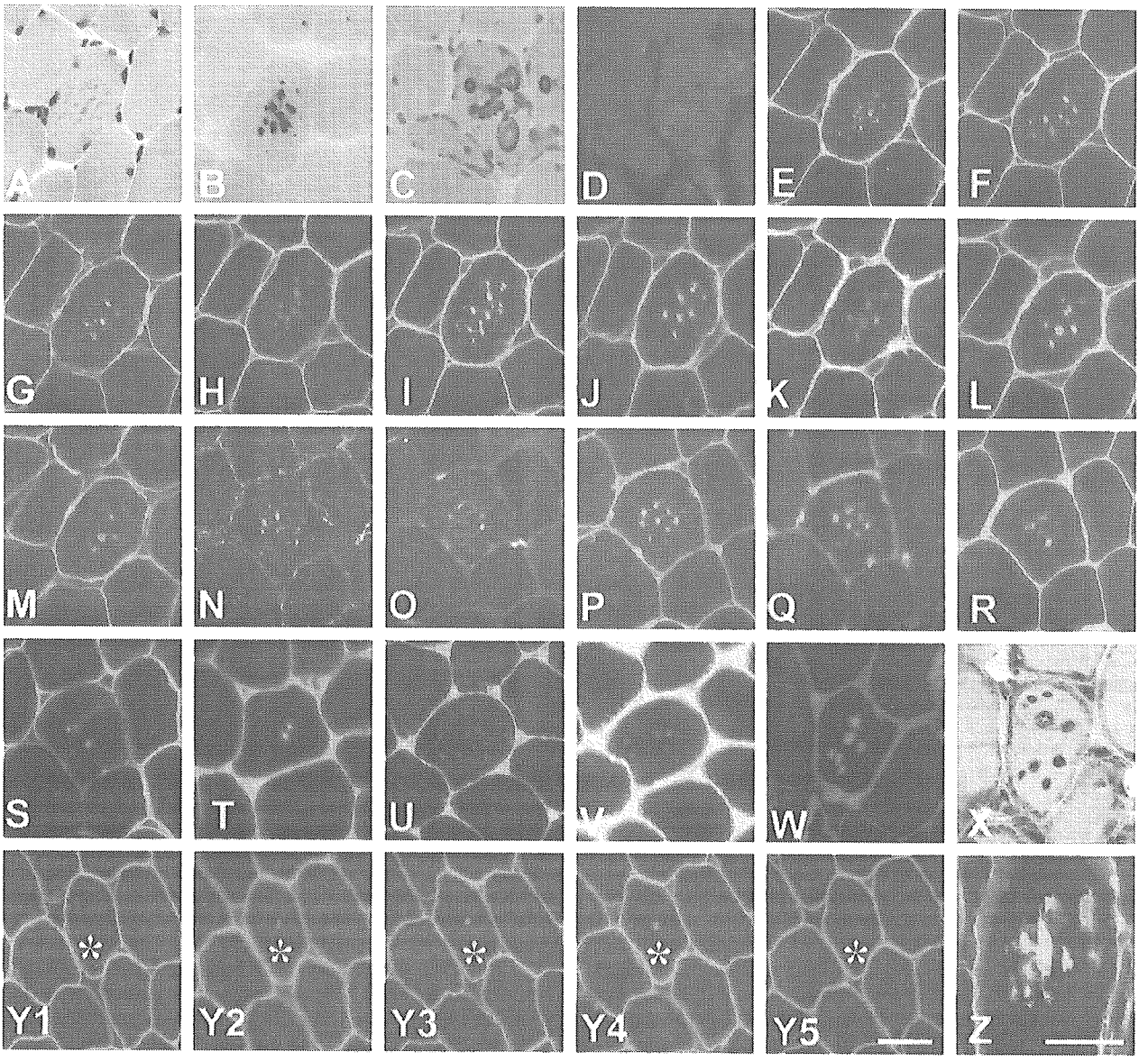

FIGURE 1. Histochemistry and immunohistochemistry. Transverse sections of skeletal muscle biopsies from Danon disease patients. Several fibers contain scattered tiny basophilic intracytoplasmic vacuoles (A): H&E. The vacuolar membrane has high nonspecific esterase (B) and acetylcholinesterase (C) activities. None of the vacuoles bind to α-bungarotoxin (D). Sections were stained with antibody against the C-terminus of dystrophin (E), the rod domain of dystrophin (F), the N-terminus of dystrophin (G), laminin α2 (H), α-sarcoglycan (I), β-sarcoglycan (J), γ-sarcoglycan (K), δ-sarcoglycan (L), dystrobrevin (M), α-dystroglycan (N), utrophin (O), dysferlin (P), β-dystroglycan (Q), perlecan (R), caveolin-3 (S), collagen IV (T), fibronectin (U), collagen VI (V), integrin β1 (W), and agrin (X). The vacuolar membranes were immuno-positive with most of the primary antibodies, although reactivity of these proteins was variable. The results are summarized in Table 1. Transverse 5-μm serial sections (Y1–Y5) and longitudinal section (Z) of muscle from Danon disease patient showing immunoreaction for dystrophin. Vacuolar membrane in muscle fiber (*) is not connected to the sarcolemma but is closed. Longitudinal section shows that the vacuoles are spherical or oval. (D–W, Y1–Y5, Z): FITC-labeled staining; (X): DAB staining. (C–S, U, V, Y1–Y5): serial sections. Scale bars: (A–W, Y1–Y5) = 20 μm; (Z) = 30 μm.

is a submembranous protein structurally similar to dystrophin and is widely expressed, albeit at low levels, in the sarcolemma (17). Integrin β1 and α7 are transmembranous proteins and form a complex with each other in the sarcolemma (18). Dysferlin and caveolin-3 are also sarcolemmal proteins and are responsible for limb-girdle muscular dystrophy (LGMD) 2B and LGMD 1C, respectively (19, 20). Extracellular proteins, collagen IV, perlecan, fibronectin, agrin, and laminin, are the

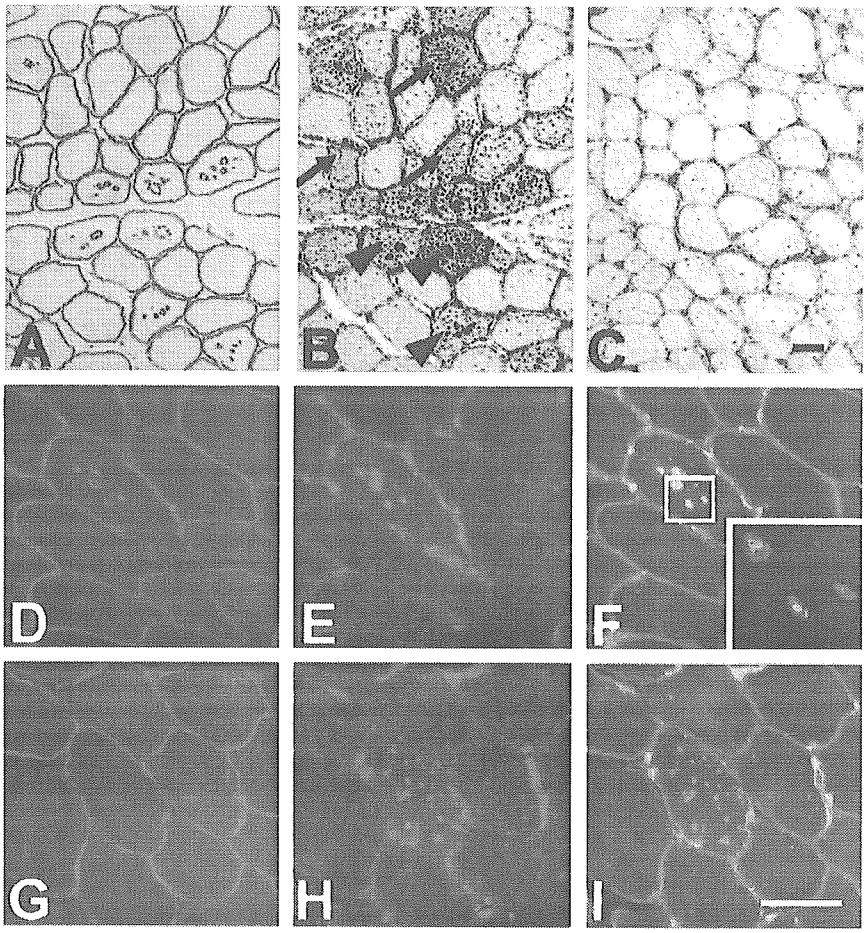

FIGURE 2. Indirect immunohistochemistry. Transverse sections of skeletal muscle stained with DAB for dystrophin (A) and LIMP-1 (B, C). (A, B) Danon disease patient; (C): Normal control. In Danon disease some muscle fibers express both LIMP-1 and dystrophin (arrowheads), whereas some muscle fibers show overexpression of LIMP-1 with absence of dystrophin (arrows). Normal control showed almost no expression of LIMP-1 (C) in muscle fibers. Scale bar = 40 μm. Double immunohistochemistry. Transverse sections of skeletal muscle from Danon disease patient, stained for dystrophin and LIMP-1. LIMP-1 is strongly accumulated inside the muscle fibers (D, G). In some muscle fibers, LIMP-1-positive accumulations are clearly surrounded by dystrophin immunopositive membrane (D–F). These vacuoles are the AVSF. In other muscle fibers, LIMP-1-positive accumulations are not surrounded by dystrophin (G–I). (D, G): dystrophin; (E, H): LIMP-1; (F, I): merged. Scale bar = 30 μm.

main components of the basal lamina. Collagen VI is present in the interstitium but is associated directly with collagen IV (21). We observed very little staining of only collagens IV and VI in vacuolar membranes, indicating that the membranes hardly contain these collagens. Based on our findings, we deduce that the vacuolar membrane of AVSFs in Danon disease and related AVMs have most of the sarcolemmal proteins ranging from cytoplasmic dystrophin to the extracellular laminin.

The vacuolar membranes of AVSF have abundant activities of AChE similar to neuromuscular junctions. Nevertheless, they are distinct from motor endplates because the membranes lacked AChRs. In the early stages of formation of the neuromuscular junction, AChE and AChRs are localized diffusely throughout the sarcolemma. When axon terminals make contact with muscle cells, postjunctional folds are quickly formed. In this process, AChE and AChRs accumulate at junctions and disappear from the extra-junctional sarcolemma (22, 23). These facts support our hypothesis that the vacuoles are intracellular enclosed spaces, because, if AVSF were derived from sarcolemma, then AChE-expressing vacuoles should be located near neuromuscular junctions rather than scattered in the cytoplasm. Furthermore, the presence of AChE without AChRs clearly indicates that the vacuolar membranes are distinct from either junctional or extra-junctional sarcolemma and suggests that they are formed through a unique process.

