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Conflicts of interest: none declared.

Cre-loxP adenovirus-mediated foreign gene expression in skin-equivalent keratinocytes

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SIR, The introduction of genes into normal human cells is a fundamental component of human gene therapy.[1] Normal human keratinocytes (NHK) are appropriate targets for gene therapy[2] because of the accessibility of the epidermis and the possibility of excising genetically modified NHK sheets from recipient skin in cases of adverse reaction. From the gene therapy safety point of view, regulated expression of introduced genes is an important consideration.[3] The Cre-loxP system is one of the most reliable gene regulation systems used in gene targeting in virus vectors.[4] We used this system in a novel way to express genes at high levels in skin-equivalent NHK.

In preliminary experiments, NHK in monolayer cultures were infected with adenovirus (Ad) and seeded on to a collagen gel. Although enhanced green fluorescent protein (EGFP) expression was detected in the epidermal sheet, the expression was mainly in the cornified layer and only weak expression was seen in the basal and spinous layers (data not shown). Therefore, we modified the method to obtain more efficient gene expression (Fig. 1). The epidermal sheet was removed from the collagen gel

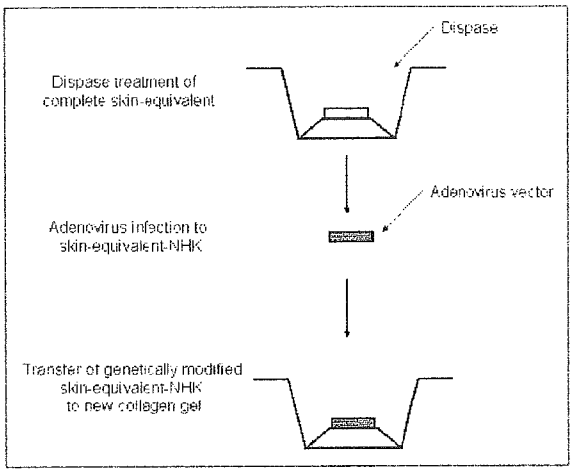

Fig 1. Experimental scheme of adenovirus (Ad) infection for shuttle-transduced skin equivalents. In the shuttle–skin equivalent method, completed skin-equivalent normal human keratinocyte (NHK) sheets were removed from the collagen gel by dispase treatment, infected with Ad, then seeded on a new collagen gel and incubated for 48 h.

once the skin equivalent had formed and was infected with the Ad; it was then placed on a new collagen gel, which was incubated for 2–3 days. In the genetically modified skin equivalent, strong EGFP expression was seen in the basal and suprabasal layers, using confocal laser microscopy (Fig. 2).

Several reports have suggested the possibility of making tissue- or tumour-specific Ad vectors[5,6] incorporating chemically inducible gene expression, such as the Tet-on/Tet-off system. These vectors would be especially applicable to Ad-mediated gene regulation in skin after implanting genetically modified skin-equivalent epidermis where topical application of the inducer chemical is possible. The Ad Cre-loxP system is an efficient method of inserting genes and regulating their expression in NHK. With this technique, we are able to induce high-level expression of different genes and to develop disease models in which the involvement of individual mutated genes in disease pathogenesis and the efficacy of gene therapies could be studied. In the past, it was possible to express toxic genes in Ad vectors; however, the virus titres tended to be low. Using the Cre-loxP system, high-titre viruses containing toxic genes could be obtained (e.g. Fas-ligand[7]). Our goal is to contribute to the quality of life of patients by developing a new method to introduce target genes with high expression levels into skin-equivalent keratinocytes. Further experiments are needed to optimize target gene expression in NHK.

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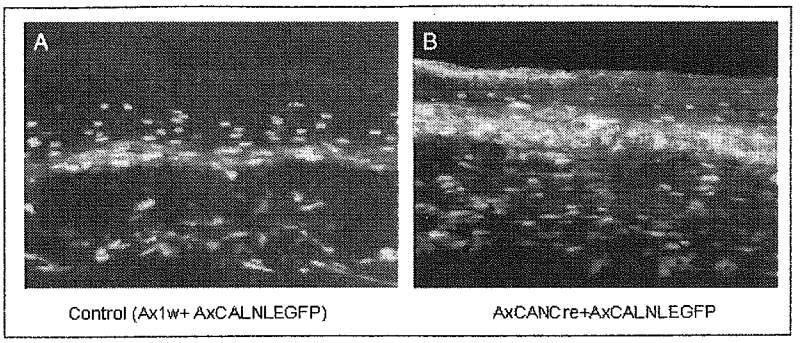

Fig 2. Confocal laser microscopic views of enhanced green fluorescent protein (EGFP) expression in shuttle–skin equivalent using the Cre-loxP adenovirus (Ad) system. (A) Control (Ax1w + AxCALNLEGFP); (B) AxCANCre + AxCALNLEGFP doubly infected shuttle-transduced skin equivalent. The cell nuclei were stained with propidium iodide. EGFP expression was detected at high levels in the basal and spinous cell layers when the Cre-loxP Ad system was used.

adenovirus Cre-loxP system and Dr Jun-ichi Miyazaki for the CAG promoter. This work was partly supported by Health Sciences Research Grants for Research on Specific Diseases from the Ministry of Health and Welfare and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Conflicts of interest: none declared.

Efficacy of issuing guidelines on acne management to general practitioners

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Sir, The objective of this study was to evaluate the practice of issuing guidelines on acne management to general practitioners (GPs) in our region. Acne can be managed in a primary care setting with appropriate training and experience.[1] However, specialist referral is required for initiation of isotretinoin. An evaluation of random referrals prior to introduction of guidelines showed that significant numbers of referrals were not in accordance with established guidelines. We used the consensus guidelines issued by the National Institute for Clinical Excellence (NICE) December 2001 as a gold standard for referral to specialist centres and based our guidelines on these criteria. NICE guidelines advocate referral to a specialist service if any of the following circumstances apply: (i) a severe variant of acne such as acne fulminans or Gram-negative folliculitis; (ii) severe or nodulocystic acne; (iii) severe social or psychological problems; (iv) risk or presence of scarring despite primary care therapies; (v) moderate acne that has failed to respond to treatment that included two courses of oral antibiotics, each lasting 3 months; and (vi) a suspected underlying endocrinological condition (such as polycystic ovary syndrome) that warrants assessment.

We also issued recommendations to optimize antibiotic prescribing which were based on established guidelines.[2,3] For example, tetracycline or oxytetracycline 500 mg twice daily were suggested as the antibiotics of choice, as they are effective and inexpensive. Doxycycline 50 mg once daily was advised if there was poor compliance with first-line agents. It was advised to reserve minocycline[2,4] and erythromycin[2] for those who fail to respond to first-line agents. Simultaneous use of different topical and oral antibiotics was discouraged.[5]

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Drug-Induced Hypersensitivity Syndrome due to Mexiletine Associated with Human Herpes Virus 6 and Cytomegalovirus Reactivation

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Abstract

A 66-year-old man developed a fever of 38°C and generalized pruritic rash about one month after mexiletine hydrochloride administration for ventricular tachycardia. The rash appeared as edematous erythema and papules with purpura on the lower extremities. Liver dysfunction, leukocytosis, and atypical lymphocytes were also present. Elevated antibody titer against human herpes virus 6 (HHV-6) was detected during the course of the disease (1:20 -> 1:640). The patient was diagnosed as having drug-induced hypersensitivity syndrome (DIHS) due to mexiletine. Discontinuation of the mexiletine administration and systemic corticosteroid treatment led to a temporary improvement, but tapering the corticosteroid dose twice led to recrudescence. Simultaneous with the recrudescence, elevated antibody titers against HHV-6 and cytomegalovirus were detected, as well as viral DNA in the blood, suggesting that these two viruses may have been involved in the recrudescence. The patient died of myocarditis, most likely related to cytomegalovirus. Our case indicates that, in addition to HHV-6, other herpes viruses such as cytomegalovirus can be reactivated in DIHS and may modify the clinical disease activity.

Key words: drug-induced hypersensitivity syndrome; human herpes virus 6 (HHV-6); cytomagalovirus

Introduction

Drug-induced hypersensitivity syndrome (DIHS) is a severe form of drug reaction characterized by high fever, facial edema, maculopapular rash and/or erythroderma, generalized lymphadenopathy, hypereosinophilia, atypical circulating lymphocytes, and abnormal liver function tests (1–6). Notably, the drugs that induce DIHS are relatively restricted; these include anticonvulsants, salazosulfapyridine, allopurinol, minocycline, and mexiletine. It has been reported that human herpes virus type 6 (HHV-6) participates in the pathogenesis of DIHS. However, reactivation of viruses other than HHV-6 has also been implicated, suggesting a complex interaction between drugs and viruses in the pathogenesis of this disease entity. Herein, we report a patient with DIHS due to mexiletine hydrochloride, in whom reactivation of both HHV-6 and cytomegalovirus (CMV) was detected.

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Case Report

A 66 year-old male with a history of acute myocardial infarction and ventricular tachycardia was started on mexiletine hydrochloride on December 15, 2001. Fever of 38–39°C and generalized pruritic rash developed from January 11,

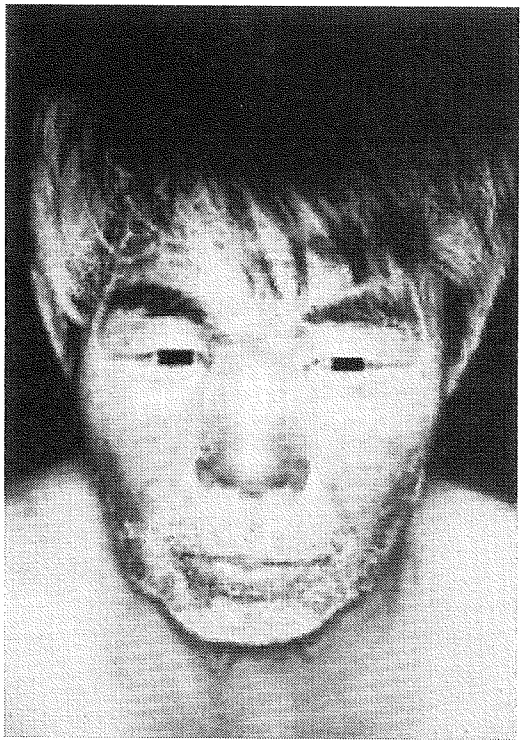

Fig. 1. Edematous erythema on the face of the patient. Similar edematous erythema was seen on almost the entire body.

