exposure to FBS should be cleaned up by chasing without Neu5Gc condition and thus might be rescued from xenogeneic rejection.

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Disclosure Statement

No competing financial interests exist.

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Cardiomyoblast-like Cells Differentiated from Human Adipose Tissue-Derived Mesenchymal Stem Cells Improve Left Ventricular Dysfunction and Survival in a Rat Myocardial Infarction Model

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Adipose tissue–derived mesenchymal stem cells (ADMSCs) are multipotent cells. Here we examined whether human ADMSCs (hADMSCs) could differentiate into cardiomyoblast-like cells (CLCs) by induction with dimethylsulfoxide and whether the cells would be utilized to treat cardiac dysfunction. Dimethylsulfoxide induced the expression of various cardiac markers in hADMSCs, such as α-cardiac actin, cardiac myosin light chain, and myosin heavy chain; none of which were detected in noncommitted hADMSCs. The induced cells were thus designated as hADMSC-derived CLCs (hCLCs). To confirm their beneficial effect on cardiac function, hCLC patches were transplanted onto the Nude rat myocardial infarction model, and compared with noncommitted hADMSC patch transplants and sham operations. Echocardiography demonstrated significant short-term improvement of cardiac function in both the patch-transplanted groups. However, long-term follow-up showed rescue and maintenance of cardiac function in the hCLC patch-transplanted group only, but not in the noncommitted hADMSC patch-transplanted animals. The hCLCs, but not the hADMSCs, engrafted into the scarred myocardium and differentiated into human cardiac troponin I-positive cells, and thus regarded as cardiomyocytes. Transplantation of the hCLC patches also resulted in recovery of cardiac function and improvement of long-term survival rate. Thus, transplantation of hCLC patches is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

Introduction

END-STAGE HEART FAILURE remains a major cause of death worldwide, with most cases due to ischemia. This is despite the remarkable progress in recent years in both medical and surgical treatments for heart failure. Cardiac transplantation and mechanical support using implantation of the left ventricular assist system were established as the ultimate means of support for these patients.[1,2] However, these treatment entities have limitations including donor shortage, rejection, and left ventricular assist system durability, and alternative strategies are needed in such circumstances.

Cellular cardiomyoplasty was developed as a new approach to restore impaired heart function,[3,4] using a variety of cell types, with encouraging initial results.[3–5] Mesenchymal stem cells (MSCs) seem particularly advantageous for cellular therapy in general because they are multipotent, potentially immune privileged,[6] and expand easily *ex vivo*. MSCs also proliferate rapidly, induce angiogenesis, and can differentiate into cardiomyogenic cells.[7–10] An MSC population was recently isolated from human adipose tissue, which is abundantly available and can be resected easily and safely in most patients.[11,12] These adipose tissue-derived cell lineages showed cardiomyocytic differentiation and rescued

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cardiac dysfunction in a myocardial infarction (MI) animal model. Thus, the adipose tissue is a convenient and preferred source of stem cell recovery for cardiac therapy. Recently, transplantation of monolayered adipose tissue-derived MSCs (ADMSCs) into MI rats reversed wall thinning in the scarred area and improved cardiac function in a short term, with the engrafted sheet of cells forming a thick stratum containing newly formed vessels and scattered cardiomyocytes derived from the implanted cells.[13] As patients with severe heart failure desire sustained and long-term recovery of cardiac function after treatment rather than short-term improvement, continued efforts should be made to develop cell transplants from ADMSCs that survive and differentiate into cardiomyocytes *in vivo* for subsequent engraftment onto scarred myocardium.

This study investigated the differentiation of human ADMSCs (hADMSCs) into cardiomyoblast-like cells (CLCs) *in vitro*, analyzed the functional and histological regeneration of damaged myocardium after transplantation of CLCs *in vivo*, and examined the effects of such transplantation on long-term patient survival.

Materials and Methods

Adipose tissues from human subjects

Excess omental adipose tissues were resected from the gastro-omental artery during coronary artery bypass graft surgery and gastrectomy in 10 subjects [4 men and 6 women; age, 55 ± 5 years, mean ± standard error of mean (SEM); range, 40–60 years]. All subjects provided informed consent, and the Review Board for Human Research of Osaka University Graduate School of Medicine approved all protocols. All subjects fasted for at least 10 h before surgery and none was on steroid therapy at the time of surgery. Ten to 50 grams of adipose tissue was obtained from each subject.

Isolation of hADMSCs and differentiation into CLCs

hADMSCs were obtained as reported previously, with modification.[11,14] Briefly, the resected excess adipose tissue was minced and then digested at 37°C for 1 h in Hank's balanced salt solution (Gibco–Invitrogen, Grand Island, NY) containing 0.075% collagenase type II (Sigma–Aldrich, St. Louis, MO). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 *g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep ($d = 1.077$; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (Gibco–Invitrogen) with 10% defined fetal bovine serum (Hyclone, Logan, UT) for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/L ethylenediaminetetraacetate solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin-coated dishes (BD BioCoat, Franklin Lakes, NJ) in 60% Dulbecco's modified Eagle's medium-low glucose, 40% MCDB-201 medium (Sigma–Aldrich), 1 × insulin-transferring selenium (Gibco–Invitrogen), 1 nM dexamethasone (Sigma–Aldrich), 100 μM ascorbic acid 2-phosphate (Sigma–Aldrich), 10 ng/mL epidermal growth factor (PeproTec, Rocky Hill, NJ), and 5% fetal bovine serum. After passaging five to six times in the same medium, the hADMSCs were used for transplantation. Cardiomyocytic differentiation was achieved by inducing hADMSCs with 0.1% dimethylsulfoxide (DMSO) for 48 h, resulting in a population named CLCs.

Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from hADMSCs and cardiomyoblasts using an RNAeasy kit (Qiagen, Hilden, Germany). As a control, excess human myocardium was resected during maze surgery from 10 matched subjects (4 men and 6 women; age, 55 ± 5 years, mean ± SEM; range, 40–60 years) with informed consent. Control subjects also fasted for at least 10 h before surgery, and none was taking steroids. Approximately 1 g of myocardium was obtained from each subject, and the same protocol was performed to obtain total RNA. After treatment with DNase, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase RNase H minus (Invitrogen, Carlsbad, CA). The absence of DNA contamination in RNA samples was confirmed with polymerase chain reaction (PCR) primers flanking an intron. Primers and the reaction conditions are described in Table 1. The PCR products were fractionated by 2% agarose gel electrophoresis.

Table 1. Primers Used in Reverse Transcriptase–Polymerase Chain Reaction

Primer		*Sequence*	*No. of cycles*	*Annealing temperature (°C)*
GAPDH	Forward	GTCAGTGGTGGACCTGACCT	35	60
	Reverse	AGGGGAGATTCAGTGTGGTG		
Islet-1	Forward	TGATGAAGCAACTCCAGCAG	35	60
	Reverse	GGACTGGCTACCATGCTGTT		
Nkx2.5	Forward	GGTGGAGCTGGAGAAGACAGA	35	60
	Reverse	CGACGCCGAAGTTCACGAAGT		
GATA-4	Forward	ACCAGCAGCAGCGAGGAGAT	35	60
	Reverse	GAGAGATGCAGTGTGCTCGT		
α-Cardiac actin	Forward	GGAGTTATGGTGGGTATGGGTC	35	60
	Reverse	AGTGGTGACAAAGGAGTAGCCA		
Myosin light chain-2v	Forward	5′-GCGCCAACTCCAACGTGTTCT	35	60
	Reverse	5′-GTGATGATGTGCACCAGGTTC		
Myosin heavy chain	Forward	GGGGACAGTGGTAAAAGCAA	35	60
	Reverse	TCCCTGCGTTCCACTATCTT		

Model animals for MI

The left anterior descending coronary artery of rats with severe combined immunodeficiency was ligated. In brief, rats were anesthetized with nembutal (40 mg/kg), before being intubated and ventilated at a rate of 60 cycles/min with a tidal volume of 5 mL under room air supplemented with oxygen (2 L/min). The hearts were exposed through the fifth left-intercostal space and the left anterior descending was ligated. After 4 weeks, the hearts were again exposed through the fifth left-intercostal space, and the infarct area was identified visually based on surface scarring and abnormal wall motion. Cell sheets were subsequently implanted onto the infarcted myocardium. The control group was treated similarly, but no cell sheets were implanted. The Osaka University Graduate School of Medicine Standing Committee on Animals approved all experimental protocols.

Preparation of monolayered cell sheets

After four to five passages, the hADMSCs were trypsinized and then replated onto 35-mm temperature-responsive dishes (CellSeed, Tokyo, Japan) in 2 mL of expansion medium at 1×10^6 cells per dish. After culture at 37°C for 2 days, 0.1% DMSO was added to the medium on half of the dishes to differentiate the hADMSCs into cardiomyoblasts. After 2 days of culture, the cells were incubated again at 20°C. Within 20 min, the hADMSCs and CLC sheets detached spontaneously and floated up into the medium for use as monolayered cell grafts.[13,15–17]

Assessment of rat cardiac function

Cardiac ultrasound studies were performed before ligation, before implantation, and at 2, 4, 8, 10, 12, 14, and 16 weeks after implantation using a SONOS 7500 (Philips Medical Systems, Andover, MA). Plasma atrial natriuretic protein (ANP) level was analyzed using an ANP ELISA system (Phoenix Pharmaceuticals, Burlingame, CA) by following the instructions supplied by the manufacturer.

