilis with unknown duration). This high treatment efficacy is surprising considering that our study population could have included asymptomatic neurosyphilis, because neurosyphilis in HIV-infected patients could occur even in early syphilis without clinical symptoms [5, 6], and most study patients (94.1%) did not undergo lumbar puncture for CSF examination. Furthermore, because increased rate of treatment failure has been reported in HIV-1–infected patients compared with noninfected patients [21, 22], the treatment efficacy shown in this study can be generalized to or could be even better among patients without HIV-1 infection. The regimen of 3 g oral amoxicillin plus probenecid was highly tolerable as well; only 28 (9.8%) patients experienced adverse events, and amoxicillin was switched to doxycycline in only 21 (7.3%) patients. It is also noteworthy that treatment of syphilis was successful in 89.3% of the patients who developed adverse events. Thus, high-dose oral amoxicillin (3 g) plus probenecid can be considered the treatment of choice for early syphilis, late latent syphilis, and syphilis of unknown duration where intramuscular BPG is not available or 3 injections of intramuscular BPG at 1-week intervals is not feasible or is inconvenient to patients with late syphilis.

In the present study, the treatment duration for most patients was either for 14–16 days (49.7%) or 28–30 days (34.3%). Comparison of treatment efficacy between early and late syphilis according to treatment duration showed that treatment efficacy was similar for both 2-week and 4-week treatment in early syphilis, whereas it tended to be lower for 2-week than 4-week treatment in late syphilis, albeit statistically insignificant ($P = .15$). Based on these results and considering that the majority of the study patients used 750 mg of probenecid, we recommend 2 weeks of treatment with 3 g oral amoxicillin plus 750 mg

probenecid for patients with early syphilis and 4 weeks of treatment using the same doses for patients with late latent syphilis and syphilis of unknown duration.

To our knowledge, this is the first study to report the treatment efficacy of high-dose oral amoxicillin plus probenecid for syphilis, regardless of HIV-1 infection status. Although the treatment of oral amoxicillin plus probenecid is described as an alternative syphilis treatment in the UK national guidelines on the management of syphilis [10], previous studies were either only pharmacokinetic studies that examined amoxicillin level in the CSF, or anecdotal, and all of these studies were published in the 1970s and 1980s [13, 14, 23, 24]. It is also noteworthy that amoxicillin, similar to aqueous penicillin, crosses the blood-brain barrier to reach the causative bacteria in the CSF [13, 25], whereas BPG, the preferred choice for early and late syphilis, does not [26]. This might be particularly important for patients with HIV-1 infection because these patients likely present with asymptomatic neurosyphilis, and progression to neurosyphilis is faster in HIV-1–infected patients than in noninfected patients [4–6]. Doxycycline and azithromycin are alternative choices for oral treatment of syphilis for patients with penicillin allergy listed in the guidelines [10]; however, evidence for treatment efficacy of these regimens is limited, particularly among patients with HIV-1 infection [27], and unfortunately, emerging resistance of azithromycin for *T. pallidum* has been reported [28, 29].

Despite several strengths of our study, such as novelty and large number of patients treated with the same regimen of oral amoxicillin plus probenecid, we acknowledge several limitations. First, cases of early latent syphilis might have been included among those with syphilis of unknown duration, because blood samples 1 year before the diagnosis of syphilis were not necessarily available for all patients. Thus, the treatment efficacy for syphilis of unknown duration might be overestimated. However, because only 5.9% of the study patients underwent lumbar puncture, neurosyphilis might be included in this group as well, suggesting that the treatment efficacy might be underestimated. Second, this study defined successful treatment as a ≥4-fold decrement in RPR titer by 24 months after starting treatment. This criterion was chosen because the serologic response after treatment in HIV-infected patients is slower than that in noninfected patients [21, 30]. Although the long observation period might increase the risk of unexpected antibiotic exposure and might contribute to overestimated treatment efficacy, in this study, 96.3% of patients with successful treatment against syphilis achieved a ≥4-fold decrement in RPR titer within 12 months after treatment (Figure 2). Third, the retrospective nature of the study could have introduced some selection and information biases. In fact, although all study patients were treated with 3 g amoxicillin plus probenecid, the duration of treatment, frequency of drug administration, and probenecid dose were not identical among the study patients. However, as described above, most patients were treated for either 2 weeks or 4 weeks, and the treatment efficacy was equally high (the efficacy of 2 weeks' treatment tended to be lower than that of 4 weeks among patients with late syphilis, though not statistically significant). Similarly, most (80.8%) patients were treated with amoxicillin 3 times a day, and 60.1% were treated with probenecid 750 mg/day. These are the reasons for recommending 2 weeks of 3 g oral amoxicillin plus 750 mg probenecid for patients with early syphilis and 4 weeks of treatment for patients with late syphilis.

In conclusion, the efficacy of 3 g oral amoxicillin plus 750 mg probenecid daily was very high in HIV-1–infected patients with early and late syphilis. This regimen was also highly tolerable and required only a single hospital visit. Two weeks of this regimen for patients with primary, secondary, and early latent syphilis, and 4 weeks of treatment for late latent syphilis and syphilis of unknown duration are suggested. Three grams of amoxicillin plus probenecid may be an acceptable replacement for intramuscular BPG. Additional prospective studies are warranted.

Notes

Acknowledgments. The authors thank Akiko Nakano, the study coordinator, and all other clinical staff at the AIDS Clinical Center for their help in completion of this study.

Financial support. This work was supported by Grants-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (grant numbers H23-AIDS-001 and H24-AIDS-003).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Patton ME, Su JR, Nelson R, Weinstock H; Centers for Disease Control and Prevention. Primary and secondary syphilis—United States, 2005–2013. MMWR Morb Mortal Wkly Rep **2014**; 63:402–6.
2. Fenton KA, Breban R, Vardavas R, et al. Infectious syphilis in high-income settings in the 21st century. Lancet Infect Dis **2008**; 8:244–53.
3. Jarzebowski W, Caumes E, Dupin N, et al. Effect of early syphilis infection on plasma viral load and CD4 cell count in human immunodeficiency virus-infected men: results from the FHDH-ANRS CO4 cohort. Arch Intern Med **2012**; 172:1237–43.
4. Bordon J, Martinez-Vazquez C, Alvarez M, et al. Neurosyphilis in HIV-infected patients. Eur J Clin Microbiol Infect Dis **1995**; 14:864–9.
5. Lukehart SA, Hook EW 3rd, Baker-Zander SA, Collier AC, Critchlow CW, Handsfield HH. Invasion of the central nervous system by *Treponema pallidum*: implications for diagnosis and treatment. Ann Intern Med **1988**; 109:855–62.
6. Ghanem KG, Moore RD, Rompalo AM, Erbelding EJ, Zenilman JM, Gebo KA. Neurosyphilis in a clinical cohort of HIV-1-infected patients. AIDS **2008**; 22:1145–51.
7. Funnye AS, Akhtar AJ. Syphilis and human immunodeficiency virus co-infection. J Natl Med Assoc **2003**; 95:363–82.
8. Janier M, Hegyi V, Dupin N, et al. 2014 European guideline on the management of syphilis. J Eur Acad Dermatol Venereol **2014**; 28:1581–93.
9. Workowski KA, Berman S, Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines, 2010. MMWR Recomm Rep **2010**; 59:1–110.