2002. The symptoms worsened gradually, and the patient was admitted for evaluation. On admission, infiltrative erythematous papules and light red edematous erythema were found scattered over the body and all extremities (Fig. 1). In some areas, the lesions had coalesced to form irregular plaques. Miliary-sized to fingertip-sized purpuric lesions were seen within the erythematous areas in the lower thigh and dorsum of the foot. Erythema with an ill-defined border was seen on the face. There was no enanthema, lymphadenopathy, or hepatosplenomegaly. Laboratory findings were as follows: WBC count 9,800/mm^3 (44% neut, 13% eos, 2% mono, 37% lymph), RBC count 444 × 10^4/mm^3, Hb 14.4 g/dL, Plt 20.7 × 10^4/mm^3, TP 6.8 g/dL, AST 51 IU/L, ALT 48 IU/L, LDH 742 IU/L, γGTP 160 IU/L BUN 15.5 mg/dL, creatinine 1.1 mg/dL, CRP 1.1 mg/dL, IgE 322 U/mL. Histopathological findings disclosed a slightly acanthotic epidermis and perivascular cellular infiltration composed mostly of lymphocytes and eosinophils. Extravasation of erythrocytes was also seen.

Based on the drug history, characteristic generalized rash, and the laboratory findings, DIHS was suspected, and mexiletine hydrochloride was stopped. The patient was treated with oral prednisolone at 40 mg/day. Initially, the fever in the upper 37°C range continued with no resolution of the systemic rash. On day 10 after the onset of illness, atypical lymphocytes were observed. On day 17, the patient developed leukocytosis with a WBC count of 41,200/mm^3 and liver dysfunction with AST 245 IU/L and ALT 561 IU/L. On day 18 after the onset of illness, the fever and rash improved with an improvement in the liver function.

On day 21, the prednisolone was tapered to 30 mg. On day 24 after the onset of illness, however, the patient experienced a recurrence of fevers in the 38–39°C range and worsening of the rash in the extremities, but these findings improved by day 26. On day 33 after the onset of illness, prednisolone was tapered to 20 mg. On day 38, the patient again experienced a recurrence of systemic erythema and fevers in the 38–39°C range. On the following day, the patient had systemic deterioration with hypotension. Medical evaluation identified myocarditis, and, despite the treatment, he died on March 3, 2002, from heart failure. Autopsy was not performed.

HHV-6 titer measured by an immunofluorescent method was negative for both IgG and IgM on day 12 after onset of illness, but the anti-HHV-6 IgG titer rapidly rose to 1:20 on day 27, 1:160 on day 32, and 1:640 on day 38 (Table 1). HHV-6 DNA was also detected in the blood by PCR. During this period the patient also showed an increase in the CMV IgG titer as detected by ELISA (6.7 to 16), and the CMV DNA in the blood also became detectable on day 27 and continued to increase to day 38 (Table 1).

Discussion

DIHS is a severe form of drug eruption in which HHV-6 reactivation is considered to be involved; it is characterized by fever and lymphadenopathy with organ injury such as hepatic and renal dysfunction (1–6). In our

Table 1. Laboratory data

	Days after onset of skin eruption									
	0	6	10	12	17	20	24	27	32	38
WBC (/mm3)		9,800	18,200	28,700	41,200	23,700	11,300	7,100	8,400	7,100
Aty-lym (%)		0	1	0	0	0	1	0	0	0
Eo (%)		13	11	22	33	19	23	4	2	8
AST (IU/l)		51	142	203	245	134	198	183	68	67
ALT (IU/l)		48	231	314	561	429	549	505	304	196
BUN (IU/l)		15	37	39	26	25	29	29	31	23
Cre (IU/l)		1.1	2.3	1.4	1.0	0.9	1.4	1.5	1.3	1.3
HHV-6										
IgG				<20			<20	20	160	640
IgM				<10			<10	<10	—	20
DNA (copy/μgDNA)				—			1,300	25,000	500	190
CMV										
IgG				6.7			12	12	13	16
IgM				<1.2			<1.2	<1.2	<1.2	<1.2
DNA (copy/μgDNA)				—			0	9,000	26,000	31,000

WBC: white blood cell, Aty-lym: atypical lymphocyte, Eo: Eosinophil, AST: L-aspartate: 2-oxoglutarate aminotransferase, ALT: L-alanine: 2-oxoglutarate aminotransfarase, BUN: blood urea nitrogen, Cre: creatinine, HHV: human herpesvirus, CMV: cytomegalovirus

case, one month after the initiation of mexiletine hydrochloride, the patient developed fever associated with generalized edematous erythema, leukocytosis, and liver function abnormalities, leading to the diagnosis of DIHS. Elevations of HHV-6 antibody titer and HHV-6 viral DNA were detected during the clinical course.

Recent reports have noted that patients with DIHS have elevations of antibody titers against viruses other than HHV-6. Arakawa et al.(7) reported a patient in whom allopurinol-induced DIHS was associated with HHV-6 reactivation and an elevation of CMV antibody titer, while Aihara et al.(8) reported a patient with DIHS with a reactivation only of CMV. In addition, cases of DIHS associated with elevated serum antibody titers against HHV-7, EB virus, parvovirus B19, rubella virus, and VZV, have also been reported (1, 9, 10).

HHV-6 IgG and HHV-6 DNA were detected in our patient, as well as elevated CMV IgG and CMV DNA in the blood. Approximately 80–90% of the adult Japanese population has been infected with CMV. Most have asymptomatic infections, but CMV reactivation in immunocompromised hosts can result in interstitial pneumonia, myocarditis, enteritis, hepatitis, cholangitis, retinitis, and encephalitis (11–13). Our patient is unique in that both HHV-6 DNA and CMV DNA were detected during the course of the disease.

In DIHS, the elevation of virus antibody titer occurs 2–3 weeks after the onset of illness and often coincides with recrudescence of symptoms. Based on this observation, Tohyama et al. suggested that the pathogenesis of DIHS involves the reactivation of HHV-6 following the drug allergy (2). Our patient experienced the first recrudescence on day 24 of illness and the second recrudescence on day 38; these dates coincide with the peaks of HHV-6 DNA and CMV-DNA levels seen on day 27 and day 38. With respect to the serum antibody titer, HHV-6 IgG antibody titer began to rise on day 27, while the

CMV IgG titer began to rise on day 24. The rise in CMV antibody titer was less than 4-fold (ELISA 6.7 -> 16); however, it was associated with increased viral DNA copy number in the blood (Table 1). As seen in our patient, the findings of a coincidental increase of the virus and the recrudescence of clinical manifestations strongly suggest the involvement of viral reactivation in the pathogenesis of DIHS. Although HHV-6 IgG antibody titer was initially negative, a finding which might suggest primary infection, the pattern of changes in the IgM antibody titer was not consistent.

Our patient developed myocarditis during the second recrudescence. CMV is known to cause myocarditis (11), and it is conceivable that the DIHS-associated CMV reactivation may have been the direct cause of death, although no autopsy could not be performed to confirm this. DIHS is a severe type of drug eruption in which the involvement of HHV-6 is strongly suspected, but as in our patient, the involvement of other herpes viruses such as CMV and HHV-7 (1) has also been reported. Because CMV can affect systemic organ function and effective treatments such as ganciclovir and CMV high titer gamma globulin preparations are available (14), patients with DIHS, which is usually treated with systemic corticosteroid administration (15), must be carefully evaluated for viruses other than HHV-6.

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Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing

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Summary

Members of the epidermal growth factor (EGF) family are the most important growth factors involved in epithelialization during cutaneous wound healing. Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family, is thought to play an important role in skin wound healing. To investigate the in vivo function of HB-EGF in skin wound healing, we generated keratinocyte-specific HB-EGF-deficient mice using Cre/loxP technology in combination with the keratin 5 promoter. Studies of wound healing revealed that wound closure was markedly impaired in keratinocyte-specific HB-EGF-deficient mice. HB-EGF mRNA was upregulated at the migrating epidermal edge, although cell growth was not altered. Of the members of the EGF family, HB-EGF mRNA expression was induced the most rapidly and dramatically as a result of scraping in vitro. Combined, these findings clearly demonstrate, for the first time, that HB-EGF is the predominant growth factor involved in epithelialization in skin wound healing in vivo and that it functions by accelerating keratinocyte migration, rather than proliferation.

Key words: Conditional knockout, HB-EGF, Keratinocytes, Migration, Wound healing

Introduction

Cutaneous wound healing requires precise coordination of epithelialization, dermal repair and angiogenesis (Singer and Clark, 1999). Epithelialization is ultimately dependent on the migratory, proliferative and differentiation abilities of keratinocytes. The growth and differentiation of keratinocytes are regulated mainly by a variety of growth factors (Hashimoto, 2000), of which the members of the epidermal growth factor (EGF) family are the most important for skin wound healing.

The EGF family consists of EGF, transforming growth factor (TGF)-α, heparin binding EGF-like growth factor (HB-EGF), amphiregulin (AR), epiregulin (EPR), betacellulin (BTC), epigen and neuregulin (NRG)-1, NRG-2, NRG-3 and NRG-4 (Falls, 2003; Harari et al., 1999). The EGF receptor (EGFR) family consists of EGFR (also called ErbB1), ErbB2, ErbB3 and ErbB4 (Jorissen et al., 2003). The mammalian ligands that bind EGFR include EGF, HB-EGF, TGF-α, AR, BTC, EPR and epigen. Recent studies using gene targeting or transgenic models have revealed that EGFR is essential for epithelial development in the skin, lung and gastrointestinal tract, whereas ErbB2, ErbB3, ErbB4 and neuregulins are essential for the development of cardiac muscle and the central nervous system (Erickson et al., 1997; Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Miettinen et al., 1995; Murillas et al., 1995; Riethmacher et al., 1997; Sibilia and Wagner, 1995).