In the intracellular degradative process called autophagy, "isolation membranes" initially sequesters portions of cytoplasm to be degraded and forms "autophagosomes," which then fuse with lysosomes and become "autolysosomes." The cytoplasm sequestered in autolysosomes is then digested by proteolytic enzymes provided by the lysosomes. Most autophagic vacuoles in Danon disease are autolysosomes rather than autophagosomes, which lack enzymatic activity. These are indicated by the demonstration of many LIMP-1-positive accumulations scattered throughout the fibers (24, 25) and the autophagic nature of the vacuoles on electron microscopy. Actually, small basophilic granules on hematoxylin and eosin are most likely these autolysosomal accumulations as suggested by their pattern of distribution and the fact that lysosomes are basophilic on H&E. Moreover, some clusters of autolysosomes are surrounded by membranes with sarcolemmal features but others are not. In support of this notion, ultrastructural studies identified 2 types of autophagic vacuoles: 1) clusters of autophagic vacuoles not surrounded by membranes or basal lamina, and 2) vacuoles containing various

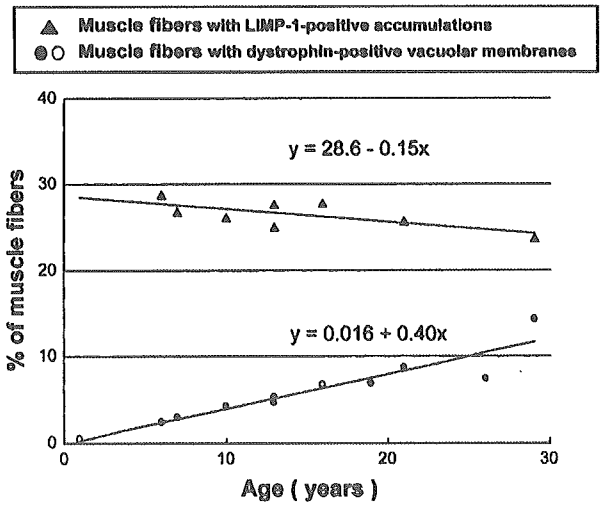

FIGURE 3. Relationship between age of the patients with Danon disease and number of muscle fibers with vacuoles highlighted with dystrophin or LIMP-1 on immunohistochemistry. The open circles show the only patient who had 2 muscle biopsies. The muscle fibers (circles) with intracytoplasmic vacuoles surrounded by dystrophin immuno-positive membrane (AVSFs) increased with age ($r = 0.936$). The muscle fibers (triangles) with overexpression of LIMP-1 showed a slight decrease with age ($r = 0.353$). r, Pearson's linear correlation coefficient.

autophagic materials encircled by membranes with basal lamina along the luminal side.

The proportion of muscle fibers with AVSFs increased with age in Danon disease and LAMP-2-deficient mice. In contrast, muscle fibers with LIMP-1-positive autolysosomal accumulations existed even in young patients and decreased slightly with age. It is most likely that the formation of these autolysosomal accumulations, which are clusters of autophagic vacuoles seen on electron microscopy, is a primary change in muscle fibers of Danon disease and related AVMs. The formation of peculiar membranes with sarcolemmal features around the autophagic vacuoles is hence a secondary phenomenon. Since muscle symptoms progress slowly in Danon disease, the development of muscle symptoms might be associated more closely with the formation of the unusual autophagic vacuoles rather than directly with the deficiency of LAMP-2.

LAMP-1 is the autosomal paralogous counterpart of LAMP-2 and both are thought to protect lysosomal membrane and cytoplasm from proteolytic enzymes within the lysosomes. LAMP-2 is tissue-specific but unlike LAMP 1, which is ubiquitously expressed, its expression is likely to be specifically regulated (26). Inhibition of LAMP-1 function results in failure of fusion of lysosomal and plasma membranes and therefore impaired exocytosis (27), a process usually by which cytoplasmic debris in the autophagosomes are extruded out from the cell through the sarcolemma (28). We therefore assume that LAMP-2 deficiency might likewise be related to dysregulation of exocytosis, leading to the development of the

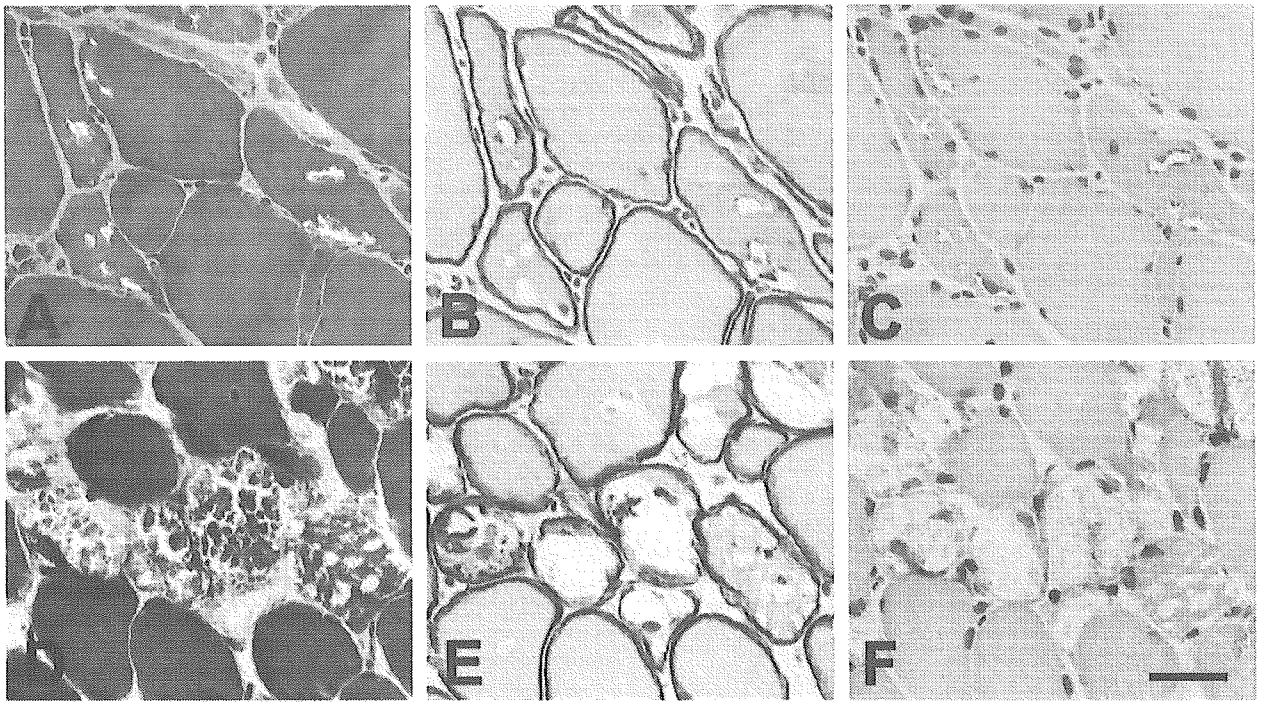

FIGURE 4. Transverse serial sections of muscle of patient with DMRV/HIBM (A–C) and with childhood AMD (D–F). Only a few rimmed vacuoles in DMRV/HIBM showed presence of dystrophin. In AMD, dystrophin is present on some vacuolar materials. However, no vacuolar membranes have AChE activity in DMRV/HIBM and AMD. (A, D): Gomori-trichrome stain; (B, E): AChE stain; (C, F): immunohistochemistry against dystrophin with DAB. Scale bar = 30 μm.

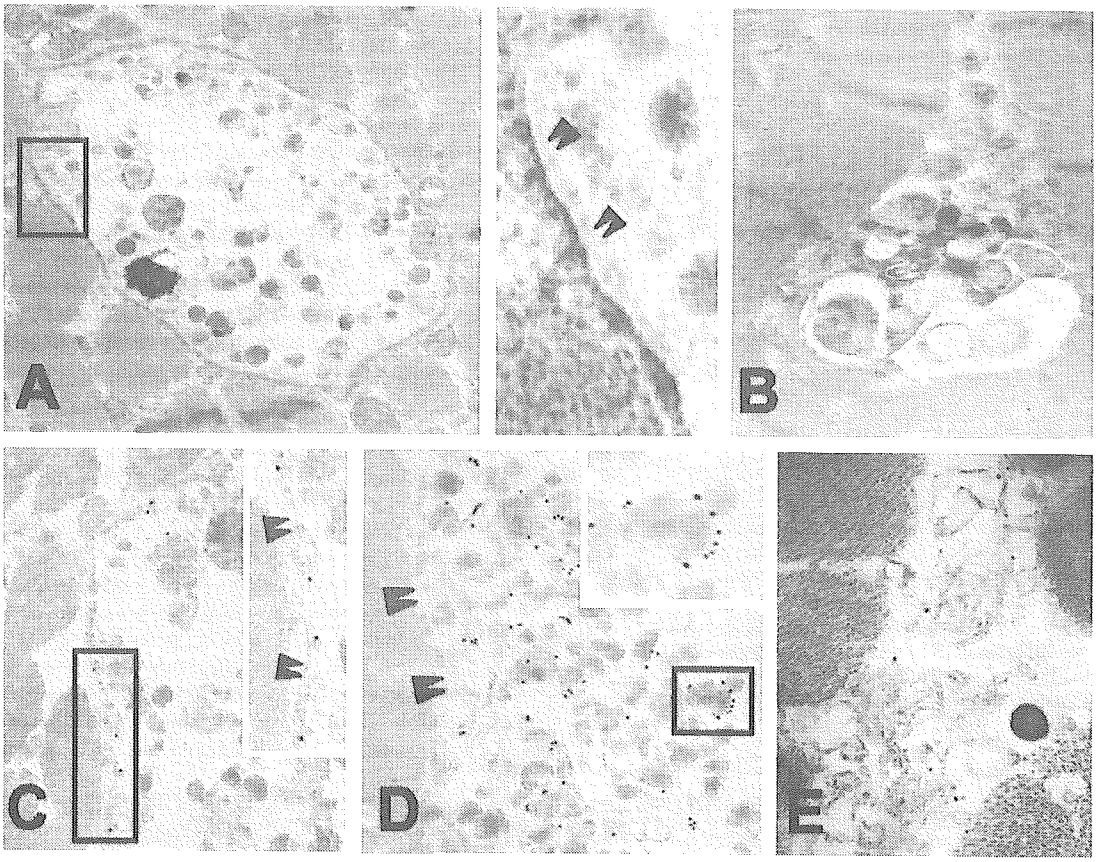

FIGURE 5. Electron micrograph in muscle from Danon disease patient. Scattered in the muscle fibers were clusters of autophagic vacuoles (A) containing cytoplasmic debris, electron dense material, and myeloid bodies. Some of these clusters were encircled by a membrane with basal lamina (paired arrowheads) on its luminal side, while other clusters are not limited by a membrane (B). Electron immunohistochemistry after single labeling with dystrophin or LIMP-1 antibody shows localization of the proteins in autophagic vacuoles (C–E). In the clusters with membranes, that is, the AVSF (A), the immunogold particles show dystrophin (C) along the vacuolar membrane (paired arrowheads), and the immunogold particles show LIMP-1 (D) around autophagic material inside autophagic vacuoles. In contrast, in the clusters not surrounded by membranes, immunogold particles show LIMP-1 around autophagic materials with absence of dystrophin (E). Original magnifications: (A) 15,000×; (B) 18,000×; (C) 20,000×; (D) 18,000×; (E) 30,000×.

unusual autophagic vacuoles with unique sarcolemmal features.

TfR and LDL-R are found in the membranes of recycling endosomes. In contrast, Rab5 and VAMP-7 are present in the membranes of early and late endosomes (29). We revealed the presence of all of these proteins in the fibers with autophagic vacuoles, indicating that in addition to the lysosomal system, the endosomal system is activated in Danon disease and related AVMs. Interestingly, VAMP-7 was increased in nonvacuolated fibers without autolysosomal accumulations, suggesting that maturation to late endosomes could prevent the formation of the unique vacuolar membranes.

Most of the vacuolar membranes were closed and were not connected to the sarcolemma in Danon disease. The autolysosomes containing cytoplasmic debris are therefore seen to be entrapped within the lumen of the vacuoles, and as such can be possibly considered to be extracellular space. Together with the observations that most AVSF did not accumulate in the subsarcolemmal region but were scattered in the cytoplasm, our findings suggest that the unique vacuolar membranes may be formed in situ in cytoplasm by a mechanism other than indentation of sarcolemma. One hypothesis is that the vacuolar membrane with basal lamina might be produced around clusters of autolysosomes (Fig. 6). The membranes surrounding the autophagic vacuoles might have originated from the lysosomal membrane or the isolation membrane that elongates and develops into the membrane of autophagosome (30), or is formed in situ and entirely de novo. If the vacuolar membranes are formed within the muscle fibers, it is compatible with the observation that the vacuolar membranes lack collagens IV and VI because collagens are thought to be produced mainly in the interstitium. Further studies are still necessary to understand the mechanism of the formation of these peculiar vacuolar membranes.