10. Kingston M, French P, Goh B, et al. UK national guidelines on the management of syphilis 2008. Int J STD AIDS **2008**; 19:729–40.
11. Hawkes S, Miller S, Reichenbach L, Nayyar A, Buses K. Antenatal syphilis control: people, programmes, policies and politics. Bull World Health Organ **2004**; 82:417–23.
12. Low N, Broutet N, Adu-Sarkodie Y, Barton P, Hossain M, Hawkes S. Global control of sexually transmitted infections. Lancet **2006**; 368:2001–16.
13. Faber WR, Bos JD, Rietra PJ, Fass H, Van Eijk RV. Treponemicidal levels of amoxicillin in cerebrospinal fluid after oral administration. Sex Transm Dis **1983**; 10:148–50.
14. Morrison RE, Harrison SM, Tramont EC. Oral amoxycillin, an alternative treatment for neurosyphilis. Genitourin Med **1985**; 61:359–62.
15. Kenyon C, Lynen L, Florence E, et al. Syphilis reinfections pose problems for syphilis diagnosis in Antwerp, Belgium—1992 to 2012. Euro Surveill **2014**; 19.
16. Marra CM, Maxwell CL, Smith SL, et al. Cerebrospinal fluid abnormalities in patients with syphilis: association with clinical and laboratory features. J Infect Dis **2004**; 189:369–76.
17. Zetola NM, Engelman J, Jensen TP, Klausner JD. Syphilis in the United States: an update for clinicians with an emphasis on HIV coinfection. Mayo Clin Proc **2007**; 82:1091–102.
18. Yang CJ, Lee NY, Lin YH, et al. Jarisch-Herxheimer reaction after penicillin therapy among patients with syphilis in the era of the HIV infection epidemic: incidence and risk factors. Clin Infect Dis **2010**; 51:976–9.
19. Nishijima T, Shimbo T, Komatsu H, Hamada Y, Gatanaga H, Oka S. Incidence and risk factors for incident hepatitis C infection among men who have sex with men with HIV-1 infection in a large urban HIV clinic in Tokyo. J Acquir Immune Defic Syndr **2014**; 65:213–7.
20. Nishijima T, Kawasaki Y, Tanaka N, et al. Long-term exposure to tenofovir continuously decrease renal function in HIV-1-infected patients with low body weight: results from 10 years of follow-up. AIDS **2014**; 28:1903–10.
21. Ghanem KG, Erbelding EJ, Wiener ZS, Rompalo AM. Serological response to syphilis treatment in HIV-positive and HIV-negative patients attending sexually transmitted diseases clinics. Sex Transm Infect **2007**; 83:97–101.
22. Rolfs RT, Joesoef MR, Hendershot EF, et al. A randomized trial of enhanced therapy for early syphilis in patients with and without human immunodeficiency virus infection. The Syphilis and HIV Study Group. N Engl J Med **1997**; 337:307–14.
23. Goldman JN. Clinical experience with amicillin and probenecid in the management of treponeme-associated uveitis. Trans Am Acad Ophthalmol Otolaryngol **1970**; 74:509–14.
24. Onoda Y. Clinical evaluation of amoxycillin in the treatment of syphilis. J Int Med Res **1979**; 7:539–45.
25. Robbins N, Koch SE, Tranter M, Rubinstein J. The history and future of probenecid. Cardiovasc Toxicol **2012**; 12:1–9.
26. Ho EL, Lukehart SA. Syphilis: using modern approaches to understand an old disease. J Clin Invest **2011**; 121:4584–92.
27. Zetola NM, Klausner JD. Syphilis and HIV infection: an update. Clin Infect Dis **2007**; 44:1222–8.
28. Workgroup AGP. Prevalence of the 23S rRNA A2058G point mutation and molecular subtypes in *Treponema pallidum* in the United States, 2007 to 2009. Sex Transm Dis **2012**; 39:794–8.
29. Wu BR, Yang CJ, Tsai MS, et al. Multicentre surveillance of prevalence of the 23S rRNA A2058G and A2059G point mutations and molecular subtypes of *Treponema pallidum* in Taiwan, 2009–2013. Clin Microbiol Infect **2014**; 20:802–7.
30. Manavi K, McMillan A. The outcome of treatment of early latent syphilis and syphilis with undetermined duration in HIV-infected and HIV-uninfected patients. Int J STD AIDS **2007**; 18:814–8.

How Does Fat Survive and Remodel After Grafting?

Takanobu Mashiko, MD, Kotaro Yoshimura, MD*

KEYWORDS

• Fat grafting • Adipose-derived stem/stromal cell • Tissue regeneration • Macrophages
• Vascular endothelial cells

KEY POINTS

- Under severe ischemia, adipocytes die within 24 hours; adipose-derived stem/stromal cells (ASCs) survive up to 3 days and are activated, contributing to the repairing process through adipogenesis, angiogenesis, and paracrine effects.
- Adipocyte fate after fat grafting is categorized into three zones depending on the distance from the surface: survival, regeneration, and necrosis.
- ASCs do not die and give rise to new adipocytes in the regenerating zone; they die in the necrotizing zone. The balance between regeneration and degeneration determines the final volume retention after fat grafting.
- Dead adipocytes under better conditions (regenerating zone) are phagocytized by macrophages and are successfully replaced by new adipocytes.
- Dead adipocytes under worse conditions (necrotizing zone) are replaced with cicatrization or oil cyst formation depending on the size of oil drops.

INTRODUCTION

Adipose tissue and adipose-derived stem/stromal cells (ASCs) obtained from liposuction were shown to have potential for regenerative therapeutic use. However, clinical outcomes of fat grafting remain unpredictable and, to improve the outcomes, it is crucial to elucidate the detailed mechanism of engraftment of fat tissue. The "cell survival theory," which maintains that transplanted adipocytes partly survive once they receive adequate nutrients and remain alive in the recipient site, had been accepted for a long time.[1–3] In contrast, our recent studies showed how ASCs work in response to microenvironmental changes, such as ischemia and applied mechanical force,[4,5] and revealed the "cell replacement theory," which holds that most adipocytes undergo ischemic death and subsequent replacement with next generation during the first 3 months after fat grafting.[6,7] Further details, such as the cellular origin of adipose regeneration and the mechanism of cicatrization and oil cyst formation, were also demonstrated.[7]

BASIC SCIENCE: FUNCTIONAL ROLES OF ADIPOSE-DERIVED STEM/STROMAL CELLS IN TISSUE REMODELING

Adipose Tissue Biology

Adipose tissue is not only an organ of energy storage, but also an endocrine organ (releasing multiple adipose-derived hormones, such as leptin and adiponectin) that regulates metabolic

Financial Disclosure: Authors report no commercial associations or financial disclosures with regard to this article.
Department of Plastic Surgery, School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-Ku, Tokyo 113-8655, Japan
* Corresponding author.
E-mail address: kotaro-yoshimura@umin.ac.jp

Clin Plastic Surg 42 (2015) 181–190
http://dx.doi.org/10.1016/j.cps.2014.12.008
0094-1298/15/$ – see front matter

homeostasis. Adipose tissue consists predominantly of adipocytes, ASCs, vascular endothelial cells (VECs), pericytes, fibroblasts, and connective tissue as well as adipose tissue-resident macrophages and lymphocytes (**Fig. 1**).[8] Our rough estimation of cellular component numbers are as follows; 1 cm^3 intact adipose tissue contains several millions cells; 1 million adipocytes, 1 million ASCs, 1 million VECs, and 1 million other cells (adipose-resident macrophages and lymphocytes, pericytes, fibroblasts, etc).[8] Adipose tissue is rich in capillary and every single adipocyte is attached to the capillary network. The size of adipocyte is 50 to 150 μm in diameter (if it becomes larger, it dies from ischemia) and its life span is several to 10 years in humans. ASCs are located perivascularly along the capillaries between adipocytes like pericytes. ASCs have been shown to release angiogenic factors responding to ischemia[4] and to differentiate physiologically into adipocytes and VECs.[9] A small subpopulation of ASCs (1%–2%) may have greater multipotency, corresponding with stem cells called multilineage differentiating stress enduring (Muse) cells.[10] The enlarged adipocytes in obese individuals occasionally die from relative ischemia and are subsequently surrounded by infiltrated M1 inflammatory macrophages (crownlike structure). The crownlike structure is seen after any types of adipocyte death (**Fig. 2**).

Adipose-Derived Stem/Progenitor Cells in Adipose Tissue Remodeling

ASCs are the main cell population contributing to adipocyte (re)generation in any types of adipose tissue remodeling/expansion, such as developmental growth, hyperplasia in obesity, repair processes after injury/ischemia,[4] or tissue expansion induced by internal/external mechanical forces.[5] These remodeling processes are in balance between adipocyte apoptosis/necrosis and adipogenesis managed by ASCs. In ASC-deficient tissues, such as irradiated or chronically inflamed tissues, any type of adipose tissue remodeling or expansion is impaired and thus fat grafting to fertilize such stem cell–depleted condition would be theoretically the right solution.[11] Adipose-tissue atrophy over aging is likely owing to a decrease in number of ASCs and consequent impaired physiologic turnover, as is commonly seen in other tissues and organs.