Previous reports have shown that TGF-α, AR, HB-EGF and EPR are autocrine growth factors in normal human epidermal keratinocytes (NHEK) (Coffey et al., 1987; Cook et al., 1991; Hashimoto et al., 1994; Shirakata et al., 2000). It has been reported that keratinocyte migration and proliferation are predominantly mediated by autocrine EGFR activation (Stoll et al., 1997). However, the importance of the role that the EGF family plays in skin wound healing has not been confirmed in vivo using knockout mice. Previously, Marikovsky et al. (Marikovsky et al., 1993) reported that HB-EGF is a major component of the mix of growth factors found in wound fluid. Therefore, we speculated that HB-EGF was an important member of the EGF family in cutaneous wound healing. To test this hypothesis, we generated keratinocyte-specific HB-EGF knockout mice, and clearly demonstrated that HB-EGF is an important growth factor for epithelialization in skin wound healing in vivo.

Materials and Methods

Cell culture

Normal human epidermal keratinocytes (NHEK) were prepared and cultured under serum-free conditions, as previously described (Shirakata et al., 2000; Shirakata et al., 2003). Third- or fourth-passage cells were used in this study.

Table 1. Primer sequences for PCR

Wild-type HB-EGF – upper	5′-CATGATGCTCCAGTGAGTAGGCTCTGATTAC
Wild-type HB-EGF – lower	5′-AGGGCAAGATCATGTGTCCTGCCTCAAGCC
lox HB-EGF – upper	5′-ATGGGATCGGCCATTGAACA
lox HB-EGF – lower	5′-GAAGAACTCGTCAAGAAGGC
cre-recombinase – upper	5′-TTACCGGTCGATGCAACGAGTGATG
cre-recombinase – lower	5′-TTCCATGAGTGAACGAACCTGGTCG
lox-out HB-EGF – upper	5′-CGGACAGTGCCTTAGTGGAACCTC
lox-out HB-EGF – lower	5′-GCTTCTTCTTAGGAGGGATCTTGGC

Table 2. Primer sequences for RT-PCR

hHB-EGF – upper	5′-CCACACCAAACAAGGAGGAG
hHB-EGF – lower	5′-ATGAGAAGCCCCACGATGAC
hEPR – upper	5′-TCGCCCGCTCCCATCGCCG
hEPR – lower	5′-GGTTCCACATATTATTTCTG
hTGF-α – upper	5′-GAGTGCAGACCCGCCCGTGGC
hTGF-α – lower	5′-CCAGGAGGTCCGCATGCTCAC
hAR – upper	5′-CCAAAACAAGACGGAAAGTGA
hAR – lower	5′-AGGATCACAGCAGACATAAAG
hGAPDH – upper	5′-ACCACAGTCCATGCCATCAC
hGAPDH – lower	5′-TCCACCACCCTGTTGCTGTA
mHB-EGF – upper	5′-GGAATTCTGGAGCGGCTTCGGAGAG
mHB-EGF – lower	5′-CAAGCTTTGCAAGAGGGAGTACGGAACT
mEPR – upper	5′-GGAATTCTGACGCTGCTTTGTCTAGGTT
mEPR – lower	5′-CAAGCTTTATGCATCCAGCGGTTATGAT
mTGF-α – upper	5′-GGAATTCCTAGCGCTGGGTATCCTGTTA
mTGF-α – lower	5′-CAAGCTTACCACCACCAGGGCAGTGATG
mAR – upper	5′-CAAGCTTACCACCACCAGGGCAGTGATG
mAR – lower	5′-CAAGCTTACCACCACCAGGGCAGTGATG

h, human; m, mouse; EPR, epiregulin; AR, amphiregulin.

Generation of HB-EGF knockout mice using a gene targeting Cre-loxP strategy and PCR

The targeting construct has been described previously (Iwamoto et al., 2003). Homozygous $HB^{lox/lox}$ mice were bred with K5 promoter-driven Cre-recombinase transgenic mice to generate K5-Cre-$HB^{lox/+}$ mice (Takeda et al., 2000). Subsequently, K5-Cre-$HB^{lox/+}$ mice were bred with $HB^{lox/lox}$ mice to generate $HB^{lox/lox}$; K5-Cre ($HB^{-/-}$) mice. The genotype of each mouse was confirmed by PCR. Primers are shown in Table 1.

RT-PCR analysis

Keratinocytes were cultured in MCDB153 complete medium on type I collagen-coated dishes until they reached confluency. Keratinocytes were treated by tip scraping and total RNA was harvested at several time points. mRNA expression of HB-EGF, TGF-α, AR, EPR and GAPDH was analyzed by RT-PCR. The absence of HB-EGF mRNA in keratinocytes from $HB^{-/-}$ mice was confirmed by RT-PCR. Primers are shown in Table 2. The RT-PCR was performed using RT-PCR High Plus (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer's instructions. cDNA was reverse-transcribed from total RNA for 30 minutes at 60°C and heated to 94°C for 2 minutes. Amplification was performed using a DNA thermal cycler (Astec, Fukuoka, Japan) for 25 cycles. A cycle profile consisted of 1 minute at 94°C for denaturation, 1.5 minutes at 60°C for annealing and primer extension.

Wound healing studies

Wound healing experiments were performed in $HB^{-/-}$ and $HB^{lox/lox}$ mice. Under sodium pentobarbital anesthesia, two full-thickness wounds were created on the skin of the backs of each of nine 9- to 10-week-old female mice using 6-mm skin biopsy punches. Each wound diameter was determined as the average of longitudinal and lateral diameter. Wound closure was monitored, and skin sections were harvested at 3, 5, 7, 9 and 11 days after wounding. For BrdU labeling, mice received intraperitoneal injections of BrdU (250 μg/g; Sigma, Tokyo, Japan) 2 hours prior to sacrifice.

Histological analysis

Mouse tissues were fixed in 4% paraformaldehyde or formaldehyde, dehydrated and embedded in paraffin. Four-μm sections were stained with Hematoxylin and Eosin. For β-gal staining, after fixation with 0.2% glutaraldehyde and 1% formalin, the tissues were stained with 5-bromo-4-chloro-3-indol β-D-galactoside (X-gal). Skin sections were stained with rabbit anti-keratin IgG or anti-BrdU IgG, and immunopositive reactions were visualized using a streptavidin-biotin-peroxidase staining kit (Nichirei Co. Inc., Tokyo, Japan) according to the manufacturer's instructions. Morphometric analysis was performed using MacSCOPE Ver2.61 software. Statistical analysis was performed using Student's *t*-test.

Results

HB-EGF mRNA induction after in vitro scrape wound

To investigate the distinct role of HB-EGF in skin wound healing of the growth factors produced by NHEK, we first examined the induction of EGFR-ligand mRNA in NHEK in an in vitro wound-healing model. Confluent cultures of NHEK were scraped with a yellow pipette tip; total RNA was harvested at several time points, and the expression of growth-factor mRNAs was analyzed by RT-PCR. HB-EGF mRNA was rapidly induced after scraping, reaching a peak of 2.6-fold induction at 1 hour, whereas AR, TGF-α and EPR mRNAs were only slightly induced, with a maximum 1.5-fold increase (Fig. 1A). This indicates that HB-EGF is the most inducible gene of the EGFR ligands in NHEK. In normal mouse keratinocytes, HB-EGF was again the EGF family member that was induced predominantly after scraping, with a maximum 4.0-fold increase at 2 hours (Fig. 1B). EPR was also induced to a lesser degree, with a maximum 2.5-fold induction. TGF-α and AR were not induced after scraping. These results indicated that HB-EGF may play an important role in skin wound healing, and led us to investigate the in vivo function of HB-EGF.

Generation of keratinocyte-specific HB-EGF-deficient mice

Since germline targeting of the HB-EGF gene resulted in severe lethality (Iwamoto et al., 2003), we generated keratinocyte-specific HB-EGF-deficient mice ($HB^{lox/lox}$; K5-Cre, which we refer to as $HB^{-/-}$) using Cre/loxP technology in combination with the keratin 5 promoter (Takeda et al., 2000). $HB^{-/-}$ mice were identified by PCR analysis (Fig. 2A-C). The keratinocyte-specific absence of HB-EGF mRNA in $HB^{-/-}$

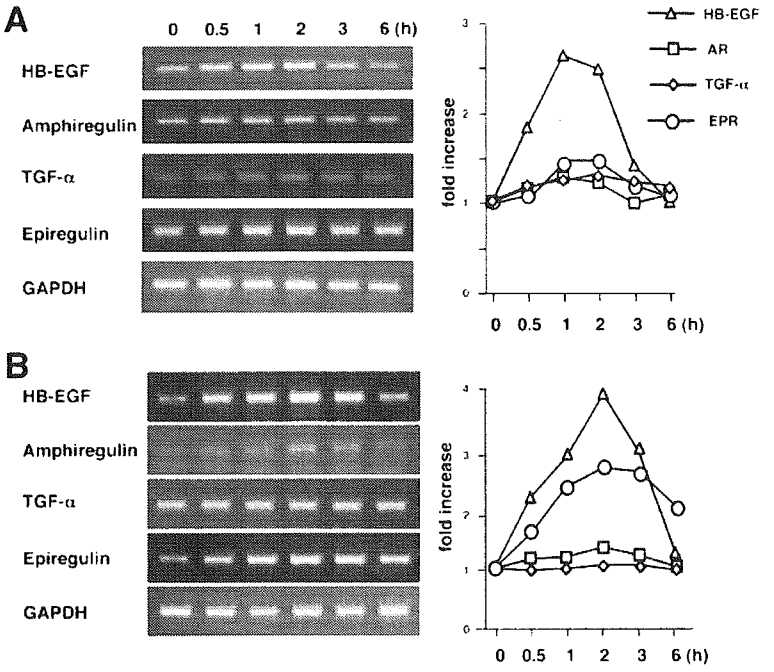

Fig. 1. Induction of expression of EGFR ligand mRNA, by scraping, in human and mouse keratinocytes. Confluent NHEK (A) and normal mouse epidermal keratinocytes (B) were scraped with a pipette tip; total RNA was harvested at several time points and mRNA expression was analyzed by RT-PCR. Right panels show densitometric analysis. In both cell types HB-EGF mRNA was rapidly and dramatically induced after scraping, whereas TGF-α, AR, and EPR mRNA were slightly induced in NHEK and the normal keratinocytes, although in the latter cells EPR was also increased.