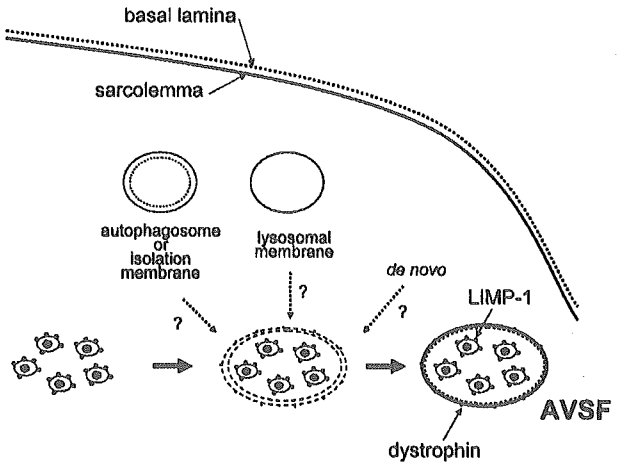

FIGURE 6. Schematic diagram of autophagic vacuoles in muscle fiber of patients with Danon disease. The membranes of the AVSFs were closed and were not connected to the sarcolemma. We suggest that the unique vacuolar membranes may be formed in situ in cytoplasm by a mechanism. One hypothesis is that the vacuolar membrane with basal lamina might be produced around clusters of autolysosomes, as illustrated.

In XMEA, vacuolar membranes have not been reported to have AChE activity (6). However, the strong similarity of the pathologic characteristics to Danon disease naturally raised the question of whether AChE activity is present in the vacuolar membranes in XMEA. Indeed, our Japanese patient with probable XMEA showed AChE activity in the vacuolar membranes. Therefore, Danon disease, XMEA, infantile AVM, and adult-onset AVM with multiorgan involvement share a common pathologic feature, namely, AVSF with AChE activity in the vacuolar membranes. Nevertheless, XMEA, infantile AVM, and adult-onset AVM are genetically different from Danon disease, as indicated by the presence of LAMP-2, which is absent in Danon disease. The observation that some features are not seen in Danon disease, like the presence of multilayered basal lamina and the deposition of C5b-9 over the surface of the muscle fiber, raise a possibility that some of these diseases might be allelic to XMEA, albeit different clinical phenotypes.

The autophagic vacuoles in AMD and rimmed vacuoles were reported to occasionally show presence of dystrophin. Nevertheless, these vacuoles are distinct from the AVSF seen in Danon disease and related AVMs, because the frequency of the vacuoles with sarcolemmal features is much less and most of sarcolemmal proteins are not consistently present in the vacuolar membranes of AMD and the rimmed vacuolar myopathies. Moreover, the activities of AChE and NSE were never found in the vacuolar membranes of these myopathies. In addition, on electron microscopy, the vacuolar membranes with basal lamina, such as those seen in Danon disease and related AVMs, were not found in AMD, DMRV/HIBM, or SIBM. According to the classification of De Bleecker et al, the AVSF may belong to type 1 vacuoles as the boundaries of type 1 vacuoles reacted for laminin, β-spectrin, and dystrophin (31). However, type 1 vacuoles were thought to be open to extracellular space and arise from invagination of the sarcolemma. Moreover, the membranes of AVSF have not only many sarcolemmal and extracellular proteins but also AChE activity, and may be formed in situ in cytoplasm as described above. Therefore, we think that the AVSF are a new, highly specific subtype of type 1 vacuoles.

Although the mechanism of their production still remains a mystery, overall, AVSF with AChE activity delineate Danon disease, XMEA, infantile AVM, and adult-onset AVM with multiorgan involvement from other AVMs. Most likely, this unique pathologic finding will probably be found in more diseases and therefore the list of AVMs in this group is likely to expand.

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IMMUNOLOGY ORIGINAL ARTICLE

The secreted form of the p40 subunit of interleukin (IL)-12 inhibits IL-23 functions and abrogates IL-23-mediated antitumour effects

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Summary

Interleukin (IL)-23 is a heterodimeric cytokine consisting of a novel p19 molecule and the p40 subunit of IL-12. Since secreted p40 can act as an antagonist for IL-12, we investigated whether p40 also inhibited IL-23-mediated immunological functions. p40 did not induce interferon (IFN)-γ or IL-17 production from splenocytes but impaired IL-23-induced cytokine production by competitive binding to the IL-23 receptors. Furthermore, a mixed population of murine colon carcinoma Colon 26 cells transduced with the *p40* gene and those transduced with the *IL-23* gene developed tumours in syngenic mice, whereas the IL-23-expressing Colon 26 cells were completely rejected. p40 also suppressed IFN-γ production of antigen-stimulated splenocytes and IL-23-mediated cytotoxic T-lymphocyte activities in the mice that rejected Colon 26 cells expressing IL-23. p40 can thereby antagonize IL-23 and is a possible therapeutic agent for suppression of IL-23 functions.

Keywords: anergy; suppression; tolerance; cytokines; interleukins; T cells

Introduction

The recently discovered cytokine interleukin (IL)-23 is a covalently linked heterodimeric cytokine that consists of a novel p19 subunit, which is structurally related to the p35 subunit of IL-12, and the p40 subunit of IL-12.[1] IL-23 is primarily secreted from activated dendritic cells and macrophages, although p19 mRNA is expressed in various tissues, and induces interferon (IFN)-γ production and proliferation of phytohaemagglutinin (PHA) T-cell blasts.[1] Although some of the immunological activities of IL-23 are similar to those of IL-12, IL-23 has distinct functions in that it induces production of a proinflammatory cytokine, IL-17, from activated T cells and promotes antigen presentation of splenic $CD8^+$ dendritic cells, which have not to date been reported as IL-12 activities.[2,3] A receptor for IL-23 is composed of IL-23R, a novel receptor subunit related to IL-12Rβ2, and the IL-12Rβ1 subunit of the IL-12 receptor.[4] IL-12Rβ2 is selectively expressed on T helper type 1 (Th1) but not Th2 cells, while IL-12Rβ1 is consistently expressed on both cell types.[5,6] IL-23R is expressed on memory and activated but not naïve $CD4^+$ T cells; thus, the responses of $CD4^+$ T cells to IL-23 or IL-12 are attributable to differential expression of IL-23R or IL-12Rβ2.[4]

Identification of IL-23 has resulted in revision of the *in vivo* roles of IL-12 in the pathogenesis of experimental autoimmune encephalomyelitis (EAE). Previous studies suggested that IL-12 was a key mediator for EAE

Abbreviations: ANOVA, analysis of variance; Con A, concanavaline A; CTL, cytotoxic T lymphocyte; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; IRES, internal ribosomal entry site; MHC, major histocompatibility complex; p19, p19 subunit of IL-23; p35, p35 subunit of IL-12; p40, p40 subunit of IL-12; PHA, phytohaemaggulutinin; RT-PCR, reverse transcriptase–polymerase chain reaction; SD, standard deviation; Th, T helper.

because administration of the neutralizing antibodies for IL-12 to animals with this autoimmune disease ameliorated symptoms.[7,8] In contrast, several reports suggested a controversial role for IL-12 in the pathogenesis of EAE; p35- and IL-12Rβ2-deficient mice still remained susceptible to EAE, whereas p40- and IL-12Rβ1-deficient mice did not develop EAE.[9–11] A recent report showed that p19-deficient mice did not develop EAE, demonstrating that IL-23 rather than IL-12 was involved in the pathogenesis of EAE.[12]

p40, the common subunit of IL-23 and IL-12, is experimentally secreted when neither p19 nor p35 is expressed, and can bind to IL-12Rβ1, acting as an antagonist for IL-12;[13–16] thus, the production of p40 *in vivo* can be a novel self-regulatory process to suppress IL-12 functions. IL-12Rβ1 is an essential signalling component of both IL-23 and IL-12 receptors; in fact, memory T-cell functions were impaired in individuals with genetic IL-12Rβ1 deficiency as a result of reduced responsiveness to both IL-23 and IL-12.[17–19] We therefore presumed that the secreted p40 also impaired IL-23-mediated responses, and examined the inhibitory effects of p40 *in vitro* and *in vivo*.

Materials and methods

Animals and cells

C57BL/6 and BALB/c mice (6- to 8-week-old females) were purchased from Japan SLC (Hamamatsu, Japan). Murine colon carcinoma Colon 26 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS). COS-7 cells and murine sarcoma Meth A cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS.

Expression of cytokines and cytokine production assay

Murine *p19*, *p35* and *p40* genes were generated by reverse transcriptase–polymerase chain reaction (RT-PCR).[20,21] The *p19* and *p35* genes were linked to the *p40* gene using an internal ribosomal entry site (IRES) to construct an expression vector with pcDNA3 (Invitrogen, Carlsbad, CA): for IL-12, p35-IRES-p40; for IL-23, p19-IRES-p40; and for p40, IRES-p40. COS-7 cells were transfected with these vectors using Lipofectin reagent (Invitrogen) and cell-free supernatants were collected after 48 hr. The amounts of p40 in the supernatants were measured with an enzyme-linked immunosorbent assay (ELISA) kit to detect p40 (Biosource, Camarillo, CA). Splenocytes from C57BL/6 mice were stimulated with 5 μg/ml concanavaline A (Con A) for 48 hr and then incubated for 24 hr with various amounts of supernatants from the transfected COS-7 cells. The amounts of IFN-γ and IL-17 were measured with respective ELISA kits (R & D Systems, Minneapolis, MN).

RT-PCR for each subunit of cytokines

First-strand cDNA was amplified for 30 cycles with the following primers and conditions: for the *p19* gene, 5′-CACAGAGCCAGCCAGATCTGAGAAGC-3′ (as a 5′-primer) and 5′-CCATGGGAACCTGGGCATCCTTAAGC-3′ (as a 3′-primer), and an annealing temperature of 60 ; for the *p35* gene, 5′-ACCTGCTGAAGACCACAGATG-3′ (5′-primer) and 5′-TTTCACTCTGTAAGGGTCTGC-3′ (3′-primer), and an annealing temperature of 57 ; for the *p40* gene, 5′-CCAGAGACATGGAGTCATAG-3′ (5′-primer) and 5′-GGGTCTGGTTTGATGATGTC-3′ (3′-primer), and an annealing temperature of 60 ; for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, 5′-ACCACAGT CCATGCCATCAC-3′ (5′-primer) and 5′-TCCACCACCC TGTTGCTGTA-3′ (3′-primer), and an annealing temperature of 60 .

Transduction of tumour cells and animal experiments

Colon 26 cells were retrovirally transduced with LXSN vectors bearing p35-IRES-p40,[20] p19-IRES-p40[21] or p40 DNA. G418 (600 μg/ml; Invitrogen)-resistant Colon 26 cells were selected and were stained with fluoresecin isothiocyanate-conjugated anti-H-2K^d (BD PharMingen, San Diego, CA), anti-H-2D^d (BD PharMingen) or anti-H-2L^d (e-Bioscience, San Diego, CA) monoclonal antibodies. The expression profiles were analysed with FACScan and Cell Quest software (Beckton Dickinson, Mountain View, CA). For animal experiments, cells (1 10^6) were inoculated subcutaneously into BALB/c mice and the tumour volume was calculated according to the formula 1/2 length width^2.

IFN-γ production and cytolytic assay in splenocytes

Mice were inoculated with tumour cells (1 10^6) and killed on day 24. Splenocytes were stimulated with 60 Gy-irradiated Colon 26 cells for 24 hr and were measured for IFN-γ production. For the cytolytic assay, splenocytes of the mice that had rejected IL-23-producing Colon 26 cells were stimulated with mitomycin C (60 μg/ml; Sigma, St Louis, MO)-treated cells for 5 days. The mitomycin C treatment inhibits cell division but not cytokine secretion up to 7 days. Cytolytic activities against Colon 26 and Meth A cells were measured with the standard 6-hr ^{51}Cr release assay at various effector:target ratios.