Histological analyses

The rat hearts were dissected out and immediately fixed overnight in 4% paraformaldehyde, washed in 70% alcohol, dehydrated through a graded ethanol series, cleared in xylene, and finally processed for embedding in paraffin wax. Paraffin sections were cut at 5 μm thickness, delineated on the microscope slide using a Dako pen (Dako, Glostrup, Denmark), deparaffinized in xylene, and then rehydrated through a graded ethanol series into distilled water. The sections were then immersed in Target Retrieval Solution (Dako) in distilled water and boiled, followed by cooling at room temperature for 20 min. The sections were then washed in two changes of Tris-buffered saline (TBS), pH 7.4, followed by 1% polyoxyethylene sorbitan monolaurate (Tween 20) in TBS (TBS-T), and then an overnight incubation with 10% Blocking One® (Nacalai Tesque) in TBS-T. The sections were then incubated in a humid chamber for 16 h at 4°C with mouse monoclonal antibodies to α-cardiac actin (α-CA) and human troponin I, diluted in the blocking solution, followed by Alexa Fluor 546-labeled donkey anti-goat IgG (Molecular Probes, Eugene, OR). The stained slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka, Japan).

Statistical analysis

All data were expressed as mean ± SEM. Differences between groups were analyzed for statistical significance by the Student's *t*-test using SPSS Statistics 17.0 (SPSS, Inc., Chicago, IL). A *p*-value less than 0.05 denoted a statistically significant difference. Survival curves were constructed by the Kaplan–Meier method and survival among groups was compared using the Log-Rank test (StatMate III for Windows; Atoms, Tokyo, Japan).

Results

Cardiac differentiation of hADMSCs into CLCs

The potential for hADMSCs to differentiate into CLCs was evaluated from the mRNA expression of several cardiac differentiation markers by reverse transcriptase-PCR before and after DMSO induction, as follows: *islet-1* is a cardiac stem cell marker; *Nkx2.5* and *GATA-4* are transcription factors required for subsequent cardiac differentiation; and α-*CA*, *myosin light chain*, and *myosin heavy chain* (*MHC*) are markers of cardiac differentiation (Fig. 1A). Preinduced hADMSCs expressed *islet-1* and *Nkx2.5* mRNA, but not that of *GATA-4*, α-*CA*, *myosin light chain*, or *MHC*. After induction by DMSO for 48 h, hADMSCs expressed all markers, indicating that DMSO treatment successfully differentiated hADMSCs into cells of the cardiac lineage, and these induced cells were named CLCs.

Preparation and transplantation of hADMSC-derived CLC patches

To evaluate the potential therapeutic usefulness of CLCs, we designed an experimental rat model of coronary ligated infarction to assess cardiac function after transplantation of CLC patches. CLC and control hADMSC patches were prepared from cell sheets, as described earlier (Fig. 1B). These patches were transplanted onto the scarred area of the left ventricular wall in the MI model Nude rats, whose left anterior descending artery had been ligated 4 weeks before graft implantation (Fig. 1C, D). Sham transplantations were also performed.

Effects of CLC transplantation on cardiac function and survival rate

Cardiac function was assessed by echocardiography at preligation, pretransplantation, and every 2 weeks after transplantation (Fig. 1D). Sixteen weeks after transplantation, the treated animals were sacrificed and cardiac tissues prepared for histological examination. Four weeks after graft implantation, wall motion was improved in both control and CLC patch-implanted hearts. However, the wall motion of control and noncommitted hADMSC patch-transplanted heart tissue was exacerbated at 16 weeks after transplantation, while improved motion was maintained with the CLC patch transplants (Fig. 2A). In the early phases of the post-transplantation period, left ventricular diastolic dimension was significantly reduced in both the transplanted groups, but by 8 weeks after implantation this parameter increased in the control hADMSC patch-transplanted group, whereas it remained unchanged in those animals that received CLC patch transplants (Fig. 2B). Likewise, left ventricular ejection

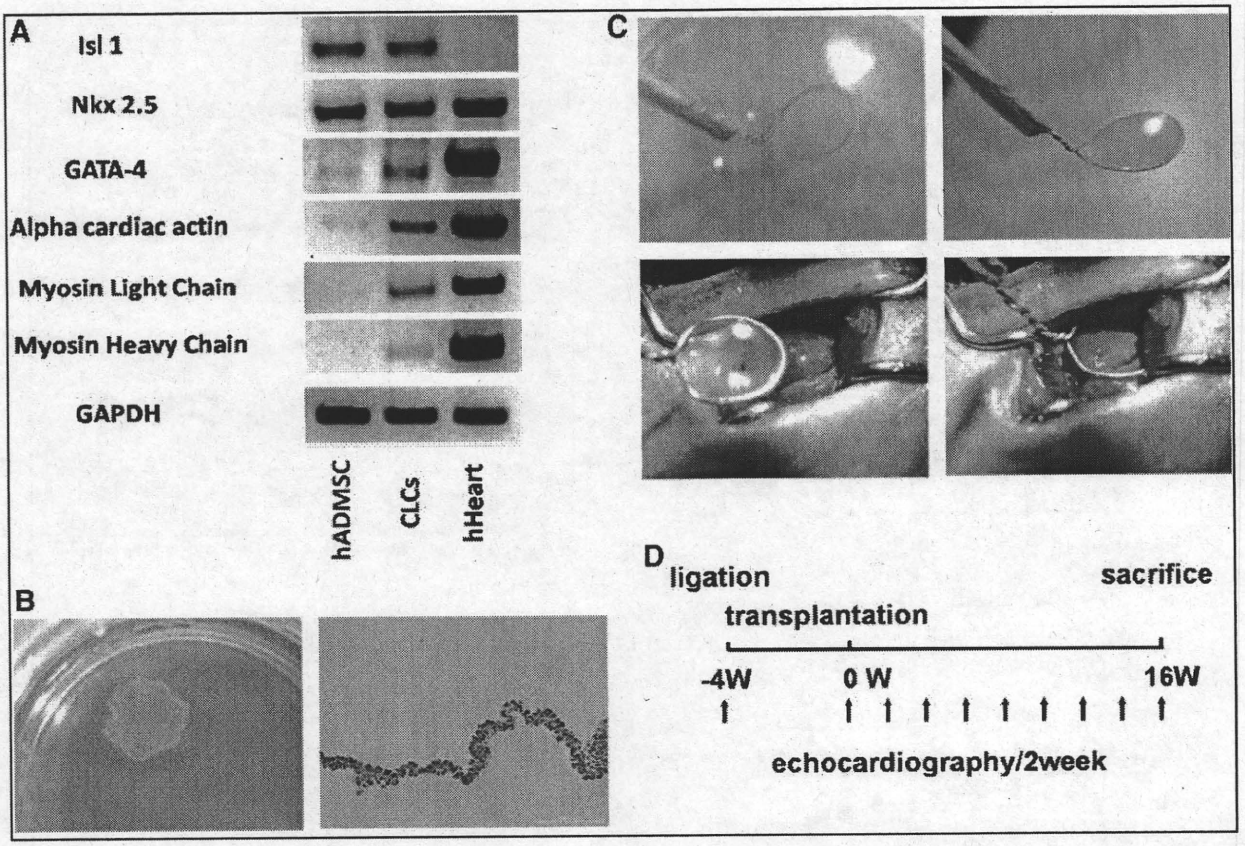

FIG. 1. Preparation and transplantation of human adipose tissue-derived mesenchymal stem cell (hADMSC)-derived cardiomyoblast-like cell (hCLC) patches. (**A**) Treatment with dimethylsulfoxide (DMSO) transformed hADMSCs into CLCs. The mRNA expressions of *islet-1*, *Nkx2.5*, *GATA-4*, *α-cardiac actin* (*α-CA*), *myosin light chain* (*MLC*), and *MHC* were analyzed by reverse transcriptase–polymerase chain reaction. The mRNAs for *islet-1* and *Nkx2.5*, with trace levels of *GATA-4* and *α-CA*, were expressed in hADMSCs, but no expression of *MLC* or *MHC* was detected. After induction with DMSO for 48 h, the hADMSCs also expressed *MLC* and *MHC*, indicating a phenotypic change into CLCs. (**B**) Preparation of hADMSC-derived CLC patches. The hADMSCs were cultured on temperature-responsive dishes for 48 h with 0.1% of DMSO for differentiation into CLCs. As the culture temperature was decreased from 37°C to 20°C, the CLCs detached spontaneously as sheets and floated up within 30 min into the culture media as a layered CLC patch. (**C**) Transplantation of hADMSC-derived CLC patches. Detached patches were transplanted onto the left ventricular wall scar in the myocardial infarction (MI) model Nude rats. (**D**) Protocol used for assessment of cardiac function. The left anterior descending artery was ligated in Nude rats at 4 weeks before graft implantation. Cardiac function was assessed by echocardiography at preligation, pretransplantation, and every 2 weeks following transplantation. The treated animals were sacrificed 16 weeks after transplantation and prepared for histological examination.

fractions improved in both the implanted groups until 8 weeks, after which time it worsened only in the group transplanted with noncommitted hADMSC patches (Fig. 2B).

ANP was then measured to confirm that chronic heart failure due to MI could be treated by CLC patch transplantation (Fig. 2C). The ANP levels were significantly increased after MI in all groups (Fig. 2C). The sham-operated MI control group showed incremental increases in plasma ANP over the time course of examination, whereas both CLC patch- and hADMSC patch-transplanted animals had low ANP levels until 8 weeks after treatment. However, ANP levels increased subsequently in the hADMSC patch-transplanted group, whereas the CLC patch-transplanted group maintained the improvement in ANP levels.

The Kaplan–Meier survival curve showed higher long-term survival rates in cell patch-transplanted groups than in sham-operated MI controls (Fig. 2D). Notably, no rat died after transplantation of an hADMSC-derived CLC patch. Survival at 16 weeks after surgery was 100% for the CLC group, 80% for the hADMSC group, and 16% for the sham-operated group, with a significant difference between the two transplanted groups. These results suggest that transplantation of hADMSC-derived CLCs has beneficial effect in rats with heart failure induced by MI.