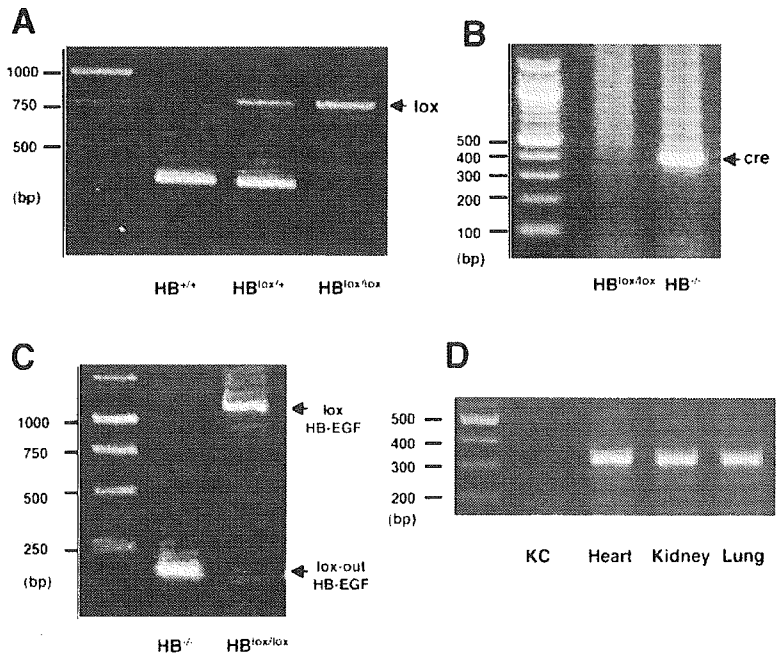

Fig. 2. Genotype of the keratinocyte-specific HB-EGF-deficient mice. (A-C) Keratinocyte-specific HB-EGF-deficient mice were confirmed by PCR as lox homozygous (A), Cre-recombinase positive (B) and lox-out (C). (D) Keratinocyte-specific disruption of HB-EGF mRNA in HB$^{-/-}$ mice was confirmed by RT-PCR. KC, keratinocytes.

mice was confirmed by RT-PCR (Fig. 2D). No apparent abnormalities were observed in the HB$^{-/-}$ mice.

Impaired wound healing in keratinocyte-specific HB-EGF-deficient mice

To examine the role of HB-EGF in skin wound healing in vivo, we performed a wound-healing assay using HB$^{lox/lox}$ and HB$^{-/-}$ mice. Two 6-mm punch skin biopsies were made in the back of each mouse and the wound diameter was measured at various times after wounding as a measure of healing. There was no difference in wound diameter up to day 3 post-wounding; however, wound healing was noticeably retarded from day 5 to 11 in the HB$^{-/-}$ mice. Wound closure was delayed significantly in HB$^{-/-}$ mice compared with HB$^{lox/lox}$ mice on day 8 (Fig. 3A). The wound diameter was reduced to 34% in HB$^{lox/lox}$ mice on day 8, whereas it was still 58% in the HB$^{-/-}$ mice (Fig. 3B). These results indicate that HB-EGF expression by keratinocytes is important for skin wound healing in vivo.

Cell proliferation was not impaired at the wound site in HB–/– mice

Since EGFR ligands promote NHEK proliferation and migration (Hashimoto, 2000), we investigated whether proliferation or migration was predominantly impaired in HB$^{-/-}$ mice. We measured the cell numbers in the leading edge of the biopsy wound and in the peripheral skin (1.2 mm from the wound margin) in HB$^{lox/lox}$ and HB$^{-/-}$ mice (Fig. 4A). After 48 hours, the total cells numbers were 90±13 and 65±19 in HB$^{lox/lox}$ and HB$^{-/-}$ mice, respectively, and after 72 hours 170±20 and 140±41 in HB$^{lox/lox}$ and HB$^{-/-}$, respectively (Fig. 4B). Since these small observed differences in cell numbers were not statistically significant, we investigated keratinocyte proliferation in HB$^{lox/lox}$ and HB$^{-/-}$ mice using a BrdU incorporation assay. Two hours before sacrifice, the mice received intraperitoneal injections of BrdU (250 μg/g). Skin samples were harvested, sectioned and stained with anti-BrdU antibody. Three days after wounding, there were no differences in the number or distribution of BrdU-positive cells between the HB$^{lox/lox}$ and HB$^{-/-}$ mice (Fig. 4C). These results suggest that delayed wound healing in HB$^{-/-}$ mice is not due to impaired cell proliferation in the epidermis.

Cell migration was impaired at the wound site in HB$^{-/-}$ mice

Since no impairment of proliferation was found, we next investigated whether migration was impaired in HB$^{-/-}$ mice. To quantify the migration

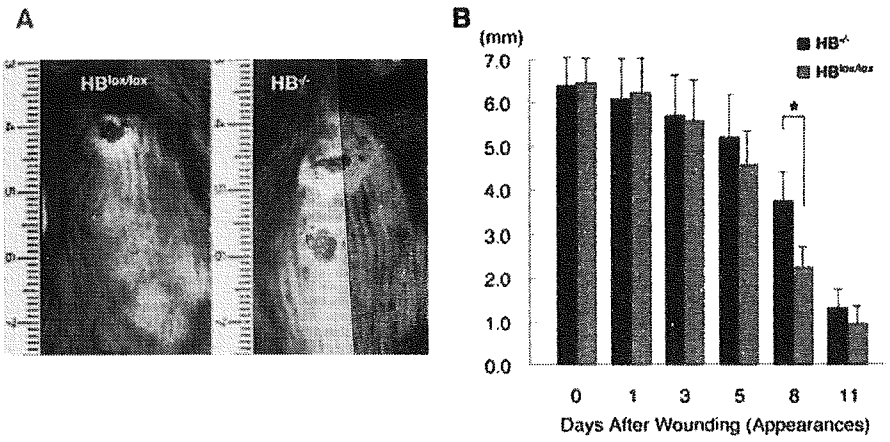

Fig. 3. Impaired wound healing in $HB^{-/-}$ mice. Two 6-mm punch biopsies were made in the skin of the backs of $HB^{lox/lox}$ and $HB^{-/-}$ mice, and wound diameter was monitored. (A) Macroscopic view of wound healing assay in $HB^{lox/lox}$ and $HB^{-/-}$ mice at day 8. (B) Measurements of wound diameter during healing. $^{*}P<0.05$.

of keratinocytes in wound healing, we measured the length of the leading edge in each wound of $HB^{lox/lox}$ and $HB^{-/-}$ mice in the wound-healing assay. Sections of skin from the wound area were stained with anti-keratin IgG (Fig. 5A). On day 7 post-wounding, the epidermis had migrated toward the center of the wound in $HB^{lox/lox}$ mice, whereas keratinocytes remained near the wound margin and the epidermis had not spread in $HB^{-/-}$ mice, suggesting that keratinocyte migration was impaired in $HB^{-/-}$ mice (Fig. 5B). We then prepared skin sections from all the samples from the wound-healing assay and calculated the ratio of leading edge to initial wound length, using computer-assisted morphometric analysis. On day 3 post-wounding, there was no difference in the leading edge ratio between $HB^{lox/lox}$ and $HB^{-/-}$ mice. However, the leading edge ratio was decreased markedly in $HB^{-/-}$ mice after day 3. The ratio was 30.7% in $HB^{-/-}$ and 44.5% in $HB^{lox/lox}$ on day 5, and 38% in $HB^{-/-}$ and 65% in $HB^{lox/lox}$ mice on day 7 (Fig. 5C). The difference on day 7 was statistically significant. These results suggest that endogenous HB-EGF is an important growth factor for the migration of epidermis in skin wound healing.

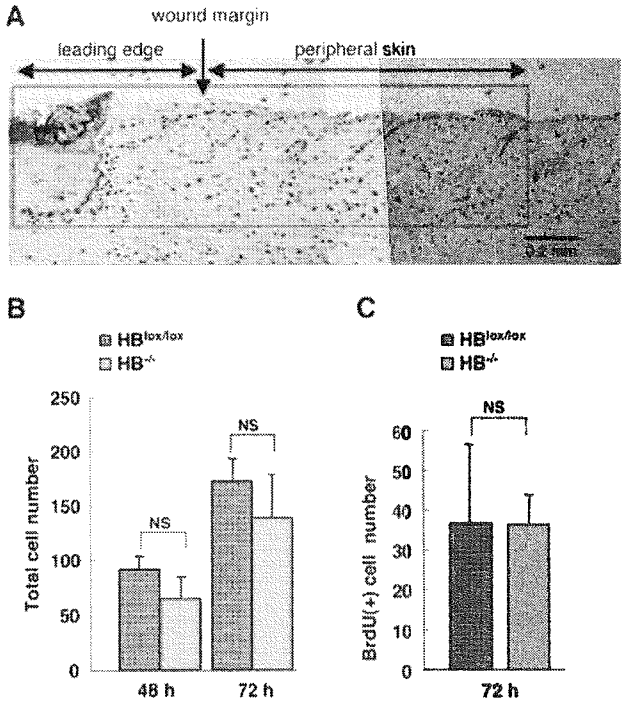

Fig. 4. BrdU-positive cell distribution at the leading edge and in the peripheral skin in $HB^{lox/lox}$ and $HB^{-/-}$ mice. (A) Skin sections were stained using anti-BrdU antibody, and cell numbers in the leading edge and in the peripheral skin were counted as indicated. (B) The total cell numbers in the peripheral skin (1.2 mm from the wound margin) and in the leading edge. (C) BrdU-positive cell number in the peripheral skin and in the leading edge. There were no differences in BrdU-positive cell numbers between $HB^{lox/lox}$ and $HB^{-/-}$ mice.