Results

p40 did not induce cytokine production in splenocytes

COS-7 cells were transfected with p35-IRES-p40, p19-IRES-p40 or IRES-p40 DNA (COS-7/IL-12, COS-7/

Table 1. Production of p40-containing proteins in COS-7 cells

Transfectants	p40-containing protein[1] (ng/ml)
COS-7/IL-12	2 48 ± 0 03
COS-7/IL-23	7 16 ± 0 85
COS-7/p40	4 88 ± 0 14

[1]Amounts of p40-containing proteins secreted in the supernatants were expressed as corresponding amounts of interleukin (IL)-12 used as standard in a p40-specific enzyme-linked immunosorbent assay (ELISA) kit. Data represent mean ± standard deviation (n 3).

IL-23 or COS-7/p40, respectively) and the amounts of p40-containing proteins secreted from the transfected cells were measured (Table 1). These transfectants, but not untransfected COS-7 cells, secreted the p40-containing proteins, and the supernatants of respective transfectants were used as IL-12, IL-23 and p40. We examined whether these molecules induced IFN-γ production in freshly isolated and Con A-activated splenocytes (Fig. 1a). Freshly isolated splenocytes produced IFN-γ in response to supernatants of COS-7/IL-12 but not COS-7/IL-23 cells; however, Con A-activated splenocytes produced IFN-γ when the cells were stimulated either with the supernatants of COS-7/IL-12 (6043 ± 391 pg/ml, mean ± standard deviation (SD), produced under the culture with 12 5% of the supernatants) or with those of COS-7/IL-23.

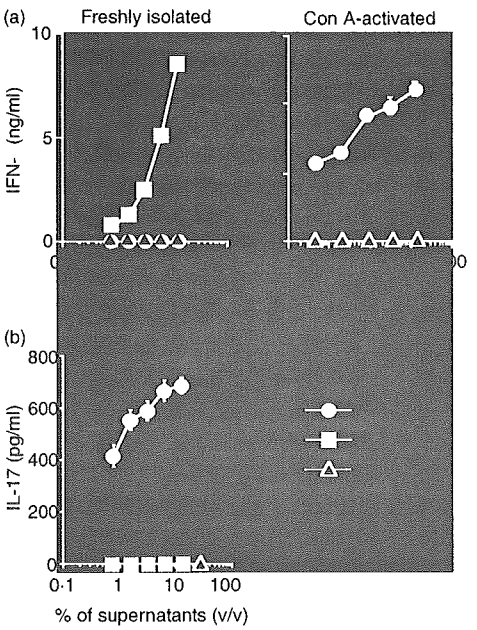

Figure 1. Cytokine production induced by interleukin (IL)-12 and IL-23 but not p40. Freshly isolated or concanavaline A (Con A)-activated splenocytes of C57BL/6 mice were cultured with various concentrations of supernatants from COS-7/IL-12 (closed squares), COS-7/IL-23 (closed circles) or COS-7/p40 (open triangles) cells. The amounts of interferon (IFN)-γ (a) and IL-17 (b) were measured in the supernatants of splenocytes. Data represent mean ± standard deviation (SD) of triplicate samples. v/v, volume/volume.

The ability of splenocytes to respond to IL-23 is attributable to Con A-mediated induction of IL-23R expression.[3] Con A-activated splenocytes also produced IL-17 when the cells were stimulated with the supernatants of COS-7/IL-23 but not those of COS-7/IL-12 (Fig. 1b). In either type of splenocyte, supernatants of COS-7/p40 cells did not induce IFN-γ or IL-17 production, showing that secreted p40 was not agonistic to IFN-γ or IL-17 production.

p40 inhibited IL-23-mediated cytokine production

We examined whether p40 inhibited IFN-γ production induced by IL-23 as well as IL-12 (Fig. 2a). IL-12- and IL-23-mediated IFN-γ production was decreased when freshly isolated and Con A-activated splenocytes were cultured with supernatants of COS-7/p40 cells. IL-23-mediated IL-17 production was also decreased when Con A-activated splenocytes were cultured with supernatants of COS-7/p40 cells (Fig. 2b). We then examined whether the timing of p40 treatment affected the level of IL-23-mediated IFN-γ production. Treatment of Con A-activated splenocytes with supernatants of COS-7/p40 cells prior to IL-23 decreased IFN-γ production to a similar level to that observed in the cells simultaneously treated with p40 and IL-23 (Fig. 3). IFN-γ production was

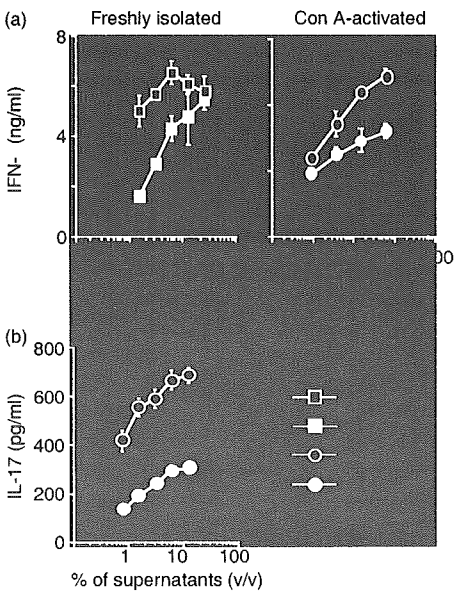

Figure 2. Decreases in interleukin (IL)-12- and IL-23-induced cytokine production mediated by p40. Freshly isolated and Con A-activated splenocytes of C57BL/6 mice were cultured for 24 hr with various concentrations of supernatants from COS-7/IL-12 (squares) or COS-7/IL-23 (circles) cells in the presence of 20% (volume/volume) of either COS-7/p40 (closed symbols) or COS-7 (open symbols) cells. The amounts of interferon (IFN)-γ (a) and IL-17 (b) in the supernatants of splenocytes are shown as mean ± standard deviation (SD) of triplicate samples. Asterisks indicate statistical significance [repeated-measures analysis of variance (ANOVA); $P < 0\ 01$]. v/v, volume/volume.

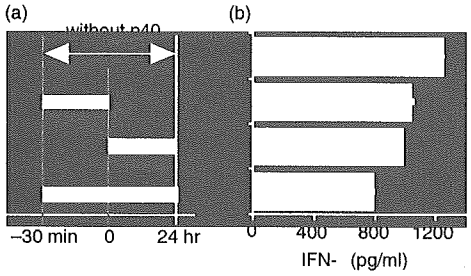

Figure 3. Transient treatment with p40 inhibited interleukin (IL)-23-dependent interferon (IFN)-γ production. (a) A schema of p40-treated periods for splenocyte culture. (b) Con A-activated splenocytes of C57BL/6 mice were stimulated for 24 hr with supernatants of COS-7/IL-23 (2% of total volume) and COS-7/p40 (20%) cells. Data represent mean ± standard deviation (SD) of triplicate samples. Asterisks indicate statistical significance compared with splenocytes cultured with IL-23 (Student *t*-test, $P < 0\ 05$).

further suppressed when the splenocytes were incubated with supernatants of COS-7/p40 cells before and during the stimulation with IL-23.

p40 abrogated IL-12- and IL-23-mediated antitumour effects

We previously reported that Colon 26 cells expressing IL-12 or IL-23 (Colon 26/IL-12 or Colon 26/IL-23) were rejected in syngeneic mice by stimulation of systemic immunity.[20,21] Here, we retrovirally transduced Colon 26 cells with the *p40* gene (Colon 26/p40) and examined whether local production of p40 impaired IL-12- and IL-23-induced antitumour effects *in vivo*. Expression of the cytokine subunit genes in the transduced cells was confirmed with RT-PCR (Fig. 4a). The proliferation rate *in vitro* of Colon 26/p40 cells was identical to that of Colon 26, Colon 26/IL-12 and Colon 26/IL-23 cells (data not shown). The expression levels of H-2K^d and H-2D^d of Colon 26/p40 cells remained the same as those of Colon 26, Colon 26/IL-12 and Colon 26/IL-23 cells (Fig. 4b). The level of H-2L^d expression of Colon 26/p40 cells was the same as those of Colon 26 and Colon 26/IL-23 cells but higher than that of Colon 26/IL-12 cells. We confirmed that the growth of Colon 26/p40 tumours developed in syngenic mice remained the same as that of Colon 26 tumours, as previously described.[21]

We mixed equal numbers of Colon 26/p40 cells with either Colon 26/IL-12 or Colon 26/IL-23 cells and then inoculated the mixtures into syngeneic mice. More than half of the mice inoculated with the mixture of Colon 26/p40 cells and either Colon 26/IL-12 or Colon 26/IL-23 cells developed tumours, whereas all the mice inoculated with the mixture of Colon 26 and either Colon 26/IL-12 or Colon 26/IL-23 cells rejected the tumours (Table 2). The tumour growth of the mixtures consisting of Colon 26/p40 cells and the IL-12 or IL-23 producers, when

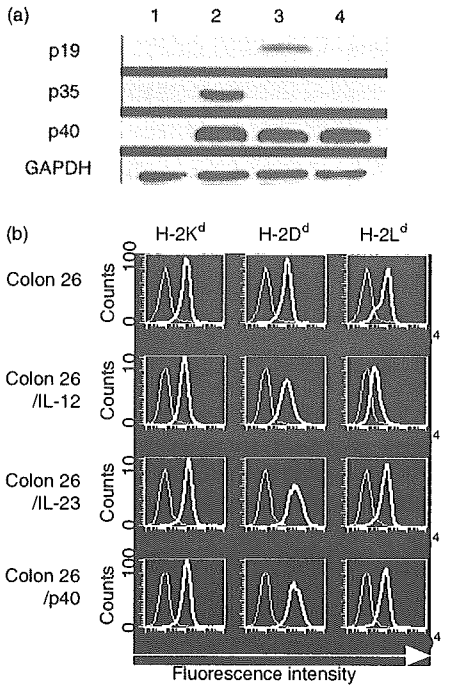

Figure 4. Expression of cytokine subunits and major histocompatibility complex (MHC) class I molecules of parent and transduced Colon 26 cells. (a) Expression of the *p19*, *p35*, *p40* and *GAPDH* genes in transduced cells: lane 1, Colon 26; lane 2, Colon 26/IL-12; lane 3, Colon 26/IL-23; lane 4, Colon 26/p40 cells. (b) Expression of MHC class I molecules. Parent and transduced Colon 26 cells were stained with anti-H-2K^d, anti-H-2D^d, anti-H-2L^d (thick line) or control antibodies (thin line).