Ischemia to Adipose Tissue

Subcutaneous adipose tissue has the highest tissue partial oxygen tension (ptO_2; 40–60 mm Hg) among organs. The high ptO_2 of adipose tissue probably reflects high density of capillaries and low oxygen consumption rate of the tissue. Diabetic adipose tissue is relatively ischemic with low-grade chronic inflammation, which causes

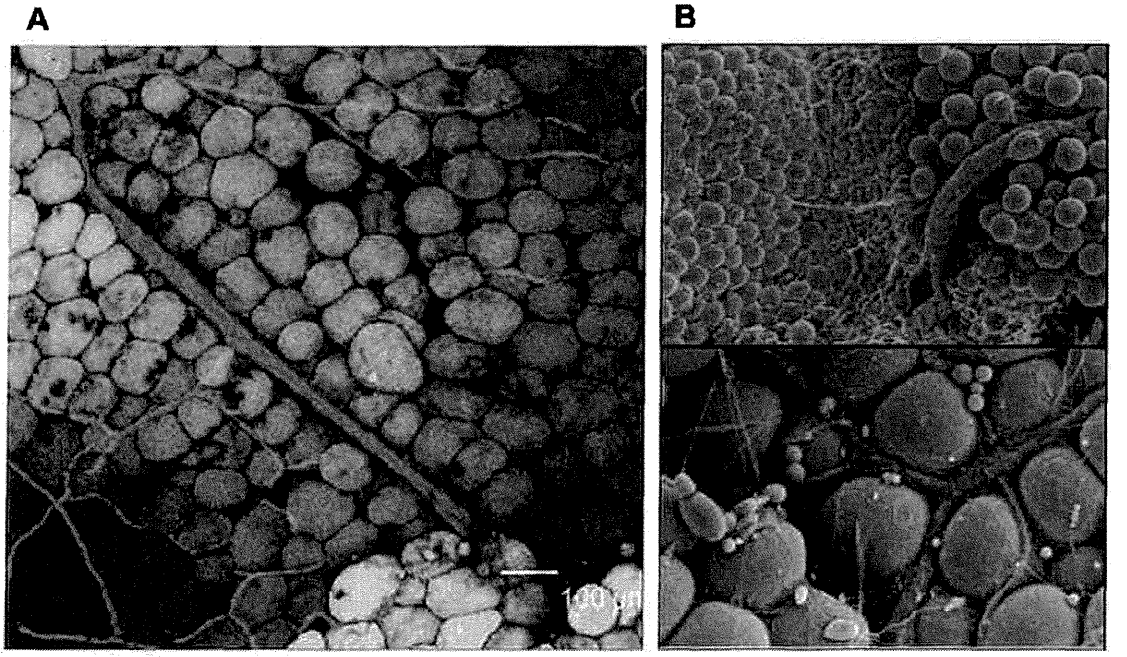

Fig. 1. Structure of human adipose tissue. (*A*) Adipose tissues are triple stained with BODIPY (adipocytes; *yellow*), lectin (endothelial cells; *red*), and Hoechst 33,342 (nuclei; *blue*). Adipose tissue is packed with adipocytes with scarce connective tissue and is rich in capillary network, though each adipocyte is exceptionally large in size. Scale bars = 100 μm. (*B*) Scanning electromicroscopic images.

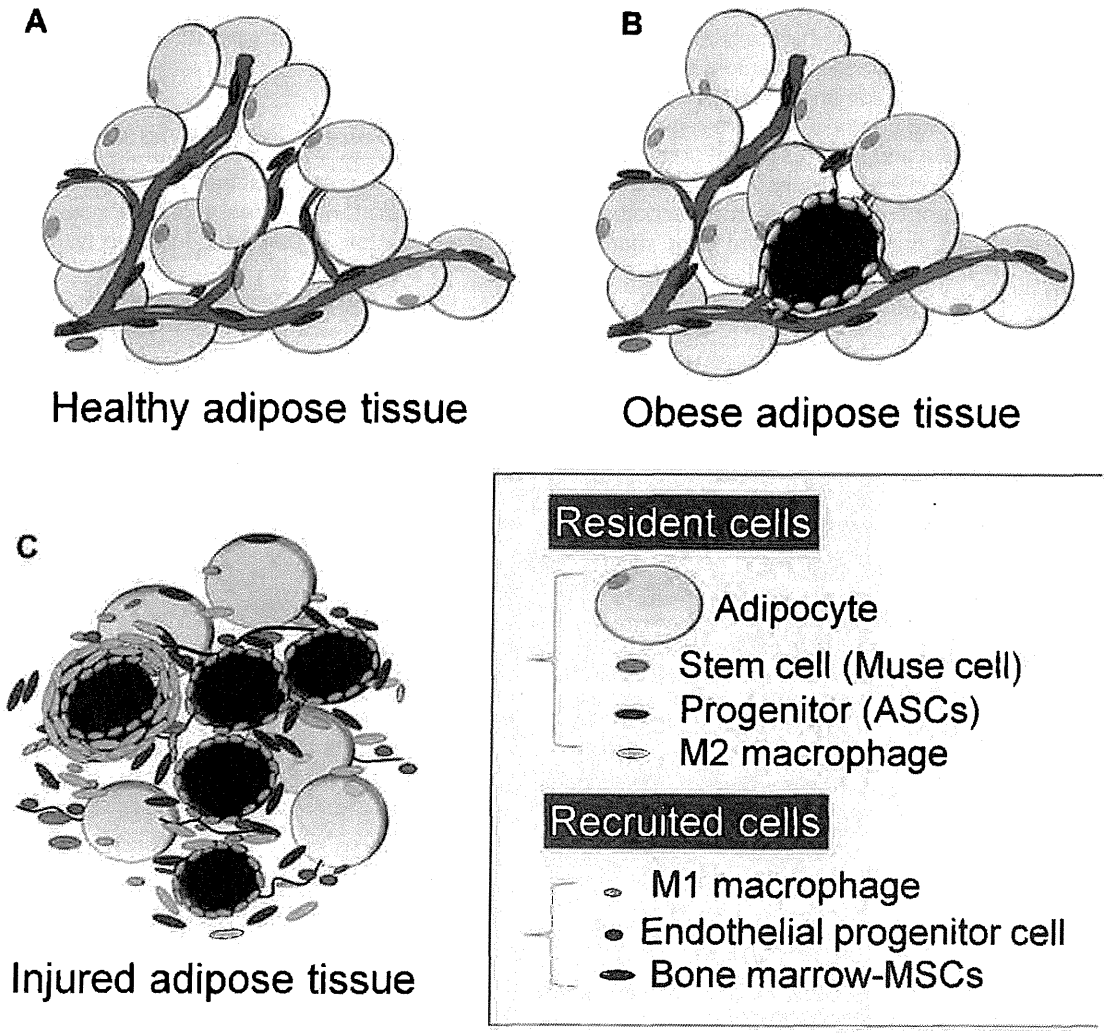

Fig. 2. Schema for structure of intact, obese, and injured adipose tissues. (*A*) Intact adipose tissue has not only adipocytes but also many other types of cells such as ASCs and vascular endothelial cells. (*B*) Obese adipose tissue has some dead adipocytes surrounded by infiltrated M1 macrophages (crownlike structure) and shows low-grade chronic inflammatory condition. (*C*) In injured adipose tissue, ASCs are activated and many types of progenitor/stem cells are recruited from bone marrow to repair the tissue damage. MSC, mesenchymal stem cell.

adipose endocrine dysfunction, insulin resistance, and the metabolic syndrome, whereas lipoma tissue is not ischemic, probably owing to upregulated angiogenesis.[12]

Among cellular components of adipose tissue, adipocytes are most susceptible to death under ischemic conditions such as 15 mm Hg of ptO_2.[6] When severe ischemia prolongs, VECs and blood-derived cells start to die next. In contrast, ASCs can remain alive up to 3 days, even under severely ischemic conditions.[6] Over the 3 days, they can be activated by signals from dying cells and contribute to the adaptive repairing process, such as by adipogenesis and angiogenesis.[6,7]

Injury to Adipose Tissue

Tissue injury also causes adipose tissue degeneration with inflammatory cell recruitment and release of inflammatory cytokines. After injury, degenerative changes such as adipocyte death occur, and primary injury factors such as basic fibroblast growth factor and other factors from aggregated platelets such as platelet-derived growth factor, epidermal growth factor, and transforming growth factor-β are first released into the injured site and trigger a cascade of wound healing processes.[4,13] Basic fibroblast growth factor is released from damaged connective tissue and

acts through a c-Jun N-terminal kinase signaling pathway to stimulate ASCs not only to proliferate, but also to secrete secondary factors such as hepatocyte growth factor and vascular endothelial growth factor, and contributes to the regeneration of adipose tissue and suppression of fibrogenesis during the first week after injury.[13] In parallel, a variety of stem/progenitor cells such as endothelial progenitor cells are recruited from bone marrow and collaborate with activated ASCs in an orchestrated repair of the damaged adipose tissue (see **Fig. 2**).