Expression of HB-EGF at wound sites

It has been reported that HB-EGF was upregulated in burn wound healing and that topical application of HB-EGF accelerated re-epithelialization of partial-thickness burns (Cribbs et al., 2002; Cribbs et al., 1998; McCarthy et al., 1996). It has been also reported that addition of HB-EGF into c-jun null keratinocyte growth medium can rescue the migration defect and induce phosphorylation of EGF receptor (Li et al., 2003). Since HB-EGF may play an important role in skin wound healing, we investigated the HB-EGF expression and keratinocyte proliferation pattern in skin wound healing using $HB^{lox/+}$:K5-Cre ($HB^{+/-}$) mice. With the targeting vector containing the *lacZ* gene as a reporter for the expression of HB-EGF, it is possible to ascertain the expression of HB-EGF by staining for β-gal in $HB^{+/-}$ mice. HB-EGF was expressed at the leading edge of the epithelium at day 2 post-wounding, and was predominantly expressed at the tip of the leading edge until day 7 (Fig. 6A). Unlike the HB-EGF expression pattern, BrdU-positive (BrdU+) cells were detected mainly within the peripheral skin on days 2 and 3. On days 5 and 7, BrdU+ cells were found toward the leading edge, although they were preferentially located near the wound margin. To quantify the distribution of HB-EGF-expressing cells and proliferating cells, we counted the β-gal-positive (β-gal+) cells and BrdU+ cells in 0.2 mm ranges in the leading edge and in the peripheral skin in $HB^{+/-}$ mice. On day 2 the peak of the β-gal+ cells was between 0 +0.2 mm into the leading edge, whereas the peak of the BrdU+ cells was −0.2 to −0.4 mm into the peripheral skin. On day 3, the peak of the β-gal+ cells was between +0.4 and +0.6 mm, whereas the peak of the BrdU+ cells was between 0

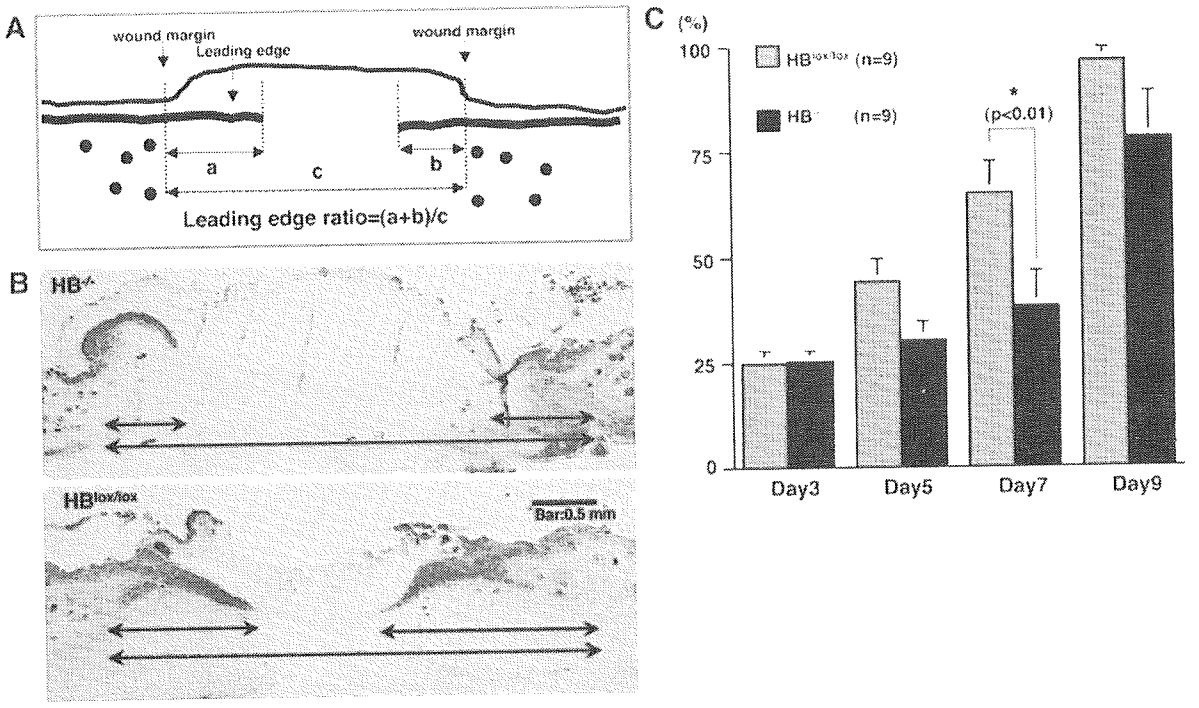

Fig. 5. Impaired keratinocyte migration in $HB^{-/-}$ mice. (A) Serial sections were prepared, and the epidermis was stained with anti-keratin antibody. Computer-assisted morphometric analysis was performed and the ratio of the leading edge to initial wound length was calculated. (B) Immunohistochemical staining of wound healing assay at day 7. Scale bar: 500 μm. (C) Measurements of leading edge ratio in $HB^{lox/lox}$ and $HB^{-/-}$ mice. The leading edge ratio was significantly decreased in $HB^{-/-}$ mice (n=9) at day 7. *P<0.01.

and +0.2 mm. From day 5 to day 7, BrdU+ cells were located at the leading edge, although they always appeared just behind the β-gal+ cells (Fig. 6B,C).

We also examined β-gal+ and BrdU+ cells in the wound-healing assay using $HB^{-/-}$ mice. As in $HB^{+/-}$ mice, in $HB^{-/-}$ mice, on day 2 post-wounding, β-gal+ cells were localized mostly 0 to +0.2 mm into the leading edge, whereas BrdU+ cells were detected mostly between –0.2 and –0.4 mm into the peripheral skin. On day 3, the peak of the β-gal+ cells were localized at 0 to +0.2 mm, whereas the peak of the BrdU+ cells were between 0 and –0.2 mm. On days 5 and 7, the peak aggregation of BrdU+ cells in $HB^{-/-}$ mice was at almost the same location as the β-gal+ cells, at the leading edge (Fig. 6D,E). The overlapping distribution patterns of BrdU+ and β-gal+ cells in these mice may be due to the impaired cell migration (Fig. 5B,C). However, the total counts of BrdU+ cells were similar in $HB^{lox/lox}$ and $HB^{-/-}$ mice (Fig. 4B,C).

Discussion

Wound healing is a complex process involving a number of coordinated events including inflammation, cell migration, cell proliferation, matrix production and angiogenesis (Singer and Clark, 1999). A complex array of cells, growth factors, cytokines and matrix components are involved in wound healing, and a number of transgenic and knockout mouse models have revealed the contribution of several molecules to wound healing (Scheid et al., 2000). Impaired wound healing was observed in mice transgenic for several molecules, such as BMP-6, follistatin, truncated FGF receptor and thorombospondin-1, as well as in mice with activin, β1 integrin, and TGF-β1 knockouts, among others (Grose and Werner, 2003; Scheid et al., 2000; Werner and Grose, 2003). EGF family members such as EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, epiregulin and their receptor EGFR mainly regulate migration, proliferation and differentiation of many cell types involved in wound healing. EGFR knockout mice showed striking abnormalities, such as wavy hair and thin skin (Miettinen et al., 1995; Sibilia and Wagner, 1995). In contrast to this striking phenotype in EGFR knockout mice, EGF-disrupted mice showed no phenotypic abnormalities (Luetteke et al., 1999). No differences in wound healing were found in TGF-α knockout mice with either excisional dorsal wounding or ear-punch wounding (Luetteke et al., 1993; Mann et al., 1993). These unexpectedly minor differences in phenotypes in wound healing in EGFR-ligand knockout mice are probably due to the known functional redundancy among the EGF family members, including HB-EGF.

HB-EGF is produced and secreted by human keratinocytes and acts as an autocrine growth factor (Hashimoto et al., 1994). HB-EGF mRNA was rapidly and dramatically induced after scrape-wounding, although slight increases in TGF-α, amphiregulin and epiregulin mRNAs were observed. Furthermore, blocking HB-EGF by addition of neutralizing antibody to the medium inhibited keratinocyte migration (Tokumaru et al., 2000). In contrast, the addition of recombinant HB-EGF to the medium accelerates keratinocyte migration (Tokumaru et al., 2000). These results indicate that