Table 2. Tumour development in mice inoculated with p40 producers

Inoculated cells (day 0)	Tumorigenesis[4] (day 30)
Colon 26[1]	6/6
Colon 26/p40[1]	6/6
Colon 26/IL-23 + Colon 26[2]	0/6
Colon 26/IL-23 + Colon 26/p40[2]	4/6
Colon 26/IL-12 + Colon 26[3]	0/6
Colon 26/IL-12 + Colon 26/p40[3]	4/6

[1]Cells (1 10^6) were inoculated subcutaneously into BALB/c mice.
[2]Colon 26/IL-23 cells (5 10^5) were mixed with Colon 26 or Colon 26/p40 cells (5 10^5) and the mixtures (1 10^6) were then inoculated subcutaneously into BALB/c mice.
[3]Colon 26/IL-12 cells (5 10^5) were mixed with Colon 26 or Colon 26/p40 cells (5 10^5) and the mixtures (1 10^6) were then inoculated subcutaneously into BALB/c mice.
[4]The number of tumour-bearing mice/the number of mice tested.

compared with that of Colon 26 cells only, was retarded in the initial phase but the growth rate remained the same thereafter (Fig. 5a). We further investigated IFN-γ production in splenocytes of the tumour-bearing mice

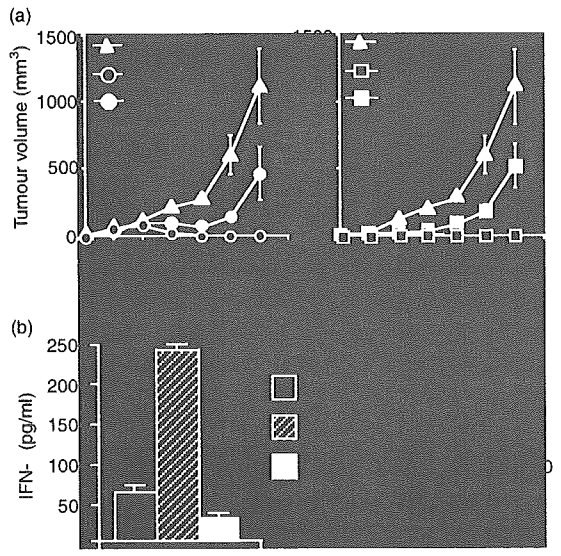

Figure 5. p40-producing tumours prevented interleukin (IL)-23- and IL-12-mediated antitumour immunity *in vivo*. (a) Tumour growth of mixtures of transduced cells in BALB/c mice. Cytokine-producing cells (5 10^5) were mixed with either Colon 26 or Colon 26/p40 cells (5 10^5) and the cells (1 10^6 in total) were inoculated subcutaneously into the flank (n 6): symbols represent Colon 26 (closed triangle), and the mixtures of Colon 26/IL-23 with Colon 26 (open circle), Colon 26/IL-23 with Colon 26/p40 (closed circle), Colon 26/IL-12 with Colon 26 (open square) and Colon 26/IL-12 with Colon 26/p40 (closed square). Data represent mean ± standard deviation (SD) for tumour-bearing mice. (b) Interferon (IFN)-γ production in the splenocytes of mice inoculated with Colon 26 cells (open bar), mixtures of Colon 26/IL-23 and Colon 26 cells (hatched bar) or mixtures of Colon 26/IL-23 and Colon 26/p40 cells (closed bar). On day 24, splenocytes were cultured for 24 hr with irradiated Colon 26 cells. Data represent mean ± SD of triplicate samples. Asterisks indicate statistical significance compared with splenocytes of mice inoculated with mixtures of Colon 26/IL-23 and Colon 26 cells (Student t-test, $P < 0\ 05$).

(Fig. 5b). Splenocytes of naïve mice did not produce any INF-γ when cultured with Colon 26 cells (data not shown), but those of Colon 26 tumour-bearing mice produced small amounts of IFN-γ. IFN-γ production increased in the splenocytes of mice inoculated with the mixture of Colon 26/IL-23 and Colon 26 cells, while production in the splenocytes of mice inoculated with Colon 26/IL-23 and Colon 26/p40 cells was completely suppressed.

p40 suppressed IL-23-mediated cytotoxic T lymphocyte (CTL) stimulation

Since $CD8^+$ T cells play a pivotal role in IL-23-dependent antitumour effects,[21,22] we examined whether p40 suppressed IL-23-mediated CTL stimulation *in vitro*. Splenocytes of mice that had rejected Colon 26/IL-23 cells or those of naïve mice were stimulated with Colon 26/IL-23

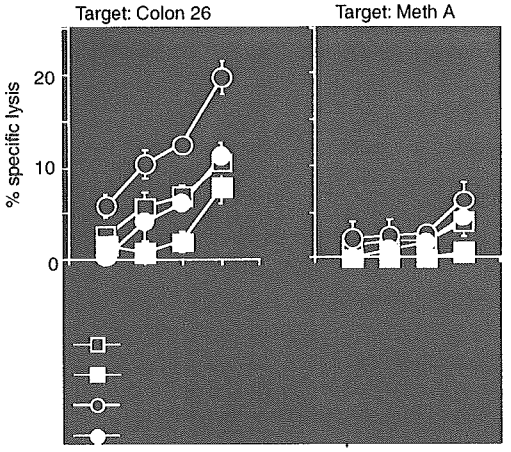

Figure 6. p40 inhibited interleukin (IL)-23-dependent cytotoxic T lymphocyte (CTL) induction *in vitro*. Splenocytes from BALB/c mice that had rejected Colon 26/IL-23 cells were cultured with mitomycin C-treated Colon 26 (open squares) or Colon 26/p40 (closed squares) cells, or equal mixtures of populations of Colon 26/IL-23 with either Colon 26 (open circles) or Colon 26/p40 (closed circles) cells. Cytolytic activity against Colon 26 and Meth A cells was measured at various effector:target (E:T) ratios. Data represent mean ± standard deviation (SD) of triplicate samples. Asterisks indicate statistical significance compared with splenocytes cultured with a mixture of Colon 26 cells [repeated-measures analysis of variance (ANOVA); $P < 0\ 01$].

and either Colon 26 or Colon 26/p40 cells, and were measured for their cytolytic activities against Colon 26 or irrelevant Meth A cells (Fig. 6). Splenocytes from naïve mice did not kill Colon 26 cells or Meth A cells, even if the cells were stimulated with Colon 26/IL-23 cells *in vitro* (data not shown). Stimulation *in vitro* with Colon 26/IL-23 cells enhanced the cytolytic activity of the splenocytes from Colon 26/IL-23-rejected mice when compared with the splenocytes stimulated with Colon 26 cells alone; however, the cytolytic activity of the splenocytes stimulated with a mixture of Colon 26/IL-23 and Colon 26/p40 cells was reduced to the level of that found with Colon 26 cells alone. The cytolytic activity of the splenocytes stimulated with Colon 26/p40 cells alone was not as great as that with Colon 26 cells. The inhibitory action of p40 was not observed in CTL activity against Meth A cells. We also found that stimulation *in vitro* with Colon 26/IL-12 cells enhanced cytolytic activity in the splenocytes from the mice that had rejected Colon 26/IL-23 cells and the presence of p40 inhibited the IL-12-mediated CTL stimulation *in vitro* (data not shown).

Discussion

This is the first study, to our knowledge, to demonstrate that a secreted p40 was inhibitory to IL-23-mediated immune responses. Since competitive binding analyses showed that p40 prevented binding of IL-12 to its

receptor,[17,18] we hypothesized that p40 could also prevent IL-23 ligation to its receptors. The splenocytes treated with p40 before IL-23 ligation to the receptors produced smaller amounts of IFN-γ, suggesting that p40 occupied IL-23 receptor complexes and prevented the binding of IL-23 to its receptor. The modest inhibitory action of p40 on IL-23-mediated cytokine production could be dependent on the molar ratio of p40 to IL-12 or IL-23 contained in supernatants of cytokine-producing COS-7 cells and on the binding affinity of p40 to respective receptor complexes. Further investigations are required to determine the differential binding of p40 to respective receptors for IL-12 and IL-23.

Inoculation of mice with mixtures of Colon 26/p40 cells and Colon 26/IL-12 or Colon 26/IL-23 cells revealed that p40 impaired IL-12- and IL-23-mediated antitumour immunity *in vivo*. We also demonstrated that IL-23 directly enhanced T cell-mediated cytotoxic activity and that p40 inhibited IL-23-mediated CTL stimulation *in vitro*. Previous studies implied that $CD8^+$ T cells could be one of the direct targets of IL-23, as IL-23 induced $CD8^+$ T cell-mediated antitumour immunity.[21,22] The present study also showed that $CD8^+$ T cells were activated by IL-23: the cytotoxic activity of splenocytes from mice primed with Colon 26/IL-23 cells increased when they were subsequently stimulated with Colon 26/IL-23 cells rather than with Colon 26 cells. This enhanced cytolytic activity was not observed in the presence of p40, and p40 also impaired the cytolytic activity induced in splenocytes stimulated with Colon 26 cells alone. We presume that macrophage and dendritic cells in the spleen produced IL-12 and/or IL-23 in the secondary CTL stimulation with Colon 26 cells and the cytotoxic activity was inhibited by p40. We did not observe any toxicity of p40 against the splenocytes in our experimental systems (data not shown).

Other studies have reported contradictory findings that the administration of p40 to $p35^{-/-}$ and $p40^{-/-}$ mice, which were susceptible to *Mycobacterium* and *Salmonella* infection, increased resistance to the infection.[23,24] However, we did not find any evidence for such agonistic effects of p40 in the present assay systems. Moreover, the forced expression of the *p40* gene suppressed Th1-mediated immune responses and consequently prevented allograft rejection in recipient animals and ameliorated the development of autoimmune diseases.[25–27] Since p40 activates macrophages and microglia to produce tumour necrosis factor-α,[28] further studies are needed to clarify the agonistic effects of p40 on macrophages.

Recent studies suggested that IL-23 was a key mediator for not only Th1-mediated immune responses but also systemic inflammation. IL-23 induced IL-17, a proinflammatory cytokine involved in the pathogenesis of collagen-induced arthritis.[2,29] Transgenic mice that aberrantly expressed either the *p19* or the *p40* gene showed inflammation in multiple organs because of excess amounts of IL-23.[30,31] The expression of IL-23 was elevated in skin lesions of patients with psoriasis.[32] These studies collectively suggest that IL-23 is a candidate target molecule for the treatment of relevant inflammatory diseases; p40 could therefore be of use in diseases with long-lasting inflammation through its inhibitory action on IL-23.

In summary, we demonstrated that p40 inhibited IL-23-dependent cytokine production and CTL stimulation *in vitro*, and IL-23-mediated antitumour effects *in vivo*. The present study implies a potential therapeutic use of p40 as an immunosuppressant for autoimmune diseases and as an anti-inflammatory agent for treatment of patients with severe systemic inflammatory diseases. p40 may circumvent non-specific immune suppression caused by current immunosuppressive medicines, which often results in adverse reactions.

Acknowledgements

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Leukemia Inhibitory Factor Induces Endothelial Differentiation in Cardiac Stem Cells*

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The importance of interleukin 6 (IL-6)-related cytokines in cardiac homeostasis has been studied extensively; however, little is known about their biological significance in cardiac stem cells. Here we describe that leukemia inhibitory factor (LIF), a member of IL-6-related cytokines, activated STAT3 and ERK1/2 in cardiac Sca-1 stem cells. LIF stimulation resulted in the induction of endothelial cell-specific genes, including VE-cadherin, *Flk-1*, and *CD31*, whereas neither smooth muscle nor cardiac muscle marker genes such as *GATA4*, *GATA6*, *Nkx-2.5*, and calponin were up-regulated. Immunocytochemical examination showed that about 25% of total cells were positively stained with anti-CD31 antibody 14 days after LIF stimulation. Immunofluorescent microscopic analyses identified the Sca-1 cells that were also positively stained with anti-von Willebrand factor antibody, indicating the differentiating process of Sca-1 cells into the endothelial cells. IL-6, which did not activate STAT3 and ERK1/2, failed to induce the differentiation of cardiac stem cells into the endothelial cells. In cardiac stem cells, the transduction with dominant negative STAT3 abrogated the LIF-induced endothelial differentiation. And the inhibition of ERK1/2 with the MEK1/2 inhibitor U0126 also prevented the differentiation of Sca-1 cells into endothelial cells. Thus, both STAT3 and ERK1/2 are required for LIF-mediated endothelial differentiation in cardiac stem cells. Collectively, it is proposed that LIF regulates the commitment of cardiac stem cells into the endothelial cell lineage, contributing to neovascularization in the process of tissue remodeling and/or regeneration.

Cardiac homeostasis is maintained by various kinds of extracellular signals through paracrine factors. Among these signals, interleukin 6 (IL-6)[2]-related cytokines have been demonstrated to play important roles in cardioprotection (1), vessel formation (2, 3), and cell-cell adhesion (4) in the heart. IL-6-related cytokines utilize glycoprotein 130 (gp130) as a common receptor. Signals through gp130 activate the signal transducer and activator of transcription (STAT) proteins and extracellular signal-regulated kinases 1/2 (ERK1/2) (5). Activated STAT proteins function as latent transcription factors and up-regulate a wide range of target genes, including *bcl-xL* (6), *VEGF* (7), metallothionein (1), *MnSOD* (manganese superoxide dismutase) (8), and *Wnt5a* (4). Recently it has been reported that cardiomyocyte-restricted ablation of the *STAT3* gene results in heart failure, accompanied by impairment of vessel growth and high sensitivity to cardiac injury (9, 10). Thus the importance of IL-6-related cytokines/gp130/STAT pathway has been established in cardiac myocytes; however, the possibility remains to be fully addressed that IL-6-related cytokines stimulate the non-myocyte population in the heart and contribute to cardiac homeostasis.