Mechanical Force to Adipose Tissue

Mechanical forces, whether external (shear, stretch, tension, distraction and compression) or endogenous (forces that are generated within the

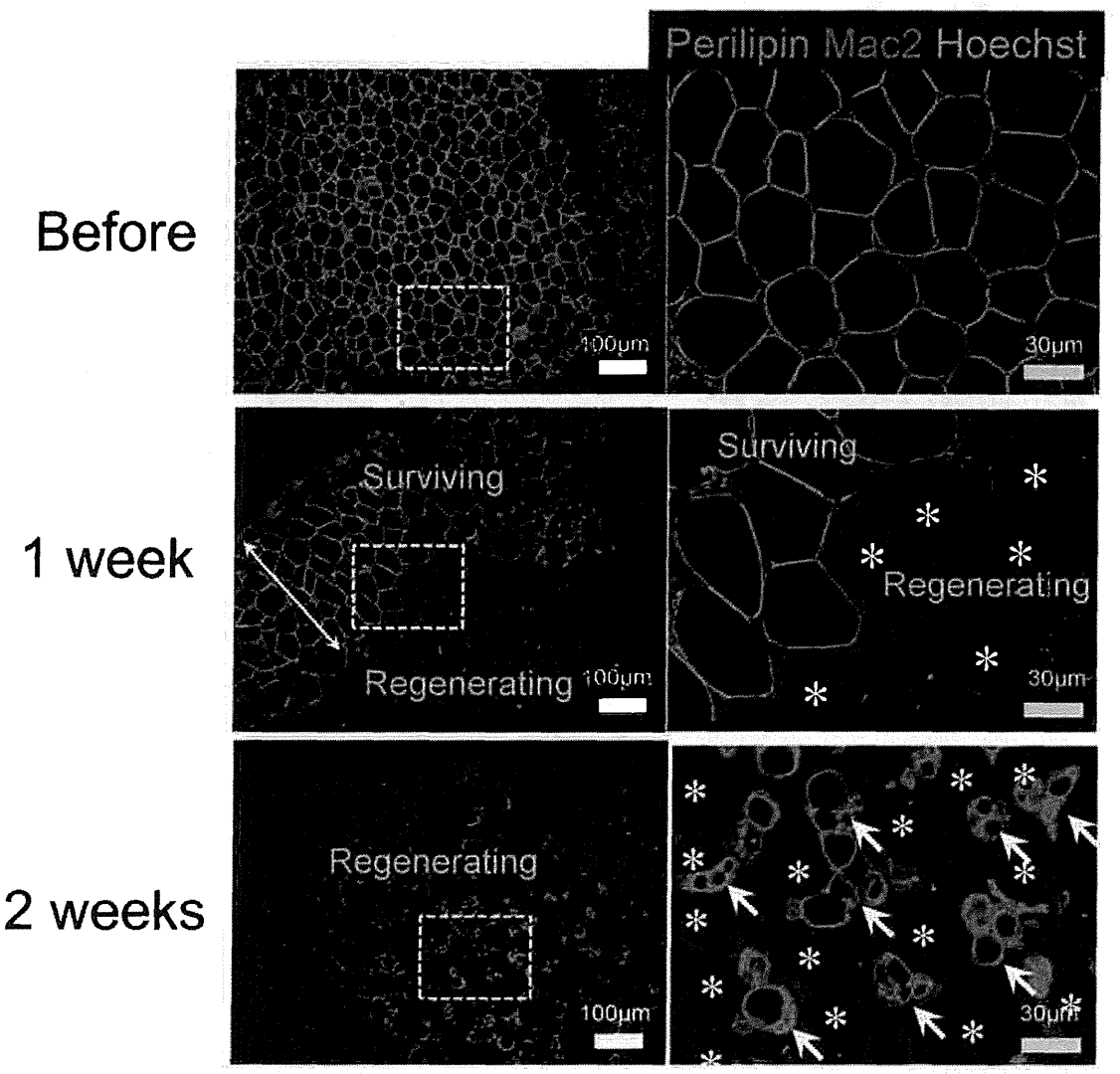

Fig. 3. Immunohistology of grafted fat tissue in mice (Before, 1 week and 2 weeks). Harvested tissue samples [before (*top*), 1 week (*middle*), and 2 weeks (*bottom*) after grafting] were immunostained for perilipin (cytoplasm of viable adipocytes; *green*), MAC2 (monocytes/macrophages; *red*) and Hoechst 33,342 (nuclei; *blue*). Rectangles in the low magnification images (*left column*; *yellow* scale bars = 100 μm) were further magnified in the right column (*white scale bars* = 30 μm). Demarcation between the surviving and regenerating zone became clear at 1 week (*interrupted line*); dead adipocytes (*asterisk*) were perilipin negative and surviving adipocytes were strongly positive for perilipin. Small-sized preadipocytes with multiple intracellular lipid droplets (*arrows*) appeared between dead adipocytes at 2 weeks; the dead adipocytes were surrounded by a single layer of macrophages (*red*). (*Adapted from* Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:306e; with permission.)

active cytoskeleton), affect tissue growth, cellular function, and even survival. Moreover, physical interactions with the extracellular matrix can significantly influence stem cell behavior.[14] Continuous external tissue expansion (Brava®) is attempted for expansion of the breast tissue.[15] Experimentally, 4 weeks of external suspension caused enlargement of the subcutaneous tissue, particularly adipose tissue, although the enlargement was reversible.[5] The regenerating potential has been attributed to the number (density) and potential of ASCs; thus, irradiated tissue has a limited potential for expansion.

RELEVANCE TO CLINICIANS: WHAT HAPPENS AFTER FAT GRAFTING?

Acute Events Immediately After Fat Grafting

The grafted nonvascularized adipose tissue is placed under ischemia (hypoxia) and is nourished only by plasmatic diffusion from the surrounding host tissue for a few days until revascularization occurs. This results in the death of many adipocytes within 24 hours and release of multiple cell death and injury-associated factors from the dying donor tissue and injured host tissue (**Fig. 3**).[6,13] Inflammatory cells, such as macrophages and lymphocytes, are infiltrated and inflammatory cytokines, such as interleukins, are secreted. Despite the death of adipocytes, ASCs, which can be functional for up to 72 hours even under severe ischemia, are activated and try to repair the damaged tissue in collaboration with infiltrated stem and progenitor cells from the bone marrow.[6,7]

Regeneration After Ischemic Tissue Damage: Three Zones with Differential Cell Fates

Based on our recent studies, the first 3 months after fat grafting is a period of tissue remodeling; adipogenesis does not occur after this period.[11] The grafted fat is categorized into three zones from the periphery to the center: (1) survival (superficial), (2) regeneration (intermediate), and (3) necrosis (central; **Fig. 4**).[7] The demarcation of the surviving zone (100–300 μm thick) from the regenerating