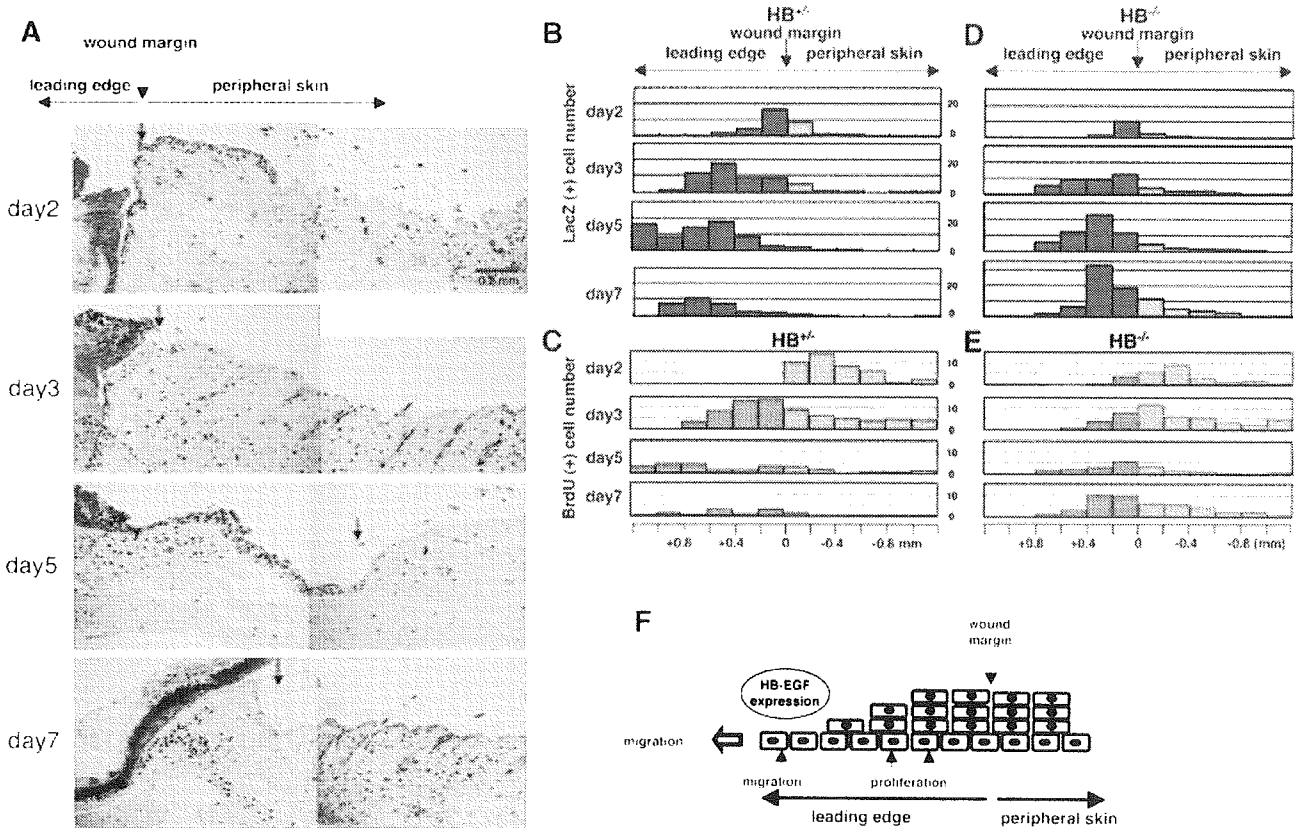

Fig. 6. HB-EGF expression in skin wound healing. The targeting vector contained the *lacZ* gene as a reporter for the expression of HB-EGF. When HB-EGF cDNA is deleted by Cre-recombinase, HB-EGF expression can be identified by X-gal staining in $HB^{+/-}$ mice. (A) Double staining for X-gal and BrdU at the wound healing stage. Scale bar: 0.2 mm. Arrow, wound margin. (B-E) Distribution of β-gal-positive [lacZ(+)] cells (B) and BrdU-positive cells (C) from day 2 to 7 in $HB^{+/-}$ mice. HB-EGF is expressed predominantly at the tip of the leading edge, whereas BrdU-positive cells were distributed mainly at wound margin. Distribution of β-gal-positive cells (D) and BrdU-positive cells (E) in $HB^{-/-}$ mice. There was no significant difference in the number of BrdU-positive cells between $HB^{+/-}$ and $HB^{-/-}$ mice at any stage of wound healing. (F) A proposed schematic illustration of skin wound healing. After injury, keratinocytes at the wound margin begin to migrate and express HB-EGF without proliferation. Next, focal release of HB-EGF may signal further migration and up-regulate HB-EGF expression in an autocrine manner. Values in B-E are number of cells per indicated area.

HB-EGF plays an important role in skin wound healing, and led us to investigate the in vivo function of HB-EGF. Since germline targeting of the HB-EGF gene resulted in embryonic lethality (Iwamoto et al., 2003), we generated keratinocyte-specific HB-EGF-deficient mice ($HB^{-/-}$) using Cre/loxP technology in combination with the keratin 5 promoter (Takeda et al., 2000). There was no difference in wound closure between $HB^{-/-}$ and $HB^{lox/lox}$ mice on day 3. However, wound closure was markedly retarded in $HB^{-/-}$ mice compared to $HB^{lox/lox}$ mice. We clearly demonstrated for the first time that endogenous HB-EGF is the most important growth factor in the epithelialization of skin wound healing in vivo, using keratinocyte-specific HB-EGF-deficient mice.

EGF family members are well known to promote keratinocyte growth in vitro (Hashimoto, 2000). It has been reported that TGF-α, amphiregulin, HB-EGF and epiregulin are autocrine growth factors in normal human keratinocytes (Coffey et al., 1987; Cook et al., 1991; Hashimoto et al., 1994; Shirakata et al., 2000). In vitro observation suggests that these EGF family members play important roles in development, epidermal morphogenesis, skin homeostasis and wound healing. In this study, we investigated HB-EGF function in cell migration and proliferation. HB-EGF stimulates keratinocyte migration in vitro and in vivo. However, there was little difference in proliferation between $HB^{lox/lox}$ and $HB^{-/-}$ mice. HB-EGF promoter activity was up-regulated at the migrating epidermal edge, whereas the distribution of proliferating cells (BrdU-positive) was not identical to that of HB-EGF mRNA-positive cells. Interestingly, the wound margin of normal epidermis expressed HB-EGF mRNA and was positive for BrdU, although HB-EGF promoter activity could not be detected in normal skin far from the wound margin. Therefore, normal skin does not require much HB-EGF, but after injury HB-EGF is induced and plays a crucial role in wound healing by up-regulating keratinocyte migration but not proliferation.

Combined, the evidence suggests that the synthesis of HB-EGF at the leading epithelial edge stimulates cells, via an autocrine loop, to migrate towards the center of the wound rather than to proliferate. Interestingly, there were few β-gal-positive cells and little HB-EGF expression in normal skin far

from the wound margin. Therefore, HB-EGF expression induced by wounding might itself stimulate further expression of HB-EGF at the leading edge via an autocrine loop. Fig. 6F shows a schematic illustration of our proposed skin wound healing mechanism. After injury, keratinocytes at the wound margin begin to express HB-EGF and migrate toward the wound site without proliferating. Next, the focal release of HB-EGF may trigger the migration of additional cells, rather than cell proliferation at the leading edge. Therefore, we conclude that HB-EGF is rapidly induced after injury and plays an important role in wound healing by up-regulating keratinocyte migration.

Nuclear transcription factors play important roles in almost all biological events resulting from growth factor signaling, and several nuclear transcription factors are thought to be involved in skin wound healing. Several mouse models with gene-targeted disruption of nuclear transcriptional factors have been analyzed for skin wound healing. Sano et al. (Sano et al., 1999) reported severe retardation of wound healing in keratinocyte-specific STAT3 knockout mice. D'Souza et al. (D'Souza et al., 2002) reported impaired skin wound healing in E2F-1 knockout mice. Recently, the development of keratinocyte-specific c-jun knockout mice was reported (Li et al., 2003; Zenz et al., 2003). These mice showed retarded wound healing, and the activation of EGFR was greatly decreased (Li et al., 2003). Since EGF itself is not produced by keratinocytes, the autocrine loop consisting of HB-EGF, EGFR and c-jun might be one of the major regulatory signal transduction mechanisms in skin wound healing.

In conclusion, HB-EGF is an important growth factor in epithelialization during skin wound healing in vivo, and acts mainly by stimulating migration, rather than proliferation, of keratinocytes.

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Novel Autologous Cell Therapy in Ischemic Limb Disease Through Growth Factor Secretion by Cultured Adipose Tissue–Derived Stromal Cells

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Objective—The delivery of autologous progenitor cells into ischemic tissue of patients is emerging as a novel therapeutic option. Here, we report the potential impact of cultured adipose tissue–derived cells (ADSC) on angiogenic cell therapy.

Method and Results—ADSC were isolated from C57Bl/6 mouse inguinal adipose tissue and showed high expression of *Sca*I and CD44, but not c-kit, Lin, CD34, CD45, CD11b, and CD31, compatible with that of mesenchymal stem cells from bone marrow. In coculture conditions with ADSC and human aortic endothelial cells (ECs) under treatment with growth factors, ADSC significantly increased EC viability, migration and tube formation mainly through secretion of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). At 4 weeks after transplantation of ADSC into the ischemic mouse hindlimb, the angiogenic scores were improved in the ADSC-treated group, which were evaluated with blood flow by laser Doppler imaging (LDI) and capillary density by immunostaining with anti-CD31 antibody. However, injected ADSC did not correspond to CD31, von Willebrand factor, and α-smooth muscle actin-positive cells in ischemic tissue.

Conclusion—These adipose tissue–derived cells demonstrated potential as angiogenic cell therapy for ischemic disease, which appears to be mainly achieved by their ability to secrete angiogenic growth factors. (***Arterioscler Thromb Vasc Biol.* 2005;25:2542-2547.)**

Key words: adipose tissue ■ angiogenesis ■ growth factors ■ HGF ■ VEGF

Therapeutic angiogenesis, a strategy to treat tissue ischemia by promoting the proliferation of collateral vessels, has emerged as one of the most promising therapies developed to date.[1,2] Therapeutic potential of various angiogenic molecules has been reported in animal models or humans with ischemic disease.[3,4] Recently, autologous transplantation of bone marrow (BM) stromal cells or endothelial cell (EC) progenitors has been shown to enhance angiogenesis or peripheral blood flow, and these cells were incorporated into sites of angiogenesis after tissue ischemia in the limb, retina, and myocardium.[5–7] However, it has also been suggested that bone marrow mononuclear cells contain various characteristics of stem cells for mesenchymal tissues[8] and secrete a broad spectrum of angiogenic or antiangiogenic cytokines, such as IL-1β, which would play a role in the process of angiogenesis.[9]

Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains stroma that is easily isolated,[10] which was classically used to investigate preadipocyte differentiation into mature adipocytes. However, this fraction was also reported to be a very convenient and nonrestrictive source of pluripotent cells such as hematopoietic progenitors and spare mesodermal stem cells able to differentiate into osteogenic, chondrogenic, myogenic, and neurogenic lineages.[11,12] Moreover, in rodents, the presence of hematopoietic stem cells in adipose tissue–derived stromal cells (ADSCs) has been suggested, and the presence of a cell population expressing the stem cell marker CD34 has been shown in ADSC from human adipose tissue.[13] These cells could differentiate into endothelial cells and participate in vessel formation, and interestingly, administration of adipose tissue–derived cells could potentially affect revascularization to a similar degree to BM-MNC administration.[14] Of importance, ADSC secrete multiple angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), at levels that are bioactive.[15,16] However, how to use the advantage of "cultured ADSC" for angiogenic therapy still needs to be determined.