Effects of CLC transplantation on cardiac structure

Cardiac structure was next examined histologically to analyze further the difference between CLC patch- and noncommitted hADMSC patch-transplanted animals in the longer term (Fig. 3). On hematoxylin and eosin and Masson trichrome staining, the sham-transplanted MI control rats

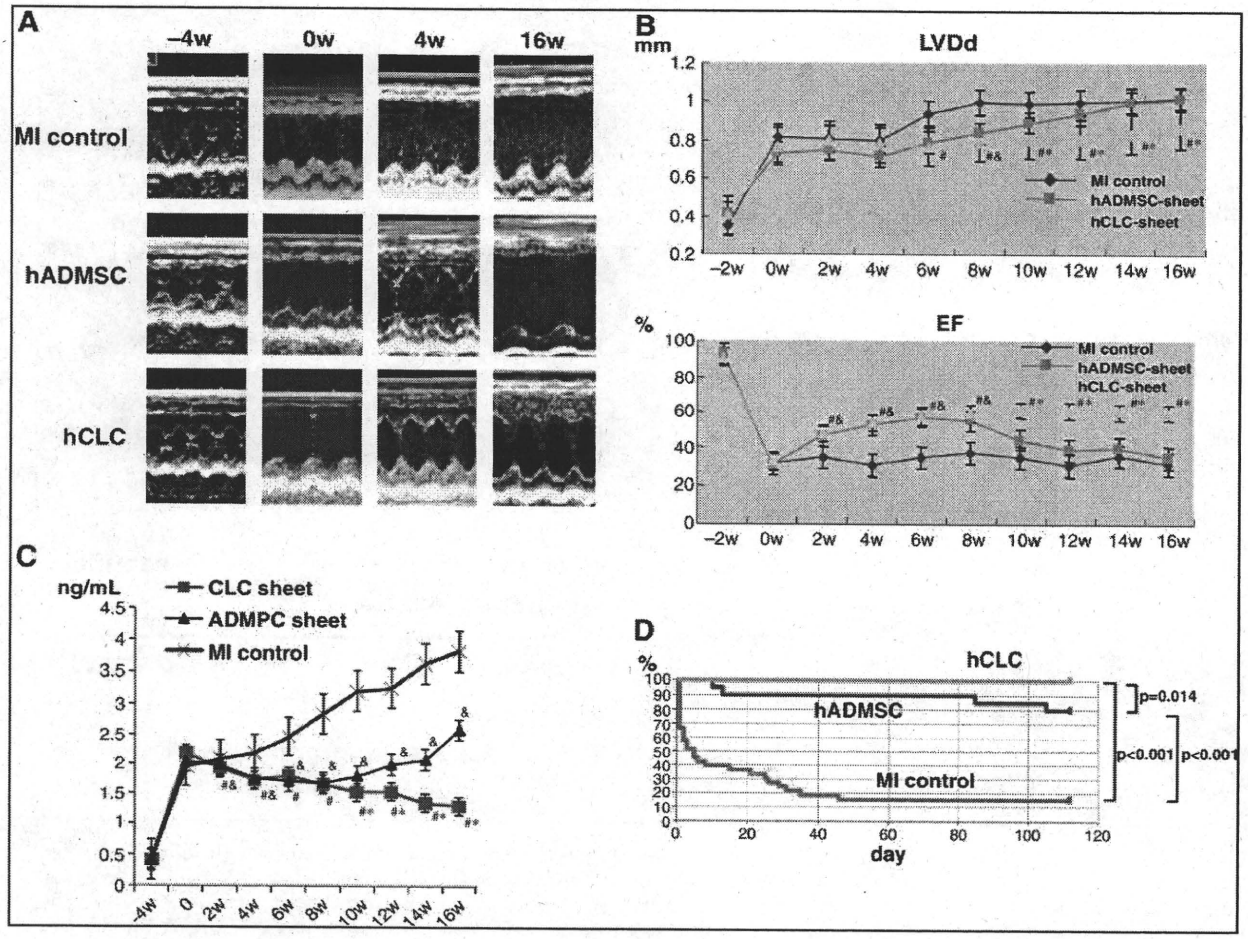

FIG. 2. Effects of CLC patch transplantation on cardiac function and long-term survival. (**A**) In both the patch-transplanted groups, echocardiography showed improved wall motion within 4 weeks of transplantation. However, at 16 weeks after transplantation, the wall motion of noncommitted hADMSC patch-transplanted rats worsened, whereas it was maintained in the CLC patch-transplanted animals. (**B**) Left ventricular diastolic dimension and ejection fractions improved significantly in both the patch-grafted groups in the early phase, as confirmed by echocardiography. However, these two parameters of cardiac function worsened at 8 weeks after implantation in the noncommitted hADMSC patch-transplanted groups, but not in the CLC patch-transplanted group. The numbers of all groups were five. Data are mean ± standard error of mean ($^{\#}p < 0.05$; MI control vs. the hCLC patch-transplanted animals; $^{\&}p < 0.05$; MI control vs. the noncommitted-hADMSC patch-transplanted rats; $^{*}p < 0.05$; hCLC patch-transplanted vs. the noncommitted hADMSC patch-transplanted rats, respectively). (**C**) Plasma ANP levels. Sham-operated MI control group showed increment of plasma ANP levels during the course of the experiment. Both the CLC patch- and hADMSC patch-transplanted groups showed suppression of ANP level increment till 8 weeks after treatment. ANP levels of the hADMSC patch-transplanted group increased from 8 weeks after transplantation, but no change in ANP levels was noted in the CLC patch-transplanted group. The numbers of all groups were four. (**D**) Long-term survival of rats with chronic heart failure that received the CLC patch ($n = 28$), noncommitted hADMSC patch ($n = 20$), or sham operation ($n = 37$). The Kaplan–Meier survival curve demonstrated that no rat died after transplantation of hADMSC-derived CLC patch. The survival rate at 16 weeks after surgery was 80% for the hADMSC group versus 16% for the sham-operated group. Log-rank test; *p*-values are indicated. LVDd, left ventricular diastolic dimension.

showed only a thin layer of cardiac muscle and fibrotic tissues in the scarred anterior left ventricular wall (Fig. 3A, B). Rats implanted with noncommitted hADMSCs showed small patches of cardiac muscles over that seen in the control MI rats (Fig. 3C, D). On the other hand, the rats transplanted with CLC patches showed significant reversal of the infracted myocardium and a full cardiac muscle layer overlaying the transplanted area (Fig. 3E, F, arrowheads).

CLCs differentiate into cardiac muscle in situ

The *in situ* differentiation capacity of the implanted cell sheets into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for α-CA and human troponin I (Fig. 4). Thin layers of α-CA-positive cells were observed on the scarred myocardium of sham-operated MI control rats (Fig. 4A). A similar but thicker

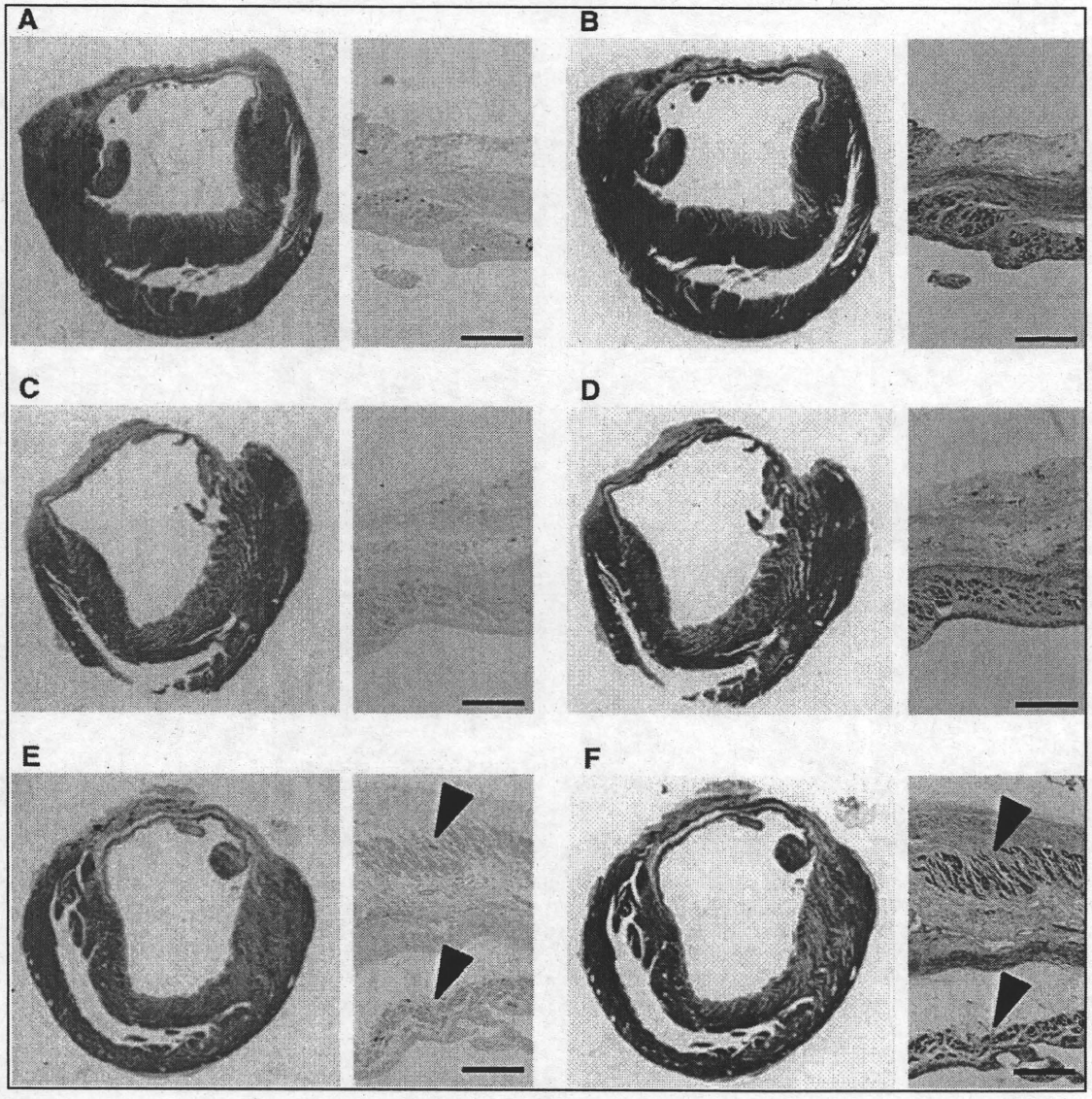

FIG. 3. Effects of CLC transplantation on cardiac structure. Photomicrographs showing representative myocardial sections stained with hematoxylin and eosin (**A, C, D**) and Masson trichrome (**B, D, F**) in the individual groups. The transplanted CLC patch reversed wall thinning of the infracted myocardium and another cardiac muscle was layered onto the transplanted area (arrowheads). (**A, B**) Sham-operated MI control group; (**C, D**) noncommitted hADMSC patch-transplanted group; and (**E, F**) hADMSC-derived CLC patch-transplanted group. Bars = 200 μm.