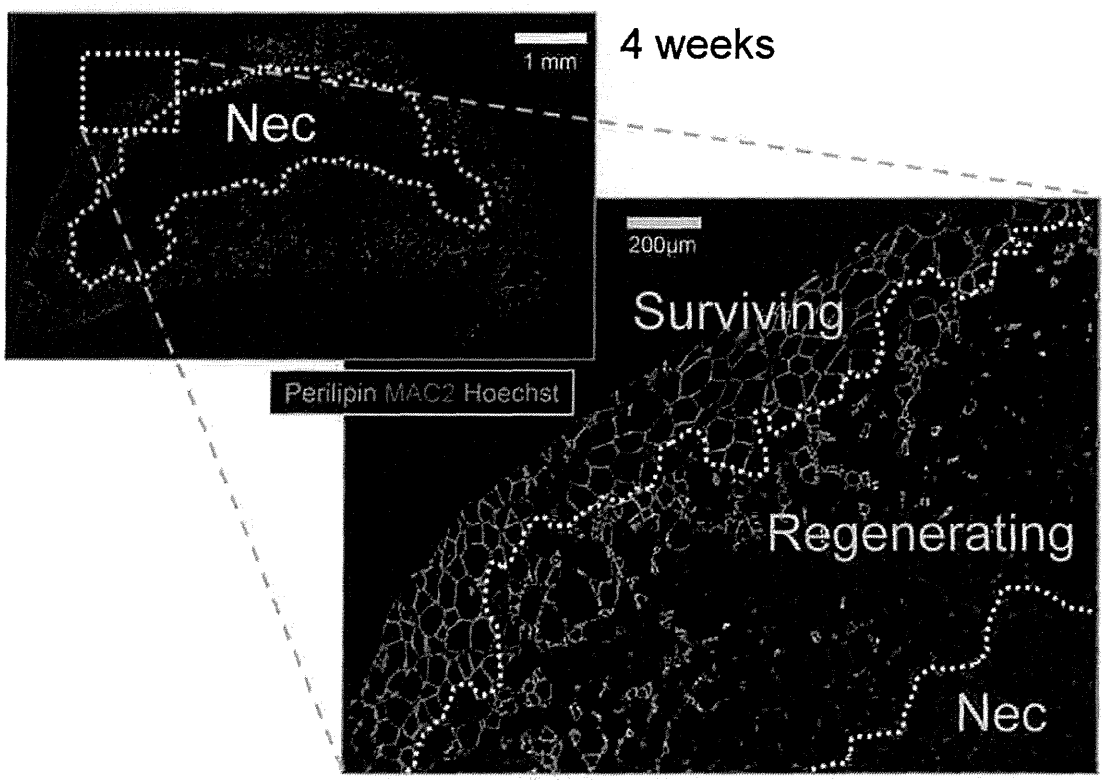

Fig. 4. Immunohistology of grafted fat tissue in mice (4 weeks). Immunohistology of a graft sample at 4 weeks showed demarcated surviving, regenerating, and necrotizing zones. (*Left, above*) A low-magnification image of perilipin staining showed the necrotizing zone (*yellow interrupted line*) with no adipogenesis. White scale bar = 1 mm. (*Right, below*) A high-magnification image showed demarcated (with *white interrupted lines*) surviving (perilipin-positive adipocytes), regenerating (perilipin-positive small adipocytes), and necrotizing zones (no viable adipocytes). Yellow scale bar = 100 μm.

zone became clear at 1 week, whereas the demarcation between the regenerating and necrotizing zones was obvious between 2 and 4 weeks (see **Fig. 3**).

Adipocytes superficially located 100 to 300 μm from the tissue edge remain alive (surviving zone), and all the rest of adipocytes (regenerating and necrotizing zones) die within 24 hours after grafting. The dead adipocytes are surrounded by M1 macrophages for phagocytosis (see **Fig. 3**), but the absorption process takes weeks or months, depending on the size and therefore the grafted fat maintains its original size for the first 4 weeks despite adipocyte death. ASCs in the regenerating and necrotizing zones are activated and start to repair the tissue. New, small preadipocytes appeared around the dead adipocytes (surrounded by a single layer of macrophages) at 1 to 2 weeks in the regenerating zone (600–1200 μm thick), whereas no adipogenesis was observed in the necrotizing zone (see **Fig. 4**). In the regenerating zone, the hypoxic condition is improved by revascularization within 3 days and ASCs give rise to new adipocytes, which finally replace the dead adipocytes by 3 months. On the other hand, in the necrotizing zone, the microenvironment is not improved within 3 days and ASCs also die, leading to central necrosis of the graft tissue.

The ratio between the necrotizing and surviving/regenerating zones, which determines the final volume retention after fat grafting, varies depending on the recipient microenvironment, based on factors such as vascularity, as well as the size of the grafted fat, grafting technique, and postoperative care. Our experimental study using a mouse model revealed that oxygenation of the recipient bed with normobaric 60% oxygen for 3 days postoperatively promotes survival, regeneration, and

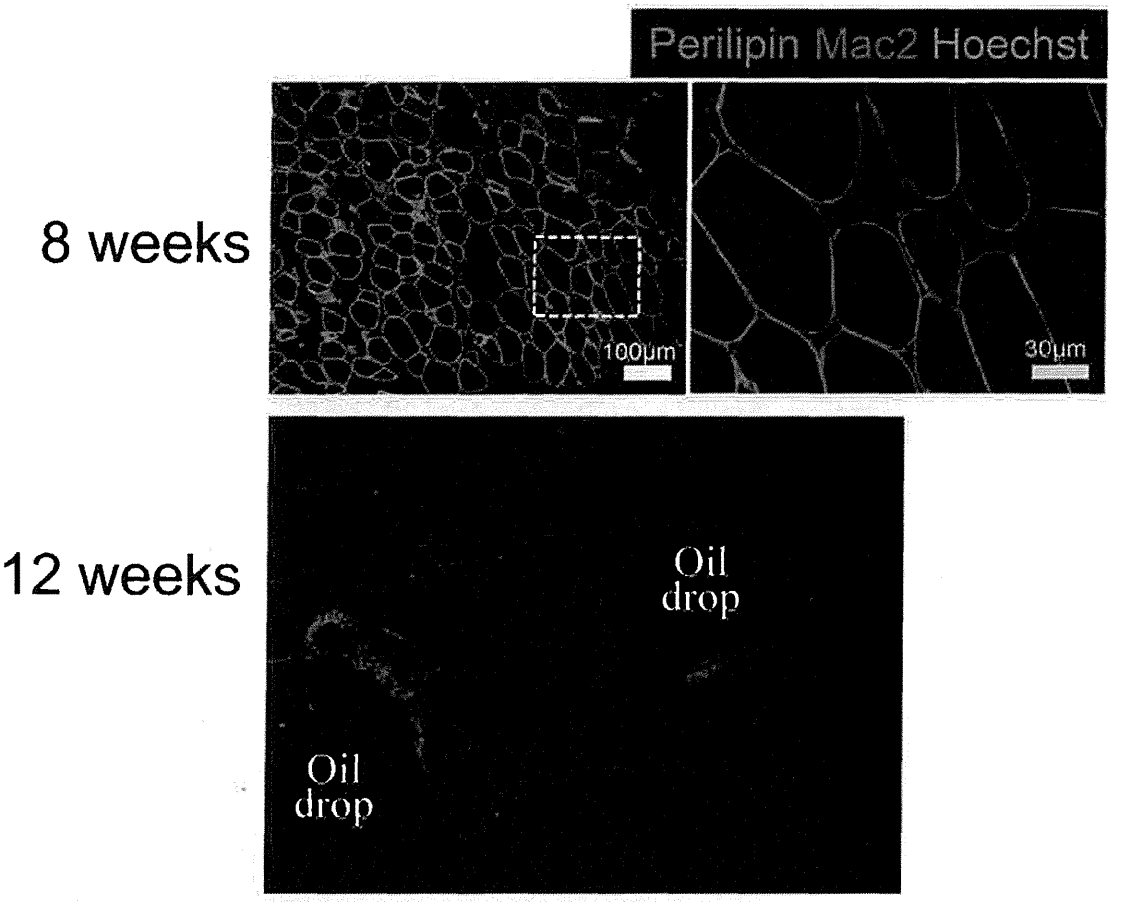

Fig. 5. Immunohistology of grafted fat tissue in mice (8 and 12 weeks). Harvested tissue samples (8 weeks [*top*] and 12 weeks [*bottom*] after grafting) were immunostained for perilipin (cytoplasm of viable adipocytes; *green*), MAC2 (monocytes/macrophages; *red*) and Hoechst 33,342 (nuclei; *blue*). There are few small new adipocytes, which means that adipose regeneration seemed to be finished by 12 weeks. Large-sized lipid drops surrounded by M1 macrophages are left in the tissue. (*Modified from* Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:306e.)