In this study, we developed cultured ADSC from mouse inguinal fat, which showed fairly homogeneous characteristics compatible with mesenchymal stem cells. ADSC can

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differentiate into endothelial cells and secrete angiogenic growth factors that strongly induce endothelial cell growth, migration, and tube formation. Transplantation of these cultured ADSC to ischemic limbs accelerated angiogenesis mainly caused by secretion of growth factors, rather than participation in vessel formation by differentiation.

Materials and Methods

Cell Culture

ADSCs were isolated from adipose tissue according to the method of Bjorntorp et al with minor modification.[17] Human aortic endothelial cells and aortic smooth muscle cells (passage 3) were obtained from Clonetics Corp (San Diego, Calif), and cultured in modified MCDB131 medium (EGM2-MV; Clonetics Corp) or smooth muscle medium supplemented with 5% fetal bovine serum and smooth muscle growth supplement (Clonetics Corp) in the standard fashion.[18] Detail information is described in Supplement (http://atvb.ahajournals.org).

Fluorescence-Activated Cell Sorting and Immunostaining

A total of 2×10^5 cells were resuspended in 200 μL phosphate-buffered saline containing 3% bovine serum albumin and incubated for 30 minutes with fluorescence-labeled antibodies against Ly-6A/E (*Sca*1)-PE (phycoerythrin), CD117 (c-kit)-fluorescein isothiocyanate (FITC) (FITC), CD31-PE, CD34-PE, CD45-PE, CD11b-PE, CD44-PE, or the respective isotype control, which were obtained from BD Biosciences (Franklin Lakes, NJ). Cells were also incubated with biotin-conjugated mouse lineage panel antibodies against CD3e, CD11b, Ly-6G and -6C, Ter-119, and CD45R/B220 (Lyn; BD Biosciences) and further incubated with streptavidin-PE. After washing steps, the labeled cells were analyzed in 3 channels: FITC in FL1, orange fluorescence (PE) in FL2, and long-wavelength red fluorescence in FL3 by flow cytometry by means of a fluorescence-activated-cell sorter (FACS) Calibur flow cytometer and CellQuest Pro software (BD Biosciences).

To immunostain cells for platelet/endothelial cell adhesion molecule 1 (PECAM) (CD31) or VE-cadherin, cells were fixed for 30 minutes at room temperature with methanol, and permeabilized for 5 minutes with 0.2% Triton X-100 and 1% goat serum. They were then incubated with an antibody to PECAM or VE-cadherin (1:100, Pharmingen, San Diego, Calif) and further incubated with Alexa Fluor 488 anti-rat antibody (1:200, Molecular Probes, Inc., Eugene, OR).

Cell Viability, Migration, and Tube Formation Assay

Cell viability of HAEC was measured by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96, Promega, Madison, Wis) as previously described.[18] Migration of HAECs was estimated in a modified Boyden chamber as previously described.[19] An angiogenesis kit (Kurabo, Tokyo, Japan) was used for tube formation assay as described by the manufacturer. The detail information was described in Supplement.

Reverse Transcription-Polymerase Chain Reaction Analysis

The conditions of the polymerase chain reaction (PCR) and design of oligonucleotide primers followed a previous report[20] with minor modification. We performed quantitative real-time PCR analysis. The detail information about reverse-transcription PCR is described in the Supplement.

Mouse Hindlimb Ischemic Model and Evaluation of Angiogenesis

Wild-type C57BL/6J mice were anesthetized with ketamine chloride (80 mg/kg) and xylidine sulfate (8 mg/kg) subcutaneously, and unilateral hindlimb ischemia was induced as described previously.[21] Blood flow by laser Doppler imaging (LDI) (Moor Instruments, Devon, United Kingdom) and capillary density within the ischemic thigh adductor skeletal muscles was performed for analysis of angiogenesis as described previously[22] All experimental protocols were approved by the Osaka University Graduate School of Medicine Standing Committee on Animals. Detailed methods are described in the Supplement.

Statistical Analysis

All values were expressed as mean±SEM. Analysis of variance with subsequent Bonferroni's/Dunnet's test was used to determine the significance of differences in multiple comparisons. Values of $P<0.05$ were considered statistically significant.

Results

Pluripotency of ADSCs

The inguinal fat pads from each mouse yielded approximately 1×10^5 nucleated cells. Freshly isolated ADSC showed adherence and expansion in culture. After second passage of cell expansion, FACS analysis of cultured ADSC indicated that *Sca*1 and CD44 antigen were expressed, but not c-kit, Lin, CD11b, CD31, CD34, or CD45 (Figure 1A). This suggests that ADSC include a large population of mesenchymal stem cells, but no hematopoietic stem cells.[23] Of importance, these characteristics of ADSC surface markers were conserved after several passages of cell expansion. These cells look like fibroblasts and can differentiate into not only adipocytes but also chondrogenic and osteogenic lineages in vitro as previous described.[12] To examine the pluripotency of ADSC, we tried to induce them to differentiate toward endothelial cells. Because murine embryonic stem cells or human endothelial progenitor cells can differentiate into endothelial cells with growth factor-rich medium in collagen-coated dishes,[24] we seeded ADSC into collagen I-coated dishes with DMEM or growth factor-rich EGM2-MV medium for 5 days. Although PECAM-positive or VE-cadherin positive cells were not observed in ADSC cultured in EBM-2 medium, we found a few PECAM-positive or VE-cadherin-positive cells in growth factor-rich medium, which showed a spindle shape similar to endothelial progenitor cells from bone marrow (Figure I, available online at http://atvb.ahajournals.org). To further confirm the differentiation of ADSC into EC, we also followed Flk-1, Tie-2, and PECAM gene expression by reverse-transcription PCR. After differentiation of murine ADSC in growth factor-rich EGM medium, we collected the PECAM-positive endothelial cells from ADSC using mouse specific anti-PECAM antibody-attached magnet beads and extracted mRNA from them as differentiated EC. As shown in Figure 1B, we confirmed the mRNA expression of Flk-1, Tie-2, and PECAM only in differentiated EC.

Effects of ADSC on EC Viability, Migration, and Tube Formation

Increasing numbers of reports highlight the possibility that adipogenesis and neovascularization are reciprocally regulated and tightly linked in rodents.[25,26] Because the proliferation and migration of endothelial cells are important aspects of angiogenesis, we examined the effect of ADSC-conditioned medium in a coculture system of human aortic endothelial cells. Cell viability of endothelial cells in the conditioned medium from ADSC was significantly higher

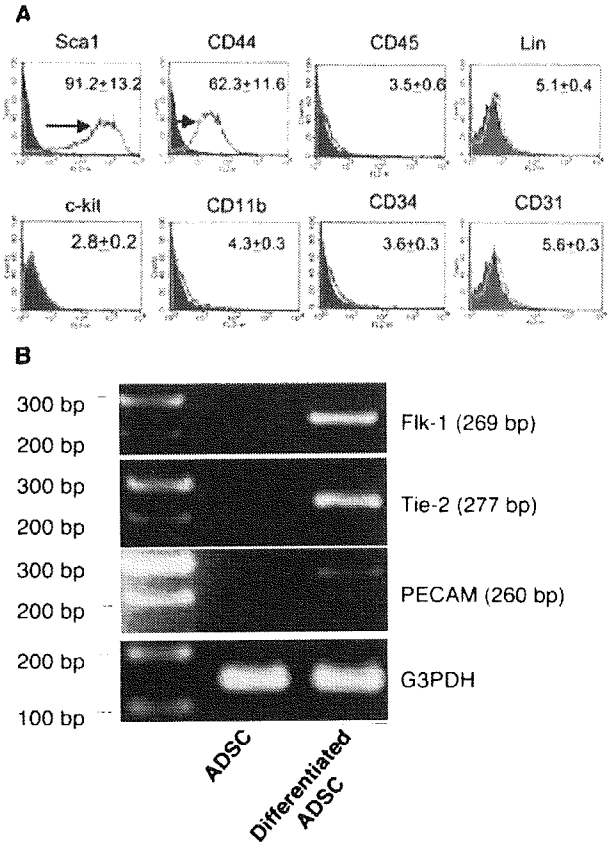

Figure 1. A, Murine ADSC were stained with anti-*Sca*l, c-kit, Lin, CD45, CD34, CD44, CD11b, or Flk1 antibody and analyzed by flow cytometry. Red lines show cells stained with anti-*Sca*l-PE, c-kit-FITC, CD34-PE, CD45-PE, CD11b-PE, CD44-PE, CD31-PE, or Lin-biotin-PE antibody, respectively, and green area shows the respective isotype control for each antibody. The means/SEM of positive signals were also shown for each antibody. B, Typical example of RT-PCR analysis of endothelial cell marker expression. RNA was extracted from ADSC or differentiated ADSC into EC, which were cultured in growth factor-rich EGM2-MV medium, and were collected with murine PECAM-positive antibody attached Dynabeads. Expression of Flk-1, Tie-2, and PECAM was examined as endothelial cell markers. G3PDH was used for normalization of total mRNA.

than that in conditioned medium from EC, and the conditioned medium from ADSC in growth factor-rich EGM2-MV medium resulted in an even greater increase in viability, assessed by MTS assay (Figure 2A). In migration assay of EC, conditioned medium from ADSC in growth factor-rich EGM2-MV medium resulted in a significant increase in viability, to a lesser extent in growth factor-withdrawal EGM2-MV medium, compared with conditioned medium from EC in EGM2-MV medium (Figure 2B). In tube formation assay, conditioned medium from ADSC in EGM2-MV medium also showed a significant increase in several parameters (length, area, joint, path) compared with the conditioned medium from EC in EGM2-MV medium (Figure 2C).