Previously it was believed that cardiac myocytes exited from the cell cycle immediately after birth and regenerated only to a lesser extent. However, recently, cardiac stem cells have been identified in the myocardium and demonstrated to differentiate into cardiomyocytes. Thus far, two kinds of cardiac stem cells, c-*kit* cells (11) and Sca-1 cells (12, 13), have been reported. It is demonstrated that c-*kit* cells differentiate into vascular smooth muscle cells and endothelial cells, as well as cardiac myocytes, whereas Sca-1 cells differentiate into osteoblasts or adipocytes. Despite the potential importance in the clinical application, the physiological signals responsible for the differentiation of the stem cells remain to be fully elucidated.

In the present study, we examined the regulatory mechanisms for the endothelial differentiation of cardiac Sca-1 cells. We then have demonstrated that leukemia inhibitory factor (LIF), an IL-6-related cytokine, promotes endothelial differentiation. Inhibition of STAT3 activity or ERK1/2 activity prevents endothelial differentiation, suggesting that both STAT3 and ERK play important roles in endothelial differentiation of cardiac stem cells. These data indicate that signals through gp130 could induce endothelial differentiation of cardiac stem cells as a potential source of endothelial cells. This study proposes a novel mechanism of gp130-mediated neovascularization.

MATERIALS AND METHODS

Preparation of Cardiac Sca-1 Cells—Sca-1 cardiac stem cells were prepared according to a previous report (13) with minor modification. Briefly, hearts from adult C57Bl/6 mice (10–12 weeks old; Japan SLC) were treated with 0.1% collagenase for 30 min (12). Cells were filtered through 80- m mesh and suspended in PBS supplemented with 3% FBS. To separate Sca-1 cells, cells were incubated with biotinylated anti-Sca-1 antibody (BD Biosciences) for 15 min on ice and washed with IMag buffer (consisting of PBS with 0.5% bovine serum albumin and 2 mM EDTA) followed by incubation with streptavidin-conjugated particles for 30 min on ice. The labeled cells were resuspended in IMag buffer, and the Sca-1 cells were separated from the cell suspension by using IMagnet (BD IMag Cell Separation System, BD Biosciences)

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[2] The abbreviations used are: IL-6, interleukin-6; LIF, leukemia inhibitory factor; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; Sca-1, stem cell antigen-1; gp130, glycoprotein 130; vWF, von Willebrand factor; mNSCM, modified neural stem cell medium; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; RT, reverse transcriptase; dnSTAT3, dominant negative STAT3;PBS, phosphate-buffered saline; FBS, fetal bovine serum.

according to the manufacturer's protocol. Flow cytometric analysis confirmed that the 97.6 1.1% of the cells were Sca-1 positive cells, which is consistent with previous studies (12, 13).

Newly isolated cardiac Sca-1 cells were incubated in Iscove's modified Dulbecco's medium supplemented with 10% FBS overnight. To maintain multipotency of the differentiation, Sca-1 cells were cultured in mNSCM (modified neural stem cell medium) consisting of Dulbecco's modified Eagle's medium and Ham's F12 (ratio 1:1) supplemented with 5 mM HEPES, ITS (5 g/ml insulin, 5 g/ml transferrin, and 5 ng/ml sodium selenite), 10 ng/ml bFGF, 20 ng/ml EGF, and 1000 units/ml LIF (11) as described under "Results." In some experiments, Sca-1 cells were amplified in mNSCM and used for the assay.

TABLE 1
PCR primers used in the present study

Genes	Direction	Sequence
VE-cadherin	Forward	5 -ATCTTCCTCTGCATCCTCAC-3
	Reverse	5 -GTAAGTGACCAACTGCTCGT-3
Flk-1	Forward	5 -TGCCGGCATGGTCTTCTGTGAGG-3
	Reverse	5 -CATTGAGCTCTGTTCTCGCTGTAC-3
CD31	Forward	5 -GAGCCCAATCACGTTTCAGTTT-3
	Reverse	5 -TCCTTCCTGCTTCTTGCTAGCT-3
Nkx-2.5	Forward	5 -CAGTGGAGCTGGACAAAGCC-3
	Reverse	5 -TTGTAGCGACGGTTCTGGAA-3
GATA4	Forward	5 -CTGTCATCTCACTATGGGCA-3
	Reverse	5 -CCAAGTCCGAGCAGGAATTT-3
Calponin	Forward	5 -GCACATTTTAACCGAGGTCC-3
	Reverse	5 -TGACCTTCTTCACAGAACCC-3
GATA6	Forward	5 -AAAGCTTGCTCCGGTAACAG-3
	Reverse	5 -GGACAGACTGACACCTATGT-3
GAPDH	Forward	5 -CATCACCATCTTCCAGGAGCG-3
	Reverse	5 -GAGGGGCCATCCACAGTCTTC-3

LIF was purchased from Chemicon International. IL-6, bFGF, and EGF were purchased from Peprotech EC (London, UK). U0126 (Cell signaling, MA), a highly selective inhibitor for MEK1/2, was used for the inhibition of ERK1/2.

Immunoblot Analyses—Immunoblotting was performed as described previously (14). Briefly, cells were stimulated with IL-6-related cytokines for the indicated time. After being washed with ice-cold PBS twice, cell lysates were prepared by the addition of SDS-PAGE sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, MA). The membrane was blocked with 2% skim milk and incubated with anti-phospho-STAT3, anti-phospho-ERK1/2, or anti-phospho-Akt (all from Cell Signaling) antibody as a first antibody. ECL system was used for detection. To quantify the extent of phosphorylation, the membranes were reprobed with anti-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERK1/2 (Cell Signaling), or anti-Akt (Cell Signaling) antibody. The band intensities of phospho-proteins were normalized with those of total proteins. The activities of phospho-specific antibodies were confirmed by using the extract from cardiomyocytes stimulated LIF as a positive control.

RT-PCR Analyses—RT-PCR was performed as described previously (6). Briefly, total RNA was prepared using the acid guanidinium thiocyanate-phenol-chloroform method (15). Total RNA (1 g) was subjected to first strand cDNA synthesis by using the oligo(dT) first strand primer. Gene-specific primers used for PCR amplification are shown in Table 1. The PCR products were size-fractionated by 2% agarose gel electrophoresis and detected by staining with ethidium bromide.

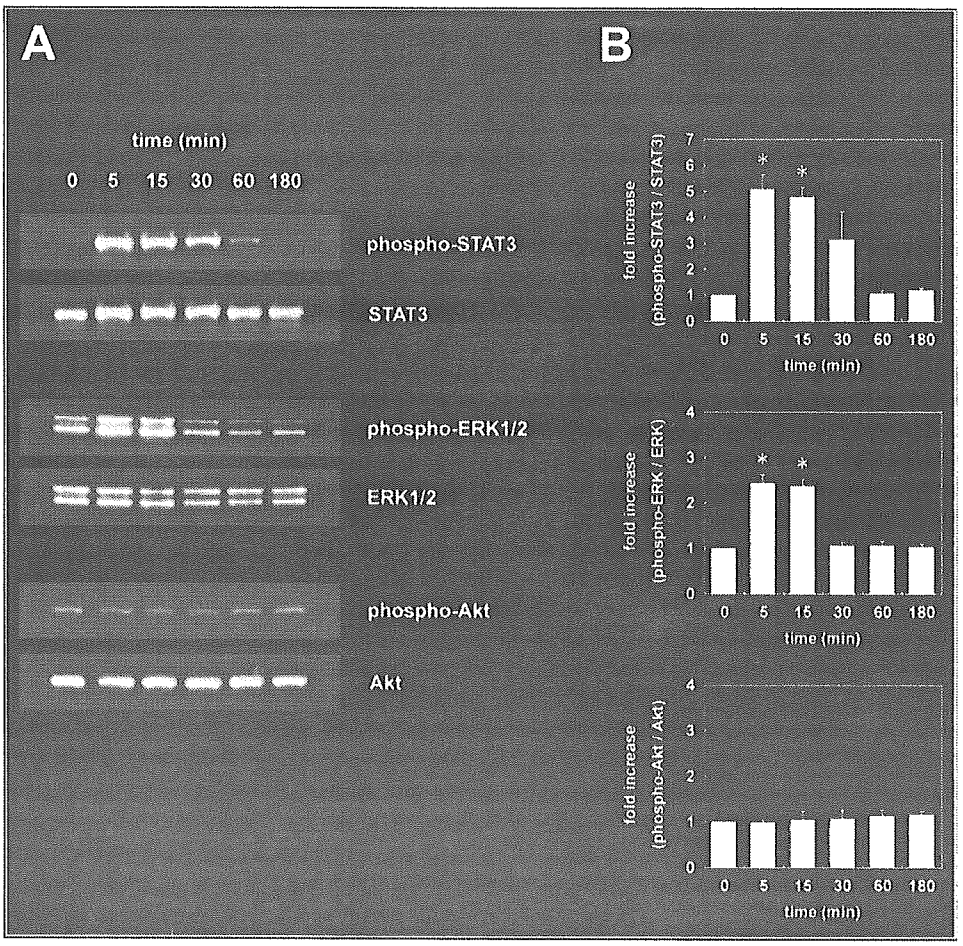

FIGURE 1. **LIF activates STAT3 and ERK, but not Akt, in cardiac Sca-1 cells.** Cardiac Sca-1 cells were stimulated by 1000 units/ml LIF for the indicated time. Cell lysates were immunoblotted with anti-phospho-STAT3, anti-phospho-ERK1/2, or anti-phospho-Akt antibody. Blots were reprobed with anti-STAT3, anti-ERK1/2, or anti-Akt antibody. *A*, representative data are shown. *B*, quantitative analysis of tyrosine phosphorylation of STAT3, ERK1/2, and Akt. The band intensities of phosphoproteins were normalized with that of total proteins. Data are presented as means S.E. (n 3). *, p 0.05 *versus* control (paired t test).