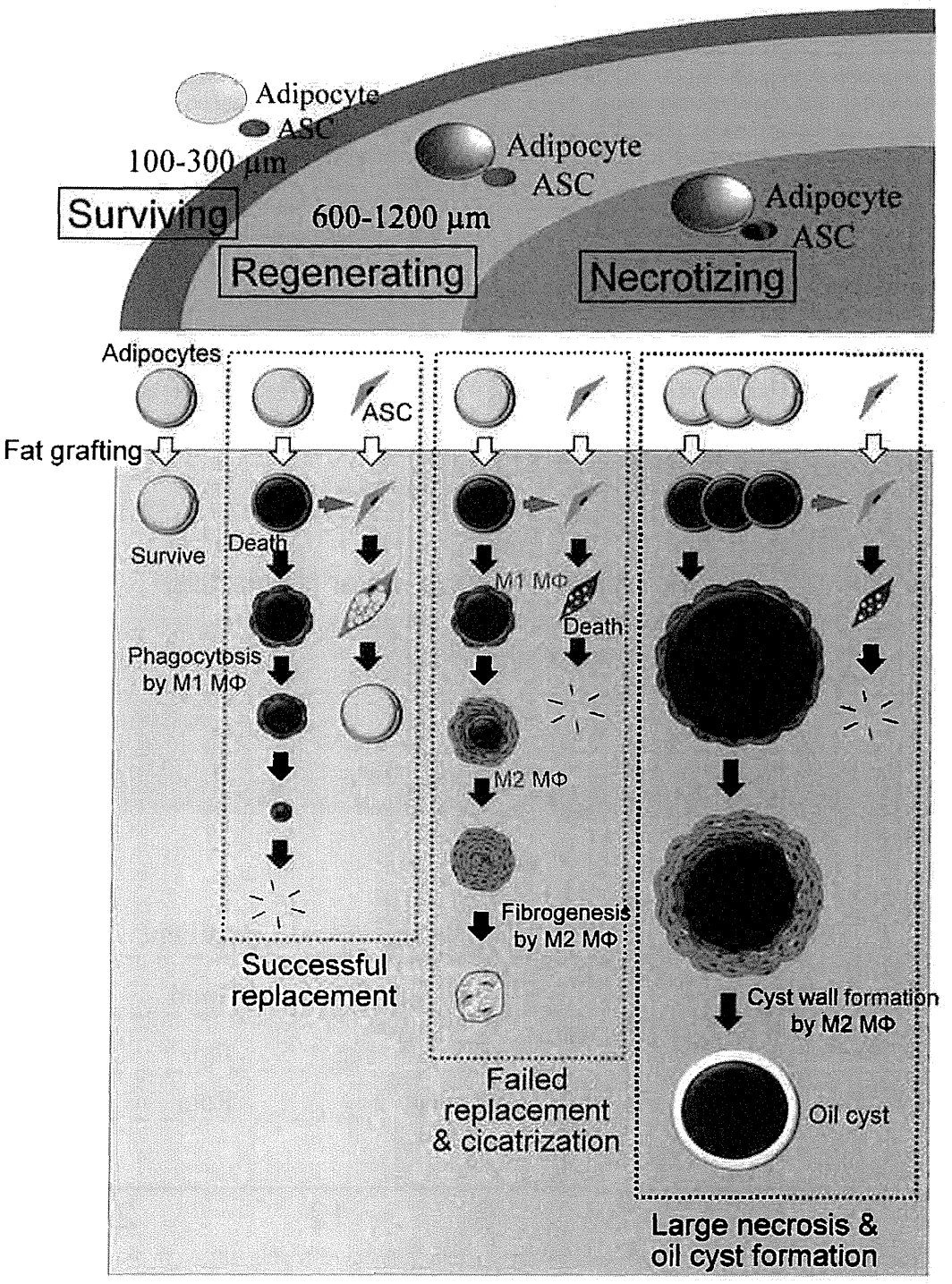

Fig. 6. Conclusive schema for the fate of adipocytes in grafted fat. During the first 3 months of adipose tissue remodeling, transplanted adipocytes have differential fates depending on their microenvironments. In this schema, complex cellular events are simplified and the adipocyte fate is categorized into 4 patterns: survival, successful regeneration, failed regeneration (cicatrization), and oil cyst formation. Cicatrization and oil cyst formation are often not complete at 3 months. The most superficial zone is the "surviving zone," which is less than 300 μm thick. In the surviving zone, both adipocytes and adipose-derived stem cells (ASCs) survive. The second zone is the "regenerating zone," the thickness of which varies (600–1200 μm) depending on the microenvironmental conditions. In this zone, adipocytes die, but ASCs survive and provide new adipocytes to replace the dead ones. The most central zone is the "necrosis zone," where both adipocytes and ASCs die, no regeneration is expected, and the dead space will be absorbed, be filled with fibrosis, or develop into an oil cyst. (*Modified from* Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:312e.)

final retention of transplanted fat.[16] The thickness of surviving and regenerating zones were increased, suggesting superior survival of adipocyte and resident ASCs, respectively.

Long-Term Stabilization Process (Lipid Absorption and Cicatrization)

In parallel with the regenerating events, stabilizing events, such as lipid absorption (phagocytosis) and lipid replacement with scar tissue (fibrosis), occur in the regenerating and necrotizing zones.[7] Although the adipogenesis/regeneration process in the regenerating zone peaks at 4 weeks and is completed by 3 months (**Fig. 5**), the stabilizing process persists for at least several more months, as suggested by clinical observations that volume reduction after fat grafting continues until the end of the first year. Small-sized oil droplets were absorbed or temporarily filled with multilayered M2 macrophages, inducing the dead space replacement with fibrogenesis in parallel with lipid absorption. On the contrary, substantially larger oil drops (>8 mm) form oil cysts in several months and remain permanently, which are considered the worst outcome of fat grafting accompanied by chronic inflammation and calcification.[7,11,17] We summarize differential fates of adipocytes depending on the microenvironment in **Fig. 6** and the postoperative time course of fat grafting in **Fig. 7**.