layer of α-CA-positive cells was apparent in the tissues from noncommitted hADMSC-transplanted rats (Fig. 4A, C), whereas the CLC patch-transplanted group showed two cardiac muscle layers positive for α-CA (Fig. 4E, arrow and arrowhead). There were no human troponin I-positive cells in the sham-operated MI control group (Fig. 4B), but some were observed in the noncommitted hADMSC patch-transplanted group (Fig. 4D). As shown in Figure 4F, large amounts of human troponin I-positive myocardium was observed in the CLC-transplanted animals (arrow) in addition to some human troponin I-negative but α-CA-positive myocardium in the internal myocardial layer (Fig. 4E, F, arrowhead). These results indicated that CLCs can efficiently differentiate into cardiomyocytes *in situ*.

Discussion

There are several advantages to hADMSC-derived CLC patch transplantation for regeneration therapy. First, the source of adipose-derived cells is easily and safely accessible and the cells can be obtained in large quantities, without serious ethical issues. Second, hADMSCs differentiate into CLCs by induction with DMSO, which is available in current good manufacturing practice grade. Third, hADMSC-derived

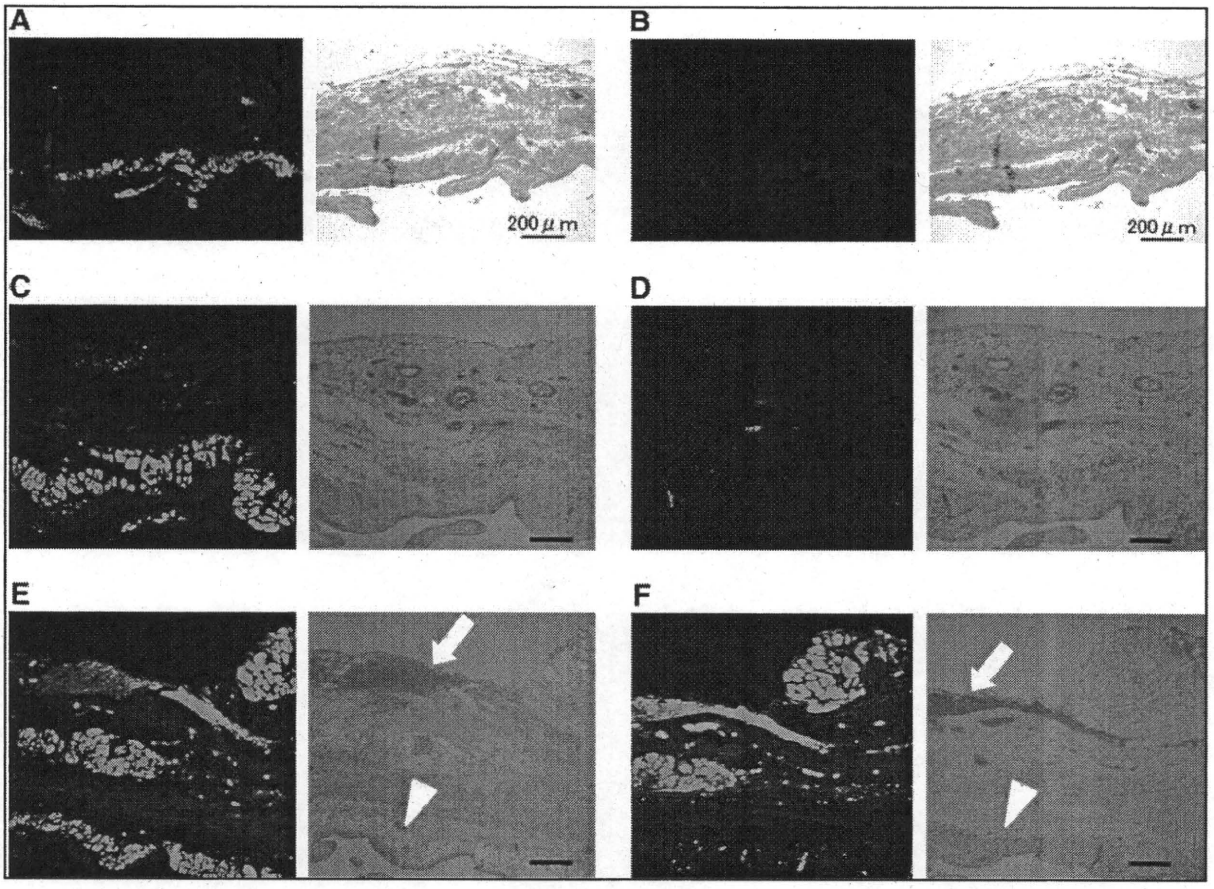

FIG. 4. CLCs differentiate into cardiac muscles *in situ*. Immunofluorescence with anti-α-CA (**A, C, E**) and anti-human-specific cardiac troponin I (**B, D, F**) antibodies, merged with phase contrast. In the hADMSC-derived CLC patch-transplanted groups, two cardiac muscle layers positive for α-CA are observed (**E**, arrow and arrowhead). A large mass of human troponin I-positive myocardium was observed in the CLC-transplanted tissues (**F**, arrows). In the same animals, the internal myocardium layer expressed α-CA but not human troponin I (**E, F**, arrowheads). (**A, B**) Sham-operated MI control group; (**C, D**) noncommitted hADMSC patch-transplanted group; and (**E, F**) CLC patch-transplanted group. Bars = 200 μm.

CLCs can differentiate into cardiomyocytes *in vivo* within the myocardial milieu, resulting in increment of myocardial muscle force. Finally, reconstruction of thick myocardial tissue rescued cardiac dysfunction after MI and improved long-term survival.

The choice of cell source is critical for realizing success in cellular therapy.[18] Liposuction surgeries yield from 100 mL to >3 L of lipoaspirate tissue.[19] The initial isolation of cells from adipose tissue was described by Bjorntorp *et al.*[14] This procedure was since modified to isolate cells from human adipose tissue specimens.[20–22] In this context, Zuk *et al.*[11] reported that the preadipocytes exhibited stem cell features as MSCs, currently known as ADMSCs. Because of the above-stated advantages of procuring cells for therapy from adipose tissues, hADMSCs present a potential and promising source for cellular therapy, even in patients with post-MI severe heart failure.

The *in vitro* differentiation of ADMSCs is now well reported, and experimental findings in recent years suggested considerable therapeutic potential for cellular replacement in the context of acute MI and chronic progressive cardiac disease.[23–27] Stem cells are differentiated into a cardiomyocyte lineage by treatment with 5-azacytidie, retinoic acid, oxytocin, and many other reagents.[28–32] We proposed that DMSO could differentiate hADMSCs into CLCs, based on the differentiation of P19 embryonic stems cells into cardiomyocytes with DMSO.[31–33] It was notable that DMSO is also available in current good manufacturing practice grade. Unfortunately, DMSO-treated hADMSCs did not show spontaneous beating as their terminal differentiation function, but the cells did express the mature markers α-*CA*, *myosin light chain*, and *myosin heavy chain* to a lesser extent. There are no reports of the use of DMSO to commit ADMSCs to a cardiomyocytic lineage. The mechanism by which DMSO elicits its effect on differentiation remains unclear. It is possible that DMSO increases intracellular calcium ion concentration, thereby elevating phosphatidylethanolamine levels in the cells and controlling the distribution of protein kinase C to commit the P19 stem cells.[33–36] These mechanisms should be investigated further in the near future.

The *in vitro* differentiation of ADMSCs has been well reported,[23–27] although only a few reports relate to the differentiation of these cells into cardiomyocytes *in vivo*. Recently,

Miyahara *et al.*[13] reported the use of monolayered ADMSCs for myocardial repair. In their study, rat ADMSCs were isolated and grown as intact monolayer sheets using temperature-responsive culture dishes. Placement of the ADMSC sheets onto a scarred myocardium in rats resulted in diminished scarring and enhanced cardiac structure and function. Histological analysis demonstrated that the engrafted ADMSC sheets grew to form a thickened layer over the infarcted muscle that included newly formed vessels and a few cardiomyocytes. In our study, hADMSC-derived CLCs differentiated into cardiomyocytes in a myocardial milieu, indicated by the immunohistological results in which transplanted cells expressed human troponin I *in vivo*. Newly developed myocardium might augment cardiac function, and thus hADMSC patch transplantation was performed as a control. Cardiac dysfunction was rescued in a short term, although the numbers of cardiomyocytes derived from transplanted cells were low. In this context, Gimble *et al.*[19] suggested that hADMSCs might secrete angiogenic factors and/or antiapoptotic factors.