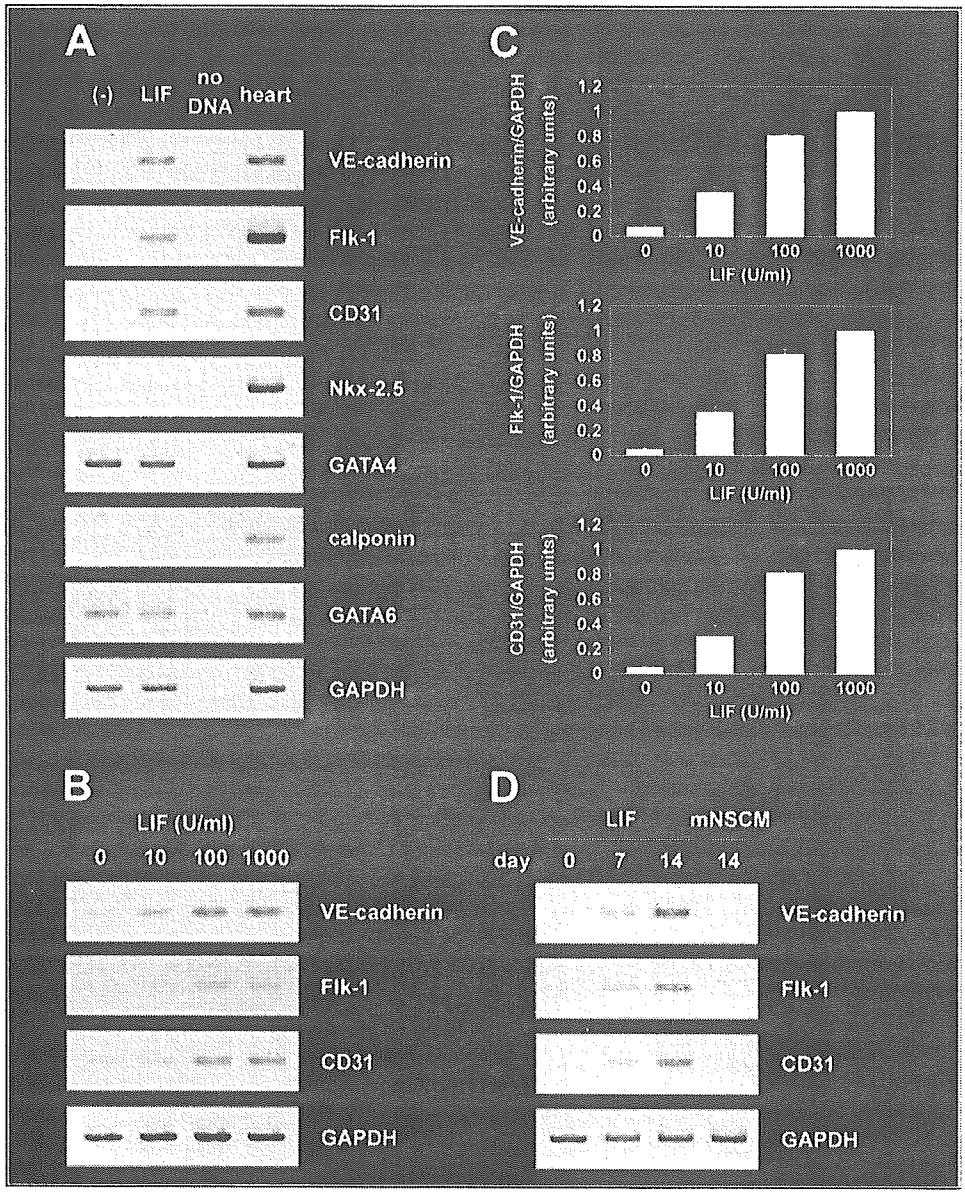

FIGURE 2. **Signals through gp130 up-regulate endothelial cell-specific marker genes in cardiac Sca-1 cells.** *A*, cardiac Sca-1 cells were stimulated with or without LIF (1000 units/ml) for 14 days. Total RNA was prepared, and RT-PCR was performed for as described under "Materials and Methods." Total RNA prepared from heart was used as positive control. GAPDH was used as an internal control. Experiments were repeated three times with similar results. *B* and *C*, Sca-1 cells were cultured with the indicated concentrations of LIF for 14 days. Total RNA was prepared, and RT-PCR was performed for the endothelial cell-specific genes. GAPDH was used as an internal control. Experiments were repeated three times with similar results. Representative data are shown in *B*. The band intensities of endothelial markers were normalized with that of GAPDH. Data are presented as means S.E. (n 3) (C). *D*, Sca-1 cells were maintained in mNSCM containing EGF, bFGF, and LIF for 10 days followed by cultivation in medium containing only LIF (1000 units/ml) or in mNSCM for the indicated time. Total RNA was prepared, and RT-PCR was performed. GAPDH was used as an internal control. Experiments were repeated three times with similar results.

Immunocytochemical Examination—Cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature and incubated with anti-CD31 antibody (BD Biosciences) followed by the incubation with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotechnology). Cells were washed with Tris-buffered saline and incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (BCIP/NBT, Sigma). After staining, positively stained cells were counted by a person who was blinded to the culture conditions.

Immunofluorescent Examination—Immunofluorescent microscopic analyses were performed as described previously (16). Briefly, cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature and stained with anti-Sca-1 (R&D Systems) and anti-von Willebrand factor antibodies (Santa Cruz Biotechnology). Alexa Fluor 488- or 546-conjugated secondary antibody (Molecular Probes) was used for detection. Nuclei were stained with Hoechst dye. Cells were examined by Olympus IX70.

Adenovirus Vectors—Generation of the adenovirus vector expressing dominant negative STAT3 (dnSTAT3) was described previously (17). Adenovirus vector expressing -galactosidase was used as a control.

Adenovirus vectors were amplified in HEK293 cells and purified by CsCl ultracentrifugation (18). Sca-1 cells were infected at a multiplicity of infection of 100 for 12 h and cultured under the indicated conditions. By this method, more than 90% of Sca-1 cells were transfected with adenovirus vectors (data not shown).

Statistical Analysis—Statistical significance was determined by paired t test or Student's t test. Data were presented as mean S.E., and p 0.05 was considered statistically significant.

RESULTS

LIF Activates STAT3 and ERK1/2 in Cardiac Stem Cells—Signals through gp130 play important roles in cardiac homeostasis. Recently, cardiac stem cells have been reported to contribute to cardiac repair and regeneration (11, 12). Thus, we examined whether signals through gp130 are transduced in cardiac stem cells. Cardiac Sca-1 cells were stimulated with LIF for the indicated time. Cell lysates were prepared, and activation of STAT3, ERK1/2, and Akt was analyzed by immunoblotting with phospho-specific antibodies (Fig. 1*A*). STAT3 and ERKs,

but not Akt, were phosphorylated by LIF stimulation. STAT3 and ERKs were activated within 5 min of LIF stimulation (Fig. 1*B*).

LIF Induces Endothelial Differentiation in Cardiac Stem Cells—We examined the effects of LIF on the differentiation of Sca-1 cells. By RT-PCR (Fig. 2*A*), it was revealed that LIF induced VE-cadherin, *Flk-1*, and *CD31*, marker genes for endothelial cells, whereas *Nkx-2.5* and calponin was not up-regulated by LIF. GATA4 and GATA6 were expressed in nonstimulated Sca-1 cells, and their expression was not affected by LIF. LIF induced the expression of endothelial marker genes in a dose-dependent manner (Fig. 2, *B* and *C*). The endothelial markers were submaximally induced by 100units/ml LIF.

Previously it was reported that cardiac c-*kit* stem cells could be maintained as undifferentiated cells in mNSCM, Dulbecco's modified Eagle's medium, and Ham's F12 (ratio 1:1) containing bFGF, EGF, and LIF (11). Thus we analyzed the expression of the endothelial markers in Sca-1 cells cultured in mNSCM. In mNSCM, cardiac Sca-1 cell population did not express endothelial marker genes; however, cells were differentiated into endothelial cells when moved to the medium containing only LIF (Fig. 2*D*). The induction of endothelial marker genes was detected 7 days after stimulation, and afterward their expression increased. These data suggest that Sca-1 cells can be maintained in the presence of bFGF, EGF, and LIF without impairing the potential for endothelial differentiation. In preliminary studies, we noticed that cardiac Sca-1 cells occasionally showed expression of CD31, an endothelial marker, as described previously (12), when cultured in the presence of FBS more than 5 days and that FBS-mediated differentiation depended on the batches of FBS (data not shown). Therefore, in the present study, Sca-1 cells were amplified in mNSCM. Sca-1 cells cultured in mNSCM without LIF showed reduced potential for differentiation into endothelial cells, suggesting that LIF could contribute to maintenance of the potential for endothelial differentiation from Sca-1 cells in the presence of bFGF and EGF (data not shown).

To estimate the frequency of endothelial differentiation from Sca-1 cell culture, cells were cultured in the presence or absence of LIF for the indicated time and stained with anti-CD31 antibody (Fig. 3*A*). At day 3, CD31 cells were not observed either in the presence or absence of LIF. Cells positively stained with anti-CD31 antibody were detected 7 days after LIF stimulation. Thereafter, the frequency of CD31 cells increased up to about 25% of total cells 14 days after LIF stimulation (Fig. 3*B*). In contrast, CD31 cells were not significantly detected when the cells were cultured in the absence of LIF. We analyzed the effects of bFGF on LIF-induced endothelial differentiation because bFGF is also known to be a potent angiogenic growth factor, and it was found that co-stimulation with bFGF and LIF did not increase the number of CD31 positive cells, as compared with LIF stimulation (data not shown).

The cardiac stem cells were cultured in the presence of LIF for 14 days and co-stained with anti-Sca-1 and anti-von Willebrand factor (vWF) antibodies (Fig. 4). Immunofluorescent microscopic analyses detected Sca-1 cells that also showed positive staining for vWF, an endothelial marker, thus suggesting the transition from Sca-1 cells to endothelial lineage.

Both STAT3 and ERK1/2 Are Required for LIF-mediated Endothelial Differentiation—To analyze the signaling pathways involved in endothelial differentiation, we tested the effects of IL-6, which utilizes gp130 as one subunit of its receptor system. In contrast to LIF, IL-6 did not activate STAT3 or ERK1/2 in cardiac stem cells (Fig. 5*A*). Consistently, the endothelial cell-specific genes, including VE-cadherin, *Flk-1*, and *CD31*, were not increased in response to IL-6 (Fig. 5*B*). We also addressed the additional effects of IL-6 to LIF-mediated endothelial differentiation; however, endothelial differentiation, induced by LIF, was not enhanced by co-stimulation with IL-6 (Fig. 5*C*). Thus, further efforts were made to address the functional significance of STAT3 and ERK1/2.

To examine whether STAT3 activity is involved in the endothelial differentiation of Sca-1 cells by LIF, we analyzed the effects of the inhibition of STAT3 by using adenovirus vectors expressing dnSTAT3 (Fig. 6*A*). LIF failed to induce the endothelial markers in dnSTAT3-expressing cells, whereas endothelial marker genes were up-regulated in

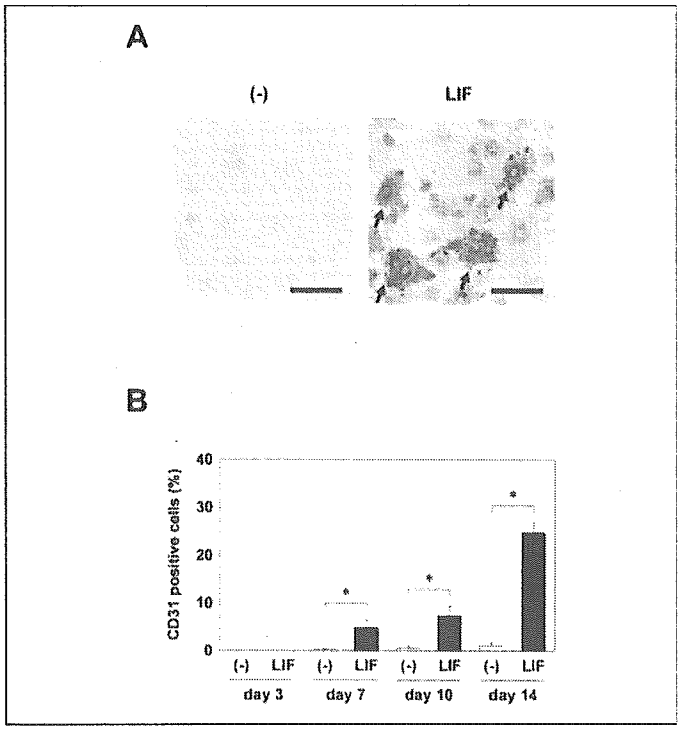

FIGURE 3. **Quantitative estimation of the frequency of the differentiation of Sca-1 cells into endothelial cells.** Cardiac Sca-1 cells were cultured in medium containing 0 or 1000 units/ml LIF for the indicated time and then stained with anti-CD31 antibody. *A*, cells were cultured in the presence or absence of LIF for 14 days. Representative immunocytochemical microscopic images were shown (magnification 100, *scale bar* 100 m). *Arrows* indicate CD31-positive cells. *B*, cells were cultured in the presence or absence of LIF for the indicated time. Cells were stained with anti-CD31 antibody. The frequency of CD31-positive cells was calculated in five fields. Each field contains 80–100 cells. Data are shown as mean S.E. *, p 0.05 (Student's t test). Experiments were repeated three times with similar results.