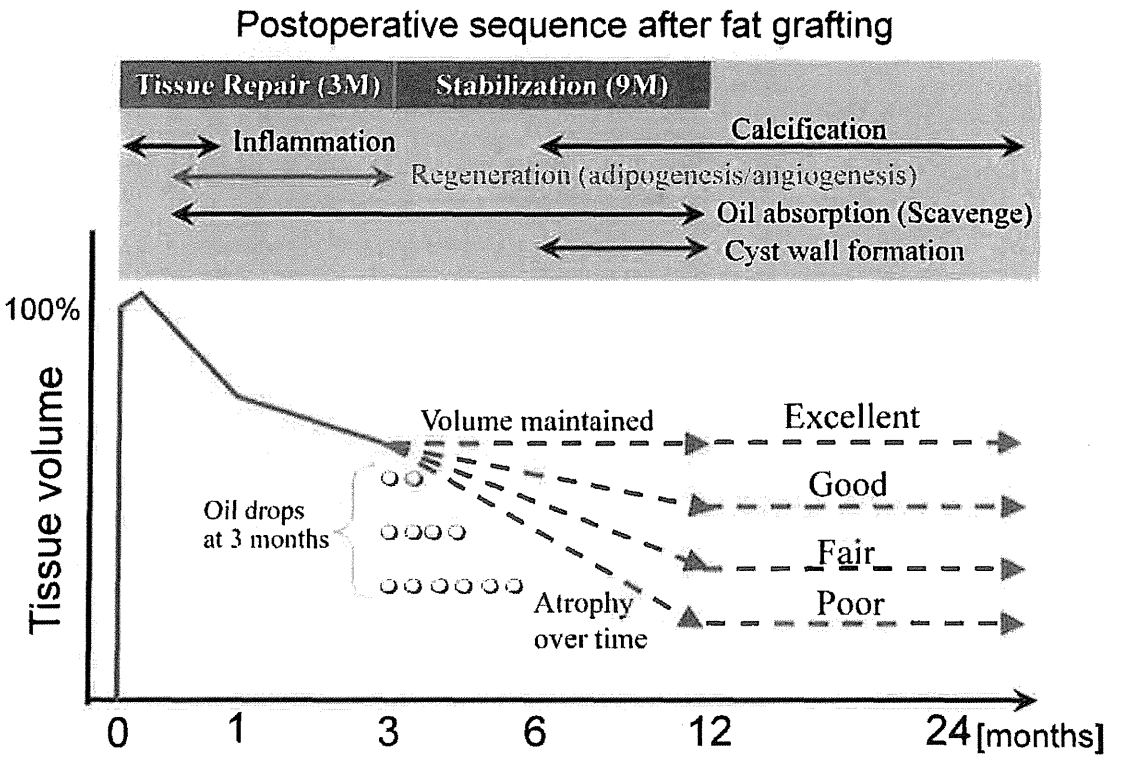

Fig. 7. Long-term postoperative sequence after fat grafting. Adipogenesis after adipocyte death is exerted by activated adipose-derived stem cells (ASCs). Some of the dead adipocytes are replaced with new adipocytes of next generation and the adipogenesis is finish by 3 months (tissue "repair" phase). Dead adipocytes remaining at 3 months are absorbed during the next 9 months (tissue "stabilization" phase). Lipid droplets (dead adipocytes) are absorbed by macrophage phagocytosis, but the absorption is very slow and the absorption period depends on the diameter of the lipid droplets; when the lipid droplet diameter was large, such as 10 mm, the cyst wall is formed before completing absorption and the cyst wall start to calcify over time. The final volume retention after fat grafting is determined by the rate of successful replacement of adipocytes. If grafted adipose has only small lipid droplets and absorption is finished by 3 months, the volume will not substantially change after 3 months (shown as "excellent"). On the other hand, many large lipid droplets remain at 3 months, tissue will atrophy between 3 and 12 months (shown as "poor"). (*Adapted from* Yoshimura K, Eto H, Kato H, et al. *In vivo manipulation of stem cells for adipose tissue repair/reconstruction*. Regen Med 2011;6(6 Suppl):38; with permission.)

What Are the Origins of Next-Generation Cells After Fat Grafting?

Our recent study using green fluorescent protein mice revealed the origin of cell components in grafted fat.[18] Mature adipocytes are mostly derived from ASCs in the graft. Although vascular wall constituents (smooth muscle cells) are chiefly graft derived; capillaries (VECs) originated equally from the graft and the host bone marrow. ASCs of the regenerated fat are an admixture of grafted, host non–bone marrow, and host bone marrow cells. These findings highlight the importance of ASCs contained in the grafted fat for regeneration of adipocytes. Also, host bone marrow and local tissues contribute substantially to capillary networks and the provision of new ASCs, which can contribute to future remodeling. Thus, although ASCs can be provided by bone marrow or other tissues, they have to get ready by staying adjacent to adipocytes in of contributing to adipocyte regeneration after adipocyte death.

Clinical Implications: How We Can Improve the Engraftment of Grafted Fat?

Recent advancements in the understanding of the underlying mechanisms provide a number of clinical implications. It is considered that the size and thickness of surviving zone are influenced by the surrounding recipient tissue. Better vascularity and greater oxygen tension of recipient tissue increase the surviving zone. Preconditioning of recipient tissue, negative pressure, and/or hyperoxygenation may help for this purpose. Excessively high internal pressure keeps the recipient tissue ischemic and reduces the surviving zone. As with skin grafts, immobilization should help the capillary to grow into the graft during the first week, which improves the oxygen tension of the regenerating zone and rescues ASCs from ischemic death. The size and surface area of grafted fat is a critical factor to minimize the central necrotizing zone; the diameter of grafted fat particles or noodles would be recommended to be as small as 2 mm. For adipogenesis after fat grafting, it is very important to have a good number of both viable adipocytes and ASCs in the graft (not helped from the outside). Adipocytes can release crucial factors to activate adjacent ASCs and lead them to differentiation into adipocytes. This finding strongly suggests that it is worth considering preparing a better number and ratio of adipocytes and ASCs during the tissue processing before grafting (discussed in the article by Kuno and Yoshimura elsewhere in this issue).

SUMMARY

ASCs act as main players in any types of adipose tissue regeneration, including after fat grafting, by differentiating into adipocytes or VECs and releasing angiogenic growth factors. The fate of grafted fat depends on its size and the microenvironment of cellular components, such as adipocytes. Adipocytes remain alive in the surviving zone, whereas they die shortly after grafting in the regenerating and necrotizing zones. Adjacent perivascular ASCs are activated by adipocyte death and begin to proliferate and differentiate to repair the damaged tissue in collaboration with infiltrated stem/progenitor cells in the regenerating zone. Dead adipocytes are phagocytized by M1 macrophages and are replaced successfully by new adipocytes without residual fibrosis in a better condition of the regenerating zone. In contrast, dead adipocytes under worse conditions in the regenerating or necrotizing zones are replaced partly with fibrosis or oil cysts; M2 macrophages act in the fibrogenesis process. Interestingly, dead adipocytes work as spacers and keep the space for new adipocytes during the regeneration process. The final volume retention after fat grafting is determined by the balance between degeneration and regeneration of adipose tissue and affected by many surgeons' factors including the microenvironments of graft regenerating zone and surrounding recipient tissue. Adipogenesis after fat grafting depends greatly on ASCs resident in the graft tissue, suggesting the importance of tissue processing before transplantation.

REFERENCES

1. Smahel J. Adipose tissue in plastic surgery. Ann Plast Surg 1986;16:444–53.
2. Billings E Jr, May JW Jr. Historical review and present status of free graft autotransplantation in plastic and reconstructive surgery. Plast Reconstr Surg 1989;83:368–81.
3. Cortese A, Savastano G, Felicetta L. Free fat transplantation for facial tissue augmentation. J Oral Maxillofac Surg 2000;58:164–9.
4. Suga H, Eto H, Shigeura T, et al. IFATS collection: fibroblast growth factor-2 induced hepatocyte growth factor secretion by adipose-derived stromal cells inhibits postinjury fibrogenesis through a c-Jun N-terminal kinase-dependent mechanism. Stem Cells 2009;27:238–49.
5. Kato H, Suga H, Eto H, et al. Reversible adipose tissue enlargement induced by external tissue suspension: possible contribution of basic fibroblast growth factor in the preservation of enlarged tissue. Tissue Eng Part A 2010;16:2029–40.

6. Eto H, Kato H, Suga H, et al. The fate of adipocytes after nonvascularized fat grafting: evidence of early death and replacement of adipocytes. Plast Reconstr Surg 2012;129:1081–92.
7. Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:e303–13.
8. Eto H, Suga H, Matsumoto D, et al. Characterization of structure and cellular components of aspirated and excised adipose tissue. Plast Reconstr Surg 2009;124:1087–97.
9. Planat-Benard V, Silvestre JS, Cousin B, et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004;109:656–63.
10. Ogura F, Wakao S, Kuroda Y, et al. Human adipose tissue possesses a unique population of pluripotent stem cells with nontumorigenic and low telomerase activities: potential implications in regenerative medicine. Stem Cells Dev 2014;23:717–28.
11. Yoshimura K, Eto H, Kato H, et al. In vivo manipulation of stem cells for adipose tissue repair/reconstruction. Regen Med 2011;6(6 Suppl):33–41.
12. Suga H, Eto H, Inoue K, et al. Cellular and molecular features of lipoma tissue: comparison with normal adipose tissue. Br J Dermatol 2009;161:819–25.
13. Eto H, Suga H, Aoi N, et al. Adipose injury-associated factors activate adipose stem/stromal cells, induce neoangiogenesis, and mitigate hypoxia in ischemic tissues. Am J Pathol 2011;178:2322–32.
14. Guilak F, Cohen DM, Estes BT, et al. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 2009;5:17–26.
15. Khouri RK, Schlenz I, Murphy BJ, et al. Nonsurgical breast enlargement using an external soft-tissue expansion system. Plast Reconstr Surg 2000;105:2500–12.
16. Kato H, Araki J, Doi K, et al. Normobaric hyperoxygenation enhances initial survival, regeneration, and final retention in fat grafting. Plast Reconstr Surg 2014;134:951–9.
17. Mineda K, Kuno S, Kato H, et al. Chronic inflammation and progressive calcification as a result of fat necrosis: the worst outcome in fat grafting. Plast Reconstr Surg 2014;133:1064–72.
18. Doi K, Ogata F, Eto H, et al. Differential contributions of graft- and host-derived cells in tissue regeneration/remodeling after fat grafting. Plast Reconstr Surg, in press.

Differential Contributions of Graft-Derived and Host-Derived Cells in Tissue Regeneration/ Remodeling after Fat Grafting

Kentaro Doi
Fusa Ogata
Hitomi Eto
Harunosuke Kato
Shinichiro Kuno
Kahori Kinoshita
Koji Kanayama
Jingwei Feng
Ichiro Manabe
AQ1 Kotaro Yoshimura, M.D.