Transplantation of the hADMSC-derived CLC regenerated the thick myocardial tissues, rescued cardiac dysfunction after MI, and improved long-term survival rate compared with the noncommitted hADMSCs and sham-operated MI controls. The existing literature suggests that ADMSCs can be engrafted and survive within an infarcted myocardial milieu, acquire phenotypic markers consistent with cardiomyocytic and vascular-related lineages, and have a positive impact on structural and functional endpoints.[19,23–27] These are desirable outcomes for cardiac function and survival. However, few reports have applied long-term observation of the transplanted animals. Our study therefore observed the three rat groups for 16 weeks after transplantation. Only CLC transplantation provided the desired outcome at the experimental endpoint. Despite these encouraging results, much progress is needed to realize the hope of cell therapies for myocardial damage. First, delivery of the cell sheets to patients should be optimized for each given disease. Second, the issue of vascularization should be considered in the infracted or affected tissues after transplantation, because many small CLC patches would be necessary for a clinical cure. Finally, the value and impact of CLC patch transplantation should be confirmed in large animal models before embarking on clinical applications.

In conclusion, we showed that the phenotype of hADMSCs could be changed to that of CLCs by induction with DMSO. These hADMSC-derived CLCs engrafted into a scarred myocardium and differentiated into cardiomyocytes. The CLC patch transplantation also resulted in recovery of cardiac function and improved survival rate. Thus, transplantation of hADMSC-derived CLC patches in heart patients might be a potentially effective therapeutic strategy for cardiac tissue regeneration in the near future.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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Original Article

HDL/Apolipoprotein A-I Binds to Macrophage-Derived Progranulin and Suppresses its Conversion into Proinflammatory Granulins

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Aim: **HDL has anti-inflammatory effects on macrophages, although the mechanism of action remains unclear. We hypothesized that HDL suppresses the conversion of macrophage-secreted factors into proinflammatory factors via binding, and tried to identify the factor that could form a complex with HDL and/or apolipoprotein (apo) A-I.**

Methods and Results: **In conditioned media obtained from human monocyte-derived macrophages, we found an apo A-I binding protein and identified the protein as progranulin/proepithelin/acrogranin/PCDGF. Co-immunoprecipitation analysis showing that progranulin binds and forms a complex with apo A-I and the presence of progranulin in the HDL fraction in the sera indicated that progranilin is a novel apolipoprotein. Conditioned media of HEK293 cells transfected with progranulin augmented the expression of TNF-alpha and IL-1-beta on macrophages, but these effects of progranulin were inhibited by co-incubation with HDL or apo A-I. Anti-progranulin antibodies also reduced the expression of TNF-alpha and IL-1-beta on macrophages. Granulins as conversion products derived from progranilin increased TNF-alpha and IL-1-beta expression and the effects were not suppressed by HDL.**

Conclusions: **Our results suggest that the anti-inflammatory effects of HDL on macrophages might be due to suppression of the conversion of progranulin into proinflammatory granulins by forming a complex.**

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Key words; HDL, Apolipoprotein A-I, Progranulin, Proepithelin, Acrogranin, PCDGF, Macrophage

Introduction

Several pathological studies have shown that low high-density lipoprotein (HDL) levels are associated with plaque instability in patients with acute coronary syndrome[1]. Accordingly, the reverse cholesterol transport system, in which excess cholesterol is extracted from atheromatous plaques, is considered important in stabilizing plaques and preventing plaque rupture[2,3]. A recent study reported that in addition to the reverse cholesterol system, HDL infusion could stabilize atheromatous plaque through its anti-inflammatory properties[4].

The acute phase of a coronary event is associated with a significant fall in serum levels of apolipoprotein (apo) A-I (a major component of HDL) and HDL-C[5]. There is evidence that plasma HDL-C measured in the initial stage of the acute phase of coronary events predicts the risk of recurrent cardiovascular events over the ensuing 16 weeks[6]. We often expe-

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rience patients with acute coronary syndrome whose HDL levels are reduced before the occurrence of plaque instability[4].

Infiltration and accumulation of foam cells (macrophages) is a characteristic feature of atheromatous plaques[7]. Once activated, macrophages secrete various pro-inflammatory cytokines and proteases, which could result in plaque instability and rupture[7]. Are the anti-inflammatory effects of HDL mediated through suppression of the secretion of such cytokines from macrophages? The main theme of our research is to determine the mechanisms underlying the reduction of serum HDL during the acute phase of coronary events. In this study, we hypothesized that HDL modulates the expression levels of pro-inflammatory cytokines secreted by macrophages. Progranulin is here described as a macrophage-derived secretory factor, which is a pluripotent protein and a precursor of its proteolytic peptides, granulins, whose functional properties were different from their intact precursor in some cases[8], and whose pro-inflammatory properties were suppressed via binding to HDL.

Materials and Methods

Lipoprotein Isolation

Apo A-I was purchased from Sigma Aldrich (St. Louis, MO). HDL3 were isolated from human serum by ultracentrifugation at a density of 1.125–1.210 g/mL[9].

Isolation of Human Monocyte-Derived Macrophages

Mononuclear cells were isolated from the buffy coats of plasma collected from healthy volunteers using density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway). The cells were then cultured for 7 days, as described previously[9].

Preparation of Conditioned Medium

A monolayer of macrophages was collected from 7-day culture and incubated in serum-free RPMI1640 for 24 h at 37℃. The conditioned medium was collected and replaced with fresh RPMI1640 every 24 hours for 5 days. The collected medium was centrifuged, and the supernatant was treated with benzamidine hydrochloride (Sigma) at a final concentration of 1 mM to protect against protease degradation.

Ligand Blotting Analysis

The concentrated conditioned medium was separated under non-reducing conditions using 10–20% polyacrylamide gradient gels, transferred onto nitrocellulose membranes, and blotted with 5 μg/mL biotinylated-apo A-I. After incubation with peroxidase-conjugated streptavidin, the blots were visualized with an ECL kit (Amersham Pharmacia Bioscience, Uppsala, Sweden).

Purification of Apo A-I Binding Protein from Macrophage-Conditioned Medium

The conditioned medium (total volume, 20 L) was collected and then treated with 80% ammonium sulfate. The precipitate was dissolved in 2.5 mL, and then desalted and equilibrated into Tris-buffered saline (20 mmol/L Tris HCl, pH 7.4, and 135 mmol/L NaCl) using a PD-10 column (Amersham Pharmacia Biotech). The eluate was added to an apolipoprotein A-I-affinity column (Amino Link; Amersham Pharmacia Biotech), and allowed to stand overnight at 4℃. After vigorous washing with Tris buffer with 1 mol/L NaCl (20 mmol/L Tris/HCl, pH 7.4, and 1 mol/L NaCl), the binding proteins were eluted with 8 mol/L urea. The eluate was concentrated with Amicon Ultra-15 50,000 MWCO (Millipore, Bedford, MA) to ensure purity, and subjected to SDS-PAGE.

Amino Acid Sequencing

The purified apo A-I-binding protein was applied for in-gel digestion with V8 endopeptidase, transferred to a polyvinylidene fluoride (PVDF) membrane, and stained with Coomassie brilliant blue (CBB) R-250. The three apparent fragmented bands were subjected to amino acid sequencing in a sequencer (Perkin Elmer-Cetus, Foster City, CA).

Construction of Progranulin, Granulin A and Granulin B Expression Vector

The expression vectors of progranulin (aa 1-593, see RESULTS), myc-His-tagged progranulin and granulin were constructed from pcDNA3.1, as described previously[10]. In short, cDNA obtained from human monocyte-derived macrophages underwent PCR using primer pairs 5'-aggaccgcggagtcggacgcaggcagacca-3' and 5'-tccgagtggggtcccgagggctgcagagtc-3', and for nested PCR, primer pairs 5'-gtcggactccggcagaccatgtggaccctg-3' and 5'-agggctcgagagtcttcagtactgtccctc-3'. The nested PCR product was digested with Bam HI and Xho I and the digested product was ligated into pcDNA3.1 pretreated with Bam HI and Xho I. The construct was used as a progranulin expression vector. To obtain myc-His tagged progranulin expression vector, cDNA obtained from human monocyte-derived macrophages underwent PCR using primer pairs 5'-aggaccgcggagtcggacgcaggcagacca-3' and 5'-tccgagtgggtcccgagggctgcagagtc-3', and for nested PCR, primer pairs 5'-gtcggactccggcagaccatgtggaccctg-3' and 5'- tccc-

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tcacctctagagcagctgtctcaagg-3', and the nested PCR product was digested with Bam HI and Xba I and the digested product was ligated into pcDNA3.1/myc-His vector pretreated with Bam HI and Xba I. Human granulin A (aa 281–337) and B (aa 206–261) vectors were constructed with secretion being driven by the human progranulin signal peptide (aa 1–17).

Immunoprecipitation

The vectors were transiently expressed in HEK293 cells using a Calcium Phosphate Transfection Kit (Invitrogen Corp., Carlsbad, CA, USA). Three days after transfection, HEK293 cells were incubated with conditioned medium containing apo A-I (final concentration 5 μg/mL) at 37℃ for 30 min. The apo A-I-containing media were collected and immunoprecipitated with anti-progranulin antibody (clone N19; Santa Cruz Biotechnology, Santa Cruz, CA), pulled-down with protein G (Amersham Pharmacia Biotech), separated by reducing SDS-PAGE, and Western blotted with anti-apo A-I antibody, or the reverse. *In vitro* translated progranulin was produced using an *in vitro* translation system (Duo, Tokyo, Japan), and mixed with apo A-I at a final concentration of 5 μg/mL. The mixture was then subjected to co-immunoprecipitation analysis.