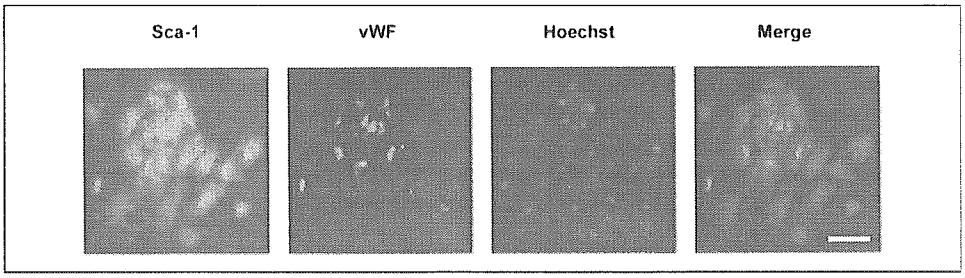

FIGURE 4. **LIF induces the expression of vWF, an endothelial marker, in cardiac Sca-1 cells.** Cardiac Sca-1 cells were cultured in the medium containing LIF (1000 units/ml) for 14 days and co-stained with anti-Sca-1 and anti-vWF antibodies. Representative immunofluorescent microscopy was shown (magnification 100, *scale bar* 100 m). Sca-1 /vWF cells were observed in three independent cultures.

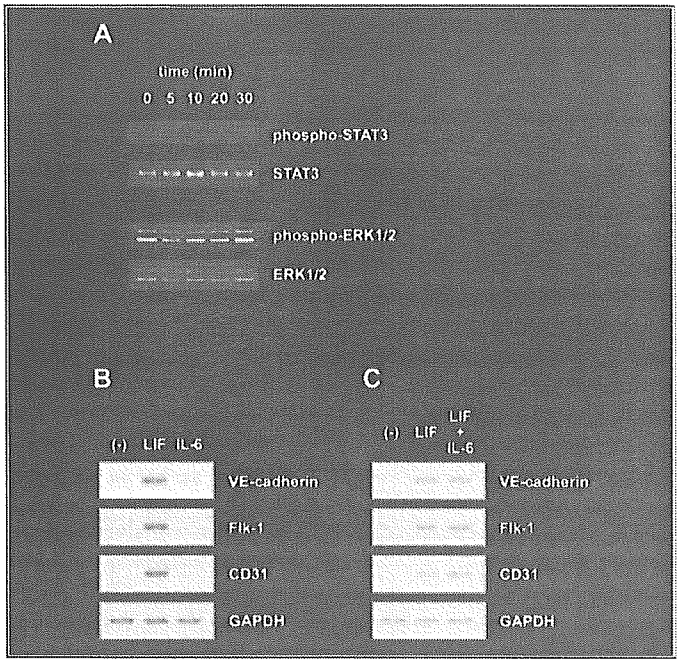

FIGURE 5. **IL-6 does not induce the expression of endothelial marker genes in cardiac Sca-1 cells.** *A*, cardiac Sca-1 cells were stimulated by IL-6 (20 ng/ml) for the indicated time. Cell lysates were prepared and immunoblotted with anti-phospho-STAT3 or anti-phospho-ERK1/2 antibody. Blots were reprobed with anti-STAT3 or anti-ERK1/2 antibody. Experiments were repeated three times with similar results. *B*, cardiac Sca-1 cells were cultured in the presence of IL-6 (20 ng/ml) or LIF (1000 units/ml) for 14 days. Total RNA was prepared, and RT-PCR was performed for endothelial cell-specific genes. GAPDH was used as an internal control. Experiments were repeated three times with similar results. *C*, to examine the additional effects of IL-6 on LIF-mediated differentiation, Sca-1 cells were cultured in the presence of LIF or LIF plus IL-6 for 14 days. Total RNA was prepared, and RT-PCR was performed for endothelial cell-specific genes. GAPDH was used as an internal control. Experiments were repeated three times with similar results.

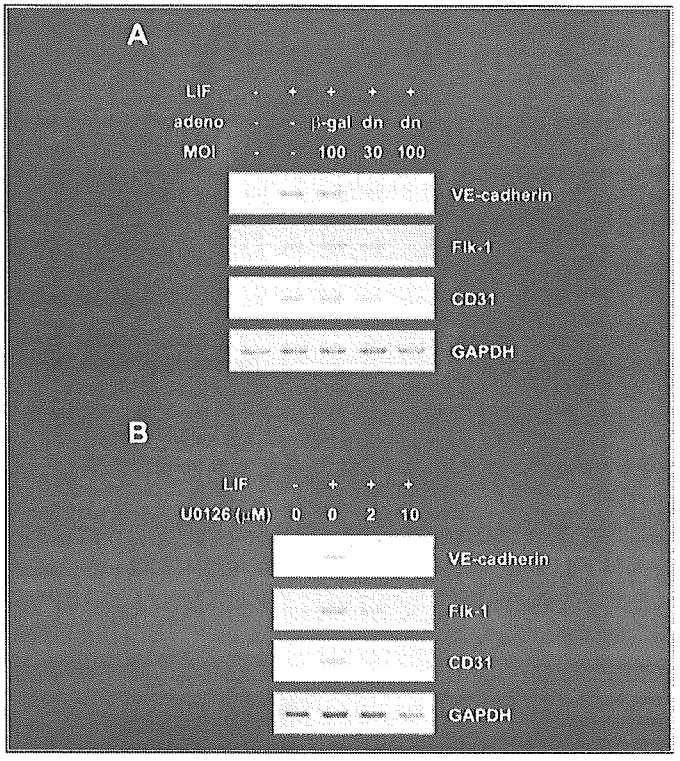

FIGURE 6. **LIF induces endothelial differentiation of cardiac Sca-1 cells through STAT3 and ERK1/2.** *A*, cardiac Sca-1 cells were transduced with adenovirus vectors (*adeno*) expressing dominant negative STAT3 (*dn*) or -galactosidase (*-gal*) as a control at the indicated multiplicity of infection (*MOI*) and cultured with ( ) or without ( ) LIF (100 units/ml) for 14 days. Total RNA was prepared, and expression of endothelial marker genes was examined by RT-PCR. GAPDH was used as an internal control. Experiments were repeated three times with similar results. *B*, cardiac Sca-1 cells were cultured in the presence or absence of LIF (100 units/ml) with or without U0126, an ERK inhibitor. Total RNA was prepared, and expression of endothelial marker genes was examined by RT-PCR. GAPDH was used as an internal control. Experiments were repeated three times with similar results.

the cells adenovirally transfected with -galactosidase, a control. Next, we tested the effects of U0126, an MEK inhibitor, on the endothelial differentiation of cardiac stem cells (Fig. 6*B*). The inhibition of ERK1/2 with U0126 inhibited the induction of endothelial marker genes by LIF. These data indicate that both STAT3 activity and ERK activity are required for the LIF-mediated differentiation of Sca-1 cells into endothelial cells. We confirmed that neither transfection of dnSTAT3 nor treatment with U0126 increased the frequency of pyknotic nuclei as analyzed by Hoechst staining, suggesting that neither inhibition of STAT3 nor of ERK affected cell viability (data not shown).

DISCUSSION

In the present study, we have demonstrated that LIF induced endothelial differentiation in cardiac stem cells. LIF stimulation rapidly activated STAT3 and ERK1/2 in cardiac stem cells. LIF up-regulated endothelial cell-specific genes without inducing either smooth muscle- or cardiac muscle-specific markers. The inhibition of STAT3 or ERK pathways abrogated endothelial differentiation, indicating that both STAT3 and ERKs are required for LIF-induced endothelial differentiation.

Initially, it was believed that postnatal neovascularization results exclusively from fully differentiated endothelial cells. However, recent studies have established that bone marrow-derived endothelial progenitor cells contribute to vessel formation (19). Endothelial progenitor cells circulate in the peripheral blood, home to the target organs, and differentiate into endothelial cells (20). In the progenitor cells, VE-cadherin and Flk-1 are highly expressed, like fully differentiated endothelial cells. In contrast, by RT-PCR we have demonstrated that neither VE-cadherin nor *Flk-1* is expressed in cardiac undifferentiated Sca-1 cells, as reported previously (12, 13). Thus, cardiac Sca-1 cells, used in the present study, are a population that is distinct from endothelial progenitors. Therefore it could be proposed that the cardiac Sca-1 population is a novel source of endothelial cells in the heart. This proposal is supported by the recent findings that skeletal muscle-derived Sca-1 cells are also differentiated into endothelial cells (21), suggesting the possible involvement of tissue-resident stem cells in vessel formation.

Cardiac Sca-1 cells are known to be differentiated into cardiomyocytes (12, 13). In the present study, we examined whether LIF induced cardiac differentiation. However, it was revealed that LIF stimulation up-regulated endothelial cell-specific genes without inducing smooth muscle or cardiomyocyte markers. In the process of *in vivo* neovascularization, differentiated endothelial cells migrate and form the capillary tubes. Therefore, we analyzed the migratory activities of LIF in cardiac Sca-1 cells; however, we could not detect an LIF-mediated increase in cell motility (data not shown). Other factors might promote the cell motility, resulting in capillary tube formation *in vivo*. LIF-induced endothelial differentiation is reminiscent of the abnormality in the placenta of LIF receptor-null mice; LIF receptor-null mice exhibit a reduction of the fetal blood vessel component in the placenta (22). It would be interesting to examine whether the tissue-resident stem cells from the peripheral organs, especially from the placenta, can be differentiated into endothelial cells by signals through gp130.

Activation of gp130 results in the rapid induction of the target genes via STAT and ERK proteins after stimulation with IL-6-related cytokines (23). Consistently, LIF rapidly activated STAT and ERK, whereas

the up-regulation of endothelial cell-specific genes such as VE-cadherin, *Flk-1*, and *CD31* was detected more than 24 h after LIF stimulation. Therefore it is unlikely that these endothelial cell-specific genes are direct targets of gp130-mediated transcriptional proteins, including STAT proteins. We examined the effects of LIF on the expression of *HoxA9* gene as an early-stage endothelial marker (24), because HoxA9 is rapidly induced by angiogenic stimuli such as shear stress and plays important roles in the expression of the endothelial genes in endothelial progenitor cells (25). However, HoxA9 was not up-regulated immediately after LIF stimulation (data not shown). Thus it is suggested that cardiac stem cells are differentiated into endothelial cells by LIF through differential mechanisms from the endothelial progenitor cells. Further studies will be required to elucidate the direct target genes of the signals through gp130 in cardiac stem cells.

The Gp130/STAT signaling pathway is activated under pathophysiological conditions in cardiac myocytes. IL-6-related cytokines are produced in cultured cardiomyocytes exposed to pathological stresses such as catecholamine, (26), mechanical stretch (27), and hypoxia (28), leading to activation of the gp130/STAT3 pathway in the myocardium. *In vivo* studies also demonstrated that LIF is up-regulated in pathological hearts, including hypertrophied hearts (29) and failing hearts (30). Moreover, LIF is produced in atrial and ventricular myocytes in failing hearts (31), suggesting the importance of the paracrine system of IL-6-related cytokines. Previously we demonstrated that activation of STAT3 promotes vessel formation through paracrine of angiogenic factors in the heart (2, 7), proposing a cardioprotective machinery via cardiomyocyte-endothelial cell interaction. Consistently, accumulating evidence has confirmed the importance of STAT3 in controlling the paracrine circuits, resulting in neovascularization in pathophysiological situations such as tumor growth (32, 33), psoriasis (34), and diabetic retinopathy (35). Here, in addition to the promotion of vessel growth through paracrine system, we propose that LIF-mediated activation of gp130 directly induces the endothelial differentiation of tissue-resident stem cells, suggesting a novel role of gp130 signaling in angiogenesis. These combined angiogenic properties may explain the prominent neovascularization induced by signals through gp130 during myocardial remodeling (3), although further efforts should be made to elucidate how much cardiac stem cells contribute to angiogenesis *in vivo*.

In summary, the present study has revealed that signals through gp130 transduce endothelial differentiation in cardiac stem cells and that IL-6-related cytokines are the paracrine factors determining the commitment of cardiac stem cells into the endothelial cell lineage. These findings propose a novel mechanism by which signals through gp130 contribute to neovascularization in the process of tissue remodeling and/or regeneration.

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