Tokyo, Japan

Background: Recent research indicates that the adipose tissue of nonvascularized grafts is completely remodeled within 3 months, although origins of next-generation cells are unclear.
Methods: Inguinal fat pads of green fluorescent protein mice and wild-type AQ2 mice were cross-transplanted beneath the scalp. At 1, 2, 4, and 12 weeks after transplantation, grafted fat was harvested, weighed, and analyzed through immunohistochemistry, whole-mount staining, and flow cytometry of cell isolates. Bone marrow of green fluorescent protein mice was transplanted to wild-type mice (after irradiation). Eight weeks later, these mice also received fat grafts, which were analyzed as well.
Results: The majority of host-derived cells detected during remodeling of grafted fat were macrophages (>90 percent at the early stage; 60 percent at 12 weeks). Cell origins were analyzed at 12 weeks (i.e., when completely regenerated). At this point, mature adipocytes were largely derived from adipose-derived stem/stromal cells of grafts. Although vascular wall constituents were chiefly graft derived, vascular endothelial cells originated equally from graft and host bone marrow. Adipose-derived stem/stromal cells of regenerated fat were an admixture of grafted, host nonbone marrow, and host bone marrow cells.
Conclusions: The above findings underscore the importance of adipose stem/stromal cells in the grafted fat for adipocyte regeneration. Host bone marrow and local tissues contributed substantially to capillary networks and provided new adipose-derived stem/stromal cells. An appreciation of mechanisms that are operant in this setting stands to improve clinical outcomes of fat grafting and cell-based therapies. (*Plast. Reconstr. Surg.* 135: 00, 2015.)

Fat grafting has been increasingly recognized for its many clinical benefits, aside from tissue volumization. Tissues depleted of stem cells may be corrected by stem cell–containing tissue grafts. Grafted fat has the potential to revitalize diseased tissues (irradiated, dystrophic, or ischemic) and painful scars, thanks to contributing adipose-derived stem/stromal cells and tissue. However, such benefits are achieved only when each procedural step of fat grafting is properly executed. Hence, it seems that there remains much room for technical improvement.

From the Departments of Plastic Surgery and Cardiology, University of Tokyo Graduate School of Medicine.
Received for publication September 17, 2014; accepted December 29, 2014.

DOI: 10.1097/PRS.0000000000001292

Adipose tissue harbors a variety of cells, namely adipocytes, adipose-derived stem/stromal cells, and blood vascular cells (endothelial and mural).[1] Isolated or cultured adipose-derived stem/stromal cells hold great therapeutic promise through their capacity for multilineage differentiation, paracrine activity, and immunomodulation.[2–6]

Disclosure: *The authors have no financial interest to declare in relation to the content of this article.* AQ11

Supplemental digital content is available for this article. Direct URL citations appear in the text; simply type the URL address into any Web browser to access this content. Clickable links to the material are provided in the HTML text of this article on the *Journal*'s Web site (www.PRSJournal.com).

Consequently, clinical investigations of adipose-derived stem/stromal cells are aimed at a multiplicity of disorders[7,8]

Adipose-derived stem/stromal cells are heavily involved in the remodeling of adipose tissue after trauma of any nature, including ischemia-reperfusion injury, mechanical force, and grafting.[9–12] Through previous efforts, we have delineated a range of events that occur during remodeling, particularly the replacement of adipocytes by next-generation cells after fat is grafted.[12,13] Although cells such as adipose-derived stem/stromal cells, vascular endothelial cells, and macrophages are known participants in remodeling,[13,14] the origins of these constituents are unclear. It is acknowledged that adipose-derived stem/stromal cells facilitate mobilization and homing of bone marrow cells (such as endothelial progenitor cells)[15]; therefore, bone marrow–derived cells are likely components of regenerating fat after grafting.

In this study, we sought to investigate the origin of cells in the grafted fat after tissue remodeling/regeneration. We removed fat from green fluorescent protein (GFP) and wild-type mice for cross-transplantation (i.e., fat exchange) to investigate the origins of regenerating cells thereafter. In addition, mice with GFP–positive bone marrow served as recipients to research the fate of bone marrow–derived cells. We believe that a better appreciation of mechanisms inherent in the remodeling of grafted fat will improve related clinical outcomes and broaden the potential of stem cell therapies.

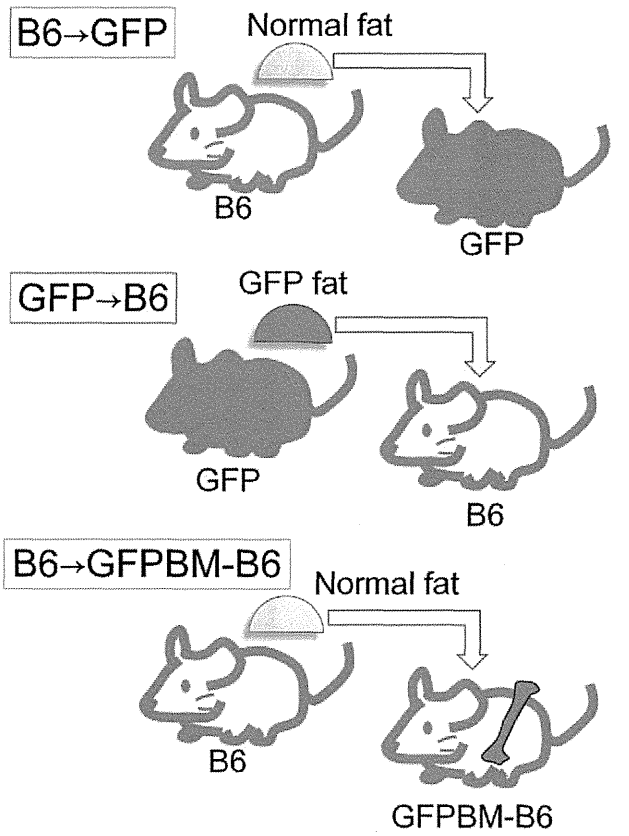

Fig. 1. Schematic diagram of experimental animal models. The inguinal fat pads of B6 mice were transplanted to GFP mice (B6→GFP), and those of GFP mice were transplanted to B6 mice (GFP→B6). GFP-positive bone marrow chimeric B6 mice (*GFPBM-B6*) were prepared by transplanting bone marrow of GFP mice into irradiated B6 mice. The fat pads of B6 mice were also transplanted to GFPBM-B6 mice (B6→GFPBM-B6).

MATERIALS AND METHODS

Animal Models

All animals were obtained from Japan SLC (Shizuoka, Japan; http://www.jslc.co.jp). Animal maintenance and experimental protocols were conducted under University of Tokyo guidelines.

Fat Exchange Models

Female 9-week-old mice of two strains, C57BL/6 (B6 mice) and C57BL/6-Tg (CAG-EGFP) (GFP mice), were anesthetized using intraperitoneal pentobarbital 50 mg/kg, and inguinal fat pads were harvested for cross-transplantation into subcutis of scalp, as described previously.[12] Fat pads of B6 mice were transplanted to GFP mice (B6→GFP), and fat pads of GFP mice were transplanted to B6 mice (GFP→B6) (Fig. 1). B6 and GFP mice are near-identical strains, so immune reactivity is negligible. At 1, 2, 4, and 12 weeks after transplantation, total body weight was recorded, and the grafted fat was harvested for further analyses, such as histology and flow cytometry. Thus, different animals were analyzed at each time point. Three animals for each group at each time point were analyzed for weight, histology, and stromal vascular fraction culture. Furthermore, nine animals for each group at 12 weeks were analyzed for fluorescence-activated cell sorting analysis of stromal vascular fraction. Bodily weight increases over time were similar in both grafted models. Graft weights were normalized (i.e., divided by body weights) to counter the effects of animal growth.

Bone Marrow Transplantation Model

GFP-positive bone marrow chimeric B6 mice (GFPBM-B6) were generated as reported previously.[16] Briefly, femoral and tibial bone marrow cells were harvested from 6-week-old female GFP mice by flushing with phosphate-buffered saline. After hemolysis with red blood cell lysis buffer (Sigma Aldrich, St. Louis, Mo.) and washing,