Immunoblotting Analysis

The conditioned medium was subjected to immunoblotting analysis with anti-progranulin monoclonal antibody (clone N-19; Santa Cruz Biotechnology). The blots were visualized after incubation with peroxidase-conjugated anti-mouse IgG antibody (DAKO, Denmark).

Quantitative Real-Time PCR

The constructed expression vectors of progranulin, granulin A and granulin B, were transiently expressed in HEK293 cells using a Calcium Phosphate Transfection Kit (Invitrogen Corp.). Three days after transfection, the conditioned media were obtained and macrophages were incubated in conditioned media with or without HDL (10 μg/mL) or apo A-I (5 μg/mL) for 24 h. Total RNA was then isolated using RNeasy MINI kits (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. For cDNA synthesis, 600 ng total RNA was reverse transcribed using SuperScript Ⅲ RTase (Invitrogen, San Diego, CA). TaqMan probe and primers for progranulin, CD14, CD36, CD68, TNF-alpha, IL-1beta and GAPDH were purchased from Applied Biosystems (Assay ID: Hs00173570_m1, Hs02621496_S1, Hs01567186_m1, Hs00154355_m1, Hs99999043_m1, Hs99999029_m1 and Hs00266705_g1, respectively). Quantitative real-time PCR was performed using the ABI Prism 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). The cDNA samples (10 ng in a total volume of 10 μL) were mixed with primers, probe and TaqMan Universal PCR Master Mix as described in the accompanying sheet supplied by the manufacturer (Applied Biosystems). PCR was conducted using the following settings: 50℃ for 2 min, 95℃ for 10 min and 40 cycles at 95℃ for 15 s and 60℃ for 1 min.

Results

Purification of Progranulin as Apo A-I Binding Protein

We first tested whether HDL suppresses the proinflammatory cytokines secreted by macrophages by forming complexes with them, and thus searched for hypothetical proteins. The conditioned media obtained from 7-day-cultured human macrophages were concentrated with the ammonium sulfate precipitation method, desalted and subjected to ligand blotting analysis using biotinylated-apo A-I as a ligand. After separating the media on SDS-PAGE under non-reducing conditions, we detected an apo A-I-binding protein with a MW of 130 kDa (**Fig. 1A**). To purify the protein, the concentrated medium was subjected to an affinity column with immobilized human apo A-I and we obtained a concentrated eluate. To ensure purity, 10 μg proteins of the conditioned medium and 0.5 μg of the eluted protein were subjected to SDS-PAGE. Silver staining showed a single band of 80 kDa protein (**Fig. 1B**).

In the next step, we obtained three polypeptide fragments after in-gel-digestion with V8 proteases. Next, the amino-terminal sequence of these polypeptides was determined. The amino acid sequences were "avacgdgh", "nattdllt" and "kapahlsl", respectively. All amino acid sequences were identical to those of progranulin (aa 89–96, 265–272 and 347–354, respectively).

To confirm that progranulin could bind to HDL, HDL fractions (0.5 μg) were applied for immunobloting with anti-progranulin antibody (clone N19). As shown in **Fig. 1C**, progranulin was observed in the HDL fraction, and a small amount was observed in serum. As indicated in **Fig. 1A**, HDL binding protein had approximately 130kDa molecular weight in non-reducing conditions; however, MW of progranulin was approximately 80 kDa, as shown in **Fig. 1B**. To clarfy these discrepancies, purified proteins by affinity column with immobilized human apo A-I were

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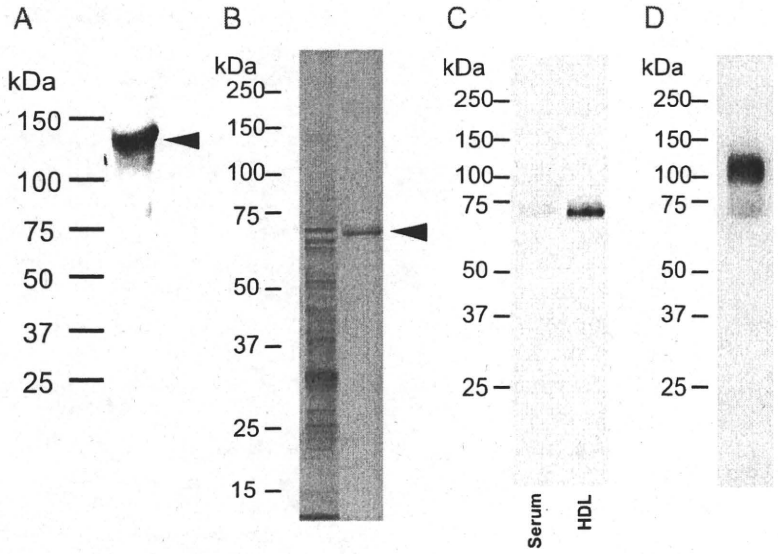

Fig. 1. Purification of apolipoprotein A-I binding protein from macrophage-conditioned medium.

(A) SDS-PAGE of purified apo A-I binding protein from macrophage-conditioned medium. The conditioned medium derived from human macrophages (150 μg/lane) was subjected to 4/20% gradient SDS-PAGE under non-reducing conditions, and subjected to ligand blotting analyses with 5 μg/mL biotinylated apo A-I.
(B) Ligand blotting of the purified apo A-I binding protein in macrophage-conditioned medium. The conditioned medium derived from macrophages was collected, concentrated, and then 10 μg proteins of the conditioned medium were subjected to SDS-PAGE and silver staining (left lane). After purification of apo A-I binding protein, 0.5 μg of the purified protein was used for SDS-PAGE and silver stained to ensure purity (right lane).
(C) Immunoblotting of HDL fraction with anti-progranulin. Human serum and HDL fractions (0.1 μg) were applied for immunobloting with anti-progranulin antibody (clone N19).
(D) Progranulin could form a homo-dimer. Proteins purified by affinity column with immobilized human apo A-I were applied for imunoblotting under non-reducing conditions. Progranulin-like immunoreactive bands were observed in 130kDa and 80 kDa.

applied for imunoblotting in non-reducing conditions. Progranulin-like immunoreactive bands was observed in 130 kDa and 80 kDa (**Fig. 1D**), indicating that progranulin could form a homo-dimer.

Formation of Progranulin-Apo A-I Complex

To confirm the formation of progranulin-apo A-I complex, we performed co-immunoprecipitation analysis (**Fig. 2**). Progranulin-expressing conditioned medium treated with apo A-I was subjected immunoprecipitated with anti-apo A-I antibody to bring down apo A-I and then Western blotted with anti-progranulin antibody or the reverse (**Fig. 2A, B**). The two proteins were recovered simultaneously, indicating that progranulin binds apo A-I. To further confirm the formation of the protein complex, progranulin was translated *in vitro*, co-incubated with apo A-I and then subjected to co-immunoprecipitation analysis (**Fig. 2C, D**). Progranulin, *in vitro* translated under non-reducing conditions (**Fig. 2C, D**), was detected in apo A-I immunoprecipitates while apo A-I was identified in progranulin immunoprecipitates or the reverse. These results indicate that progranulin and apo A-I could bind each other.

Progranulin-Expressing Macrophages

Although we purified and identified progranulin as an apo A-I binding protein from conditioned media derived from macrophages, it is important to confirm that macrophages express and produce progranulin. As shown in **Fig. 3A**, macrophages expressed progranulin and the expression level was dependent on mac-

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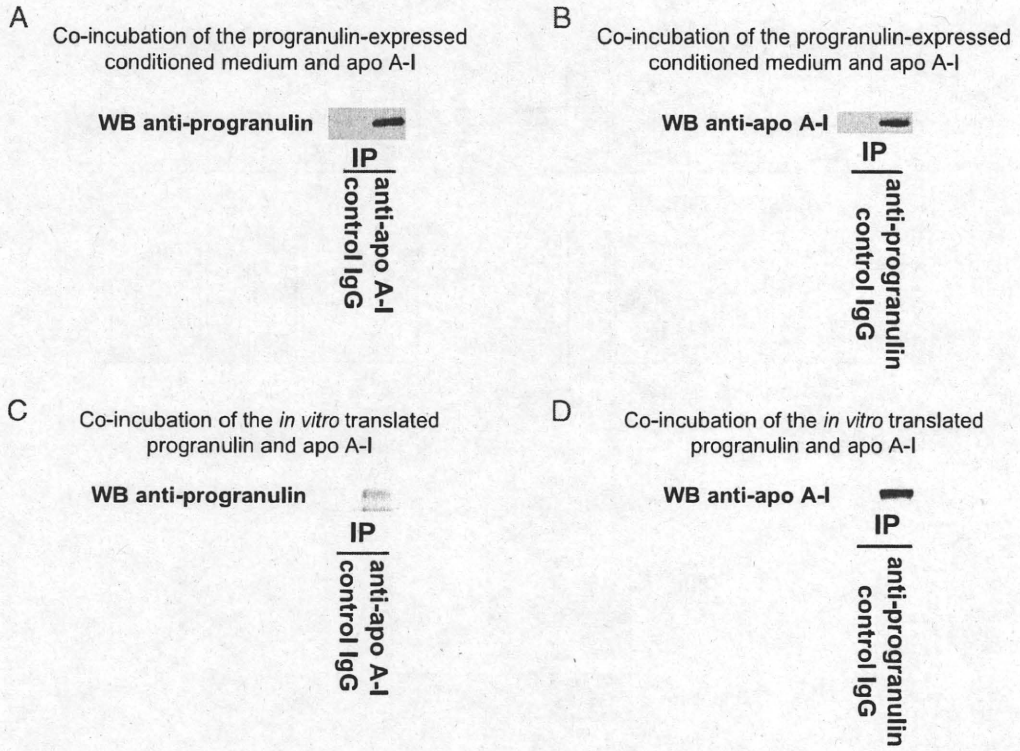

Fig. 2. Progranulin and apolipoprotein A-I form a complex.