Secretion of Angiogenesis-Related Cytokines From ADSC

It is known that ADSC secrete multiple angiogenic growth factors, such as VEGF and HGF, at levels that are bioactive.[15,16] We quantified the expression level of angiogenesis-related cytokines by real time PCR from human ADSC and vascular smooth muscle cells (VSMCs). As shown in Figure 3A, we observed the relatively high expression of HGF, VEGF, placental growth factor (PGF), and transforming growth factor (TGF)-β, the moderate expression of fibroblast growth factor (FGF)-2 and angiopoietin (Ang)-1, and the low level of Ang-2 from ADSC, and the expression level of HGF and VEGF were markedly higher than that of VSMC. In the analysis of enzyme-linked immunoassay, ADSC secreted a significant amount of HGF, and to a lesser extent VEGF, in EBM-2 medium for 72 hours. When ADSC were cultured in growth factor-rich EGM2-MV medium, consistent and prominent augmentation of both HGF and VEGF secretion was observed (Figure II, available online at http://atvb.ahajournals.org). To examine the effects of these growth factors on EC viability and migration, we used VEGF and HGF neutralizing polyclonal antibodies in a human ADSC and EC coculture system. We used human ADSC from human adipose tissue only in these experiments. Because anti-murine HGF neutralizing antibody is not available and anti-VEGF neutralizing antibody cross-reacts with both murine and human VEGF, it is better to use human cells to compare the contribution of these growth factors. Treatment with anti-HGF neutralizing antibody (10 μg/mL) in conditioned medium from ADSC resulted in $\approx$25% inhibition of EC viability (MTS assay) and 48% inhibition of EC migration, and treatment with anti-VEGF neutralizing antibody (10 μg/mL) resulted in $\approx$23% inhibition of EC viability and 26% inhibition of EC migration. The both treatments with these 2 antibodies could return the EC viability and migration to the baseline level. (Figure 3B). These results suggest that these 2 growth factors have a great contribution to EC viability and migration induced by conditioned medium from ADSC.

Angiogenic Effect of ADSC in Mice Ischemic Hindlimb Model

To determine whether transplantation of ADSC could induce an angiogenic effect, we evaluated the angiogenic effect of injected murine ADSC in the mouse ischemic limb. At 10 days after unilateral ligation of femoral artery of the mouse hindlimb, we performed LDI and separated the mice into three groups: injection of PBS or 1×10^6 ADSC cultured with growth factor-rich or withdrawal EGM2-MV medium. Figure 4A shows representative LDI images of hindlimb blood flow at 14 and 28 days after transplantation. Although serial LDI examination disclosed natural recovery of hindlimb blood flow in the control group, transplantation of ADSC resulted in more rapid recovery of the ratio of ischemic/normal blood flow. Interestingly, transplantation of ADSC in growth factor-rich EGM2-MV medium further improved it.

To investigate the degree of angiogenesis at the microcirculation level, we measured capillary density in histological sections harvested from the ischemic tissues. The representative photomicrographs of tissue immunostained with anti-CD31 antibody were shown in Figure III (available online at http://atvb.ahajournals.org). Quantitative analysis revealed that the capillary density was significantly increased by ADSC in growth factor-rich EGM2-MV medium, and to a lesser extent by

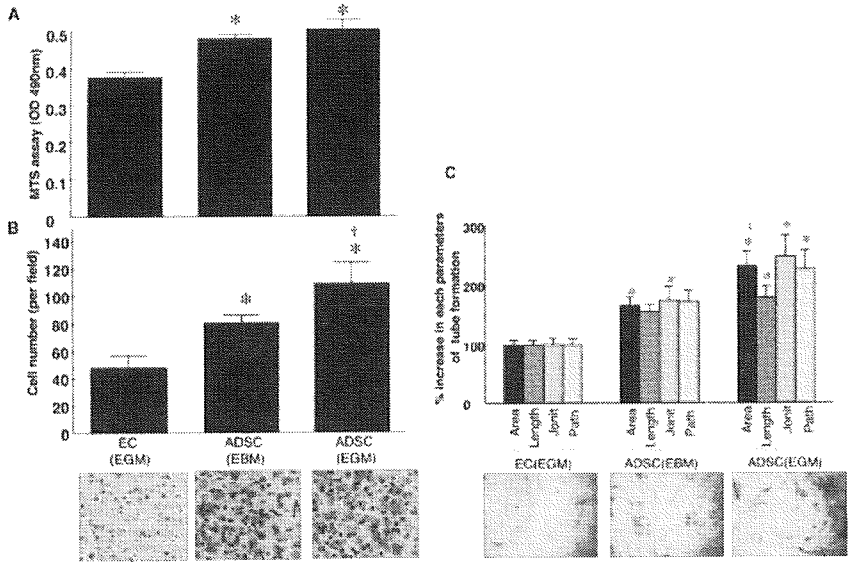

Figure 2. Effect of conditioned medium from HAEC or ADSC on (A) HAEC viability (MTS assay), (B) HAEC migration, and (C) tube formation under coculture with HAEC and murine ADSC. A, HAEC viability by MTS assay under coculture condition with HAEC or ADSC for 48 hours. B, HAEC migration for 4 hours in conditioned medium from EC or ADSC, and typical picture of each group (×400 magnification). C, Tube formation at 4 days induced by conditioned medium from EC or ADSC, and typical picture of each group (×400 magnification). N=4 per group calculated from 4 independent experiments. EC (EGM) indicates HAEC cocultured with HAEC and maintained in EGM2-MV medium (with growth factor); ADSC (EBM), HAEC cocultured with ADSC in EBM-2 medium (without growth factor); ADSC (EGM), HAEC cocultured with ADSC in EGM2-MV medium. *P < 0.01 vs EC (EGM), †P < 0.01 vs ADSC (EGM).

ADSC in growth factor-withdrawal EGM2-MV medium, compared with the control group (Figure 4B).

We further performed the comparative study with bone marrow nuclear cells (BMN) and HGF gene therapy that had been already performed in human clinical trial for peripheral arterial diseases.[4,7] In the analysis of blood flow by LDI at 28 days after each treatment, there was no significant difference between three groups (Figure III).

Involvement of ADSC in Vasculogenesis

To confirm whether ADSC are directly involved in the development of vasculogenesis in the ischemic limb, we isolated ADSC from mice engineered to constitutively express GFP and injected ADSC cultured in EGM2-MV medium into the mouse ischemic limb as described. At 3, 14, or 28 days after injection, we isolated muscle and observed GFP-positive cells. As shown in Figure 5, we identified a few clusters of GFP-positive cells in the ischemic area, which may have been the injected area, at 3 days after cell transplantation, and fewer GFP-positive cells at 14 days, and almost no GFP-positive cells by immunostaining with anti-GFP antibody at 28 days, which did not correspond to the distribution of CD31, von Willebrand factor and α-smooth muscle actin-positive cells. It suggests that ADSC might be not directly involved in the development of vasculogenesis in this model.

Discussion

The present study demonstrated that transplantation of ADSC induced angiogenesis accompanied by an increase of blood flow and capillary density in the ischemic limb.

Despite the promising potential for regenerative applications, the fundamental scarcity of endothelial progenitor cell populations in the hematopoietic system constitutes an important

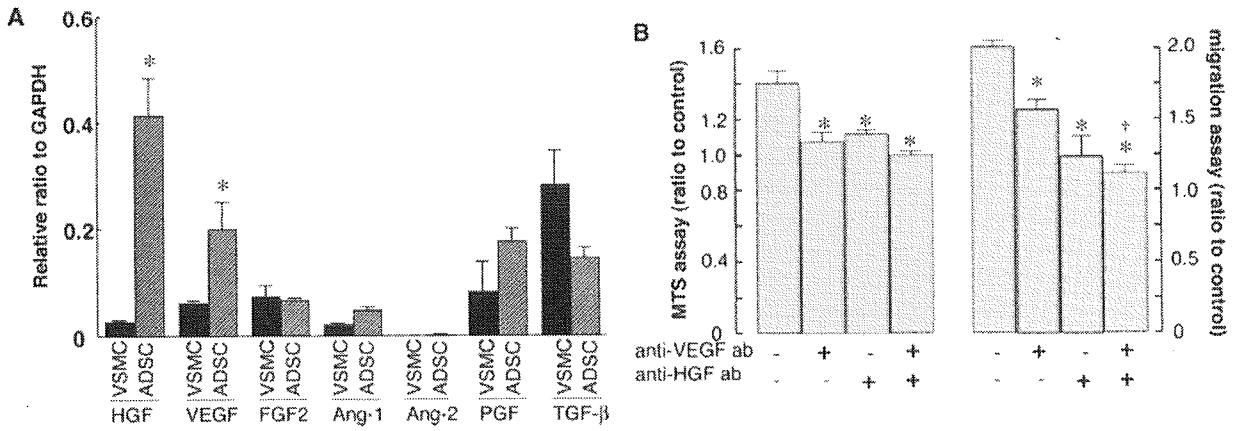

Figure 3. A, Real-time quantitative reverse-transcriptase polymerase chain reaction of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiopoietin-1, 2 (ANGPT-1, 2), placental growth factor (PGF), TGF-β, and transcripts in human ADSC and VSMC. Results are expressed as relative ratio to GAPDH for copy numbers of each mRNA. B, Effect of neutralizing anti-VEGF or anti-HGF antibody (10 μg/mL) on EC viability (MTS assay), and migration induced by conditioned medium from ADSC. Data are shown as ratio compared with control (conditioned medium from EC). N=4 per group calculated from 4 independent experiments. VEGF ab (+ or −) indicates anti-VEGF antibody or goat serum IgG (10 μg/mL) added to conditioned medium from ADSC in EGM2-MV; HGF ab (+ or −), anti-HGF antibody or rabbit serum IgG (10 μg/mL) added to conditioned medium from ADSC in EGM2-MV. *P < 0.01 vs VEGF ab(−) and HGF ab(−), †P < 0.01 vs VEGF ab(+) and HGF ab(−).