(A) Formation of a protein complex from progranulin, transfected and secreted from HEK293 cells, and apolipoprotein A-I. The conditioned medium of HEK293 cells, transfected with pcDNA3.1 expression vector of progranulin, and apo A-I, was immunoprecipitated using rabbit anti-apo A-I antibody. Similar samples were immunoprecipitated using control rabbit IgG as a negative control. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-progranulin antibody, and then visualized.

(B) Formation of a protein complex from progranulin, transfected and secreted from HEK293 cells, and apolipoprotein A-I. The conditioned medium of HEK293 cells, transfected with pcDNA3.1 expression vector of progranulin, and apo A-I, was immunoprecipitated using rabbit anti-progranulin antibody. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized.

(C) Progranulin, translated under native but not reducing conditions, and apolipoprotein A-I form a complex. The *in vitro* translated progranulin and apo A-I were immunoprecipitated using rabbit anti-apo A-I antibody. Similar samples were immunoprecipitated using control rabbit IgG as a negative control. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-progranulin antibody (N19), and then visualized.

(D) Progranulin, translated under native but not reducing conditions, and apolipoprotein A-I form a complex. The *in vitro* translated progranulin and apo A-I were immunoprecipitated using rabbit anti-progranulin antibody. Similar samples were immunoprecipitated using control rabbit IgG as a negative control. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized.

rophage differentiation; gene expression was higher after 5-day culture. Next, we examined progranulin protein production by macrophages (**Fig. 3B**). After cultivation for the indicated time, macrophages were incubated without serum for 24 h and their conditioned media were blotted with anti-progranulin antibody. The production of progranulin protein by macrophages increased in a macrophage differentiation-dependent manner, similar to the gene expression. To examine whether progranulin could augment the expression of progranulin itself, TNF-alpha and IL-1-beta, 7-day-cultured human macrophages were cultured for 24 hours with progranulin. As shown in **Fig. 3C**, progranulin increased the expression of pro-

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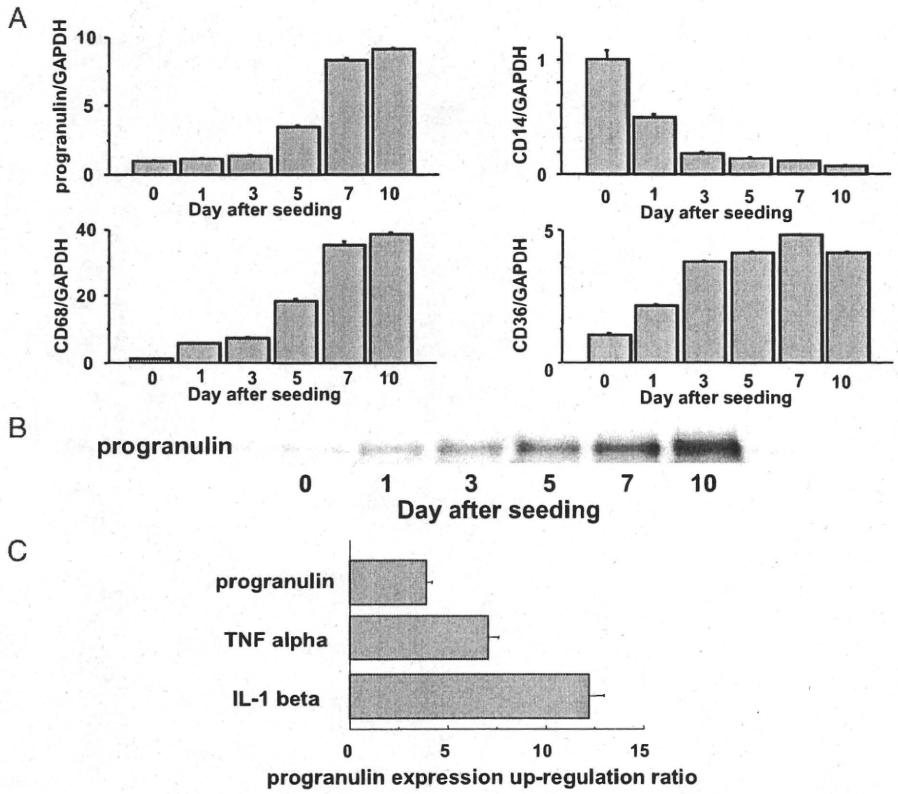

Fig. 3. Human monocyte-derived macrophages express and secrete progranulin into conditioned media.

(A) Expression of progranulin on macrophages is macrophage maturation-dependent. Human monocyte-derived macrophages expressed progranulin and the expression level was dependent on macrophage differentiation by quantitative PCR. Maturation of monocytes-macrophages was associated with over-expression of CD36 and CD68 and under-expression of CD14. Data are the mean ± SE of 5 experiments.

(B) The amount of progranulin secreted by macrophages is macrophage maturation-dependent. Human monocyte-derived macrophages secreted progranulin into the conditioned media and the amount of progranulin protein increased with the differentiation of these cells.

(C) Autocrine regulation of progranulin production. Progranulin could augment the expression of progranulin itself, TNF-alpha (10 ng/mL) and IL-1-beta (10 ng/mL) on macrophages cultured with progranulin. Up-regulation of progranulin expression by progranulin itself, TNF-alpha, or IL-1-beta was observed. Data are the mean ± SE of 5 experiments.

granulin itself, TNF-alpha and IL-1-beta (3.9-, 7.1- and 12.2-fold expressions, respectively).

Progranulin Activates Macrophages and HDL/Apo A-I Suppress as Such Activation

The production of cytokines by macrophages is often regulated in a paracrine or autocrine manner[8]. Next, we examined whether progranulin had effects on macrophages and whether HDL and apo A-I suppress such effects of progranulin. TNF-beta and IL-1-alpha were selected in the present study as representative pro-inflammatory cytokines. Seven-day-cultured macrophages were incubated with progranulin-expressing conditioned medium for 24 h, and TNF-alpha and IL-1-beta gene expression levels were examined by quantitative PCR using a TaqMan Probe (**Fig. 4A**). Progranulin augmented the expression levels of TNF-alpha and IL-1beta, indicating that progranulin affects macrophages. To confirm the binding of progranulin and HDL/apo A-I, the supernatants of macrophages with progranulin-expressing conditioned medium, and apo A-I or HDL under the same conditions were applied for immunoprecipitation with anti-progranulin. The precipitated samples were resolved

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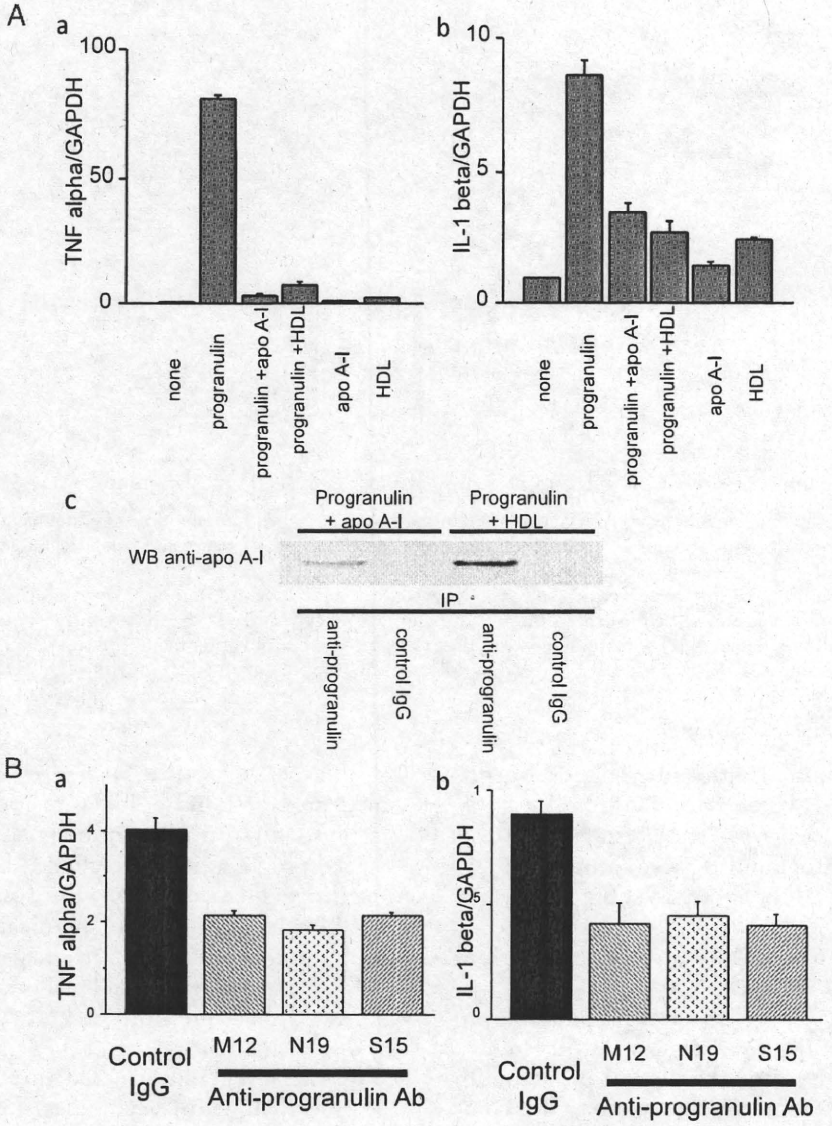

Fig. 4. Effects of progranulin on the expression levels of TNF-alpha and IL-1-beta in macrophages.

(A) Progranulin activated macrophages and HDL/apo A-I suppressed the effects. Seven-day-cultured macrophages were incubated with progranulin-expressing conditioned medium for 24 h and the expression levels of TNF-alpha (a) and IL-1beta (b) were examined with quantitative PCR using TaqMan Probe. Progranulin augmented the expressions of TNF-alpha and IL-1-beta. These properties of progranulin were suppressed by co-incubation with apo A-I (5 μg/mL) or HDL (10 μg/mL). Data are the mean ± SE of 5 experiments. The supernatants of macrophages with progranulin-expressing conditioned medium, and apo A-I or HDL under the same conditions were applied for immunoprecipitation with anti-progranulin. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized (c).

(B) Anti-progranulin antibodies reduced the expression of TNF-alpha and IL-1beta. Macrophages were cultured in the conditioned medium with anti-progranulin antibody for 24 h. Compared to incubation with control IgG (100 μg/mL), anti-progranulin antibody (clone M12, N9 and S15, 100 μg/mL each) suppressed the expressions of TNF-alpha (a) and IL-1-beta (b). Data are the mean ± SE of 5 experiments.

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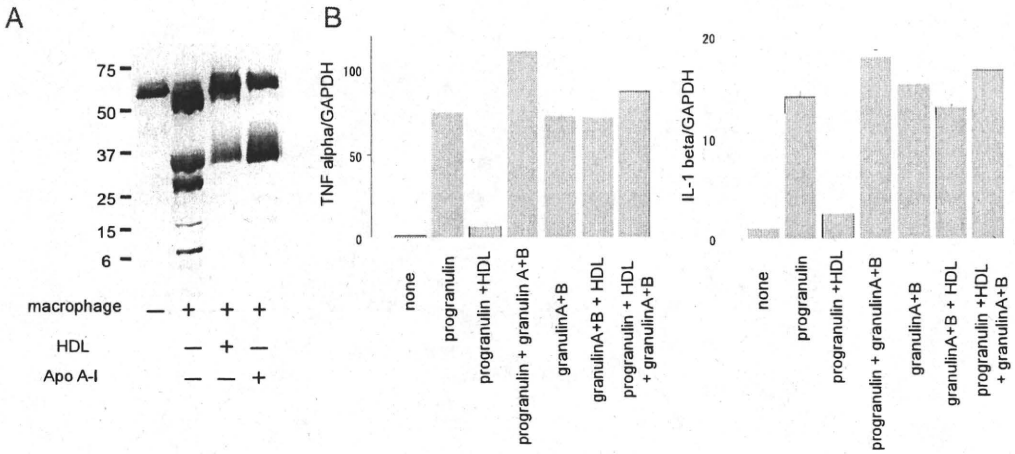

Fig. 5. Progranulin exerts its proinflammatory properties via conversion into granulins.

(A) Progranulin conversion by macrophages. C-terminal-myc-His-tagged progranulin was incubated with or without human macrophages for 24hr. Progranulin was degraded by macrophages and small degraded products were observed, but conversion was suppressed by incubation with HDL or apo A-I.

(B) Suppression of pro-inflammatory properties of progranulin by HDL via inhibition of conversion into granulins. Progranulin could increase the expression of pro-inflammatory cytokines, TNF-alpha and IL-1 beta, and this augmentation was suppressed by incubation with HDL (10 μg/mL). On the other hand, the augmentation effect on the expression of TNF-alpha and Il-1 beta of granulin was not blocked by incubation with HDL. Data are the mean ± SE of 4 experiments.

by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized (c). These results indicated that the effects of progranulin were suppressed by co-incubation with HDL or apo A-I via binding.

Anti-Progranulin Antibodies Reduce the Expression of TNF-Alpha and IL-1Beta

To examine whether the effect of progranulin on macrophages is autocrine in nature, fully differentiated macrophages were cultured with anti-progranulin antibody for 24 h. Compared to incubation with control IgG, anti-progranulin antibody (clone M12, N9 and S15) suppressed the expression of TNF-alpha and IL-1-beta (**Fig. 4B**). These results suggest that progranulin is secreted by macrophages and its effect is autocrine in nature, indicating that progranulin is an autoactivating molecule.

Progranulin Exerts its Properties Via Conversion into Proinflammatory Granulins

We examine whether progranulin could be converted into fragments by macrophages and exerted its properties via conversion into proinflammatory granulins. C-terminal-myc-His-tagged progranulin construct was transfected into HEK293T, and the conditioned media were incubated with or without human macrophages for 24 hr. Probond beads were added to the media to capture His-tagged proteins. Next, the incubated probond beads were applied for immunoblotting with anti-myc antibody. C-terminal-myc-His-tagged progranulin was degraded by macrophages and small degraded products were observed but not with HDL incubation (**Fig. 5A**), indicating that prgranulin could be converted by macrophages.

Next, we examined whether granulin could increased the expressions of TNF-alpha and IL-1-beta, and the increment could be suppressed by HDL (**Fig. 5B**). Progranulin could increase the expressions of pro-inflammatory cytokines, TNF-alpha and IL-1-beta, and this augmentation was suppressed by incubation with HDL. On the other hand, the augmentation effect on the expressions of TNF-alpha and Il-1-beta of granulin was not blocked by incubation with HDL (**Fig. 5B**, left and right panels, respectively). These results suggested that progranulin could exert its pro-inflammatory properties via conversion into granulins, and that HDL could suppress the pro-inflammatory properties of progranulin by inhibiting the conversion into granulins.

Discussion

Progranulin, a PC-cell-derived growth factor (PCDGF), or acrogranin, was purified from the conditioned media of transformed cell lines as an auto-

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crine growth factor[11]. It is reported to be involved in cancer progression[8], development[12], wound healing[13], and myeloid cell proliferation[14], whereas mutation of progranulin causes frontotemporal dementia[15, 16]. In the present study, we demonstrated that the macrophage-secreted factor was approximately 130 kDa, while the purified protein identified as progranulin was 80 kDa. The 130-kDa HDL binding protein might be a homodimer or heteromer that includes progranulin, which is known to be glycosylated and to have disulfide bonds[11].

Ong and colleagues[14] used myeloid cell lines and reported the overexpression of progranulin in macrophages and monocyte-derived dendritic cells, and that the level of expression was dependent on cell differentiation. Our results also demonstrated that the differentiation of human monocyte-derived macrophages was associated with increased expression levels of progranulin and that progranulin expression was regulated in an autocrine fashion in human monocyte-derived macrophages. We also demonstrated that apo A-I, the major component of HDL, suppressed the conversion of progranulin into pro-inflammatory granulins on human peripheral monocyte-derived macrophages. This is in agreement with others, who indicated that the protein precursor (progranilin) and its processed fragments (granulins) are both bioactive and pro-inflammatory[8].

The role of progranulin in the inflammatory process was initially explored in research on the functions of secretory leukocyte protease inhibitor (SLPI) in wounds[10]. Using a yeast two-hybrid approach, with SLPI as the bait, Zhu and colleagues[10] demonstrated that progranulin is associated with SLPI[10]. This interaction was confirmed by immunoprecipitation experiments demonstrating that progranulin regulates inflammation through a tripartite loop with SLPI, which protects progranulin from proteolysis, and elastase, which digests progranulin between granulin/epithelin domains, generating smaller granulin/epithelin peptides. SLPI blocks this proteolysis, by inhibiting both elastase activity directly and by binding progranulin and sequestering it from the enzyme[10]. Intact progranulin is anti-inflammatory through the inhibition of certain actions of TNF-alpha, while proteolytic peptides may stimulate the production of pro-inflammatory cytokines, such as IL-8[8]. We suppose that HDL/apo A-I have anti-inflammatory effects on macrophages through the formation of a complex with progranulin and prevent the conversion of progranulin into granulins by elastase secreted by macrophages such as SLPI, which is reported as a neutrophil-derived anti-inflammatory factor[10]. In this study, the possibility could be not rejected that progranulin binds HDL particles itself but free apoA-I is dissociated from HDL, which is just a reserve of apoA-I *in vivo* according to the limitations of the experimental conditions. In the near future, our colleagues will demonstrate which of apo A-I, lipid-free apo A-I dissociated from HDL, apo A-I in pre-beta HDL or apo A-I in HDL will bind to progranulin *in vivo*.

An unstable and subsequently ruptured atherosclerotic coronary plaque superimposed on thrombosis constitutes the most common pathological background of acute coronary syndrome[7]. High levels pro-inflammatory cytokines have been found in unstable angina, possibly supporting their role in acute coronary syndrome[7]. Cytokines induce their own expression in an autocrine fashion and also the expression of various adhesion molecules via the cellular transcription factor NF-kappaB[17]. Monocytes adhering to the endothelium and penetrating the plaque (macrophages) are activated by several paracrine/autocrine pro-inflammatory mediators. At this crucial stage, activated macrophages then synthesize and secrete pro-inflammatory cytokines TNF-alpha and IL-1-beta[7]. The human and murine progranulin promoter contains potential inflammation-related promoter elements[18, 19]. Furthermore, TNF-alpha and IL-1-beta activate progranulin gene expression through the NF-kappaB system[18, 19]. The progranulin-granulin loop might play a role in autoactivation of macrophages. Given its actions in atherosclerosis, progranulin may prove a useful clinical target, both for prognosis and therapy.

In summary, we identified progranulin/proepithelin/acrogranin/PCDGF as a macrophage-derived factor whose properties were suppressed by binding to HDL/apo A-I. The normal function of progranulin is complex; the full-length form of the protein has both trophic and anti-inflammatory activities, whereas proteolytic cleavage generates granulin peptide that promotes inflammatory activity. Based on our results, we propose that HDL prevents the conversion of progranulin into its degraded proinflammatory products, granulins; however, the mechanisms involved in HDL-induced suppression of the pro-inflammatory effects of progranulin have not fully elucidated. Further studies are necessary to identify these mechanisms.

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