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Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice

(interleukin 6 receptor)

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ABSTRACT **To investigate the physiological roles of gp130 in detail and to determine the pathological consequence of abnormal activation of gp130, transgenic mice having continuously activated gp130 were created. This was carried out by mating mice from interleukin 6 (IL-6) and IL-6 receptor (IL-6R) transgenic lines. Offspring overexpressing both IL-6 and IL-6R showed constitutive tyrosine phosphorylation of gp130 and a downstream signaling molecule, acute phase response factor/signal transducer and activator of transcription 3. Surprisingly, the distinguishing feature of such offspring was hypertrophy of ventricular myocardium and consequent thickened ventricular walls of the heart, where gp130 is also expressed, in adulthood. Transgenic mice overexpressing either IL-6 or IL-6R alone did not show detectable myocardial abnormalities. Neonatal heart muscle cells from normal mice, when cultured *in vitro*, enlarged in response to a combination of IL-6 and a soluble form of IL-6R. The results suggest that activation of the gp130 signaling pathways leads to cardiac hypertrophy and that these signals might be involved in physiological regulation of myocardium.**

gp130 was initially identified as a signal-transducing receptor component that associates with the interleukin 6 receptor (IL-6R) when the receptor is occupied with interleukin 6 (IL-6). It has been revealed that the receptor complexes for IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M, and ciliary neurotrophic factor (CNTF) utilize this gp130 protein as a common signal-transducing component (1–10). From the study on cytokine receptor complexes sharing gp130, it is now clear that a general first step in the signaling processes of cytokines appears to be ligand-induced dimerization of receptor components whose cytoplasmic regions interact to activate downstream molecules (2, 5). These molecules include members of a JAK family of nonreceptor tyrosine kinases, JAK1, JAK2, and TYK2, and a latent cytoplasmic transcription factor, acute phase response factor/signal transducer and activator of transcription 3 (APRF/STAT3) (11–16).

In the case of the IL-6R system, a complex of IL-6 and IL-6R [either in a membrane-anchored form or in an extracellular soluble (s) form] associates with gp130 to induce its homodimerization (1, 17, 18). Thus, the addition of sIL-6R to IL-6-responsive cells, whose phenotype is IL-6R$^+$/gp130$^+$, enhances their responsiveness to IL-6. The IL-6–sIL-6R complex, when added to IL-6R$^-$/gp130$^+$ cells, which normally are nonresponsive to IL-6, confers their responsiveness to IL-6. This complex has been observed to mimic the actions of not only IL-6 but also IL-11, LIF, oncostatin M, and CNTF at least *in vitro* in cells expressing gp130 but not cytokine-specific receptor molecules (19, 20).

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gp130 is ubiquitously expressed in almost all the tissues examined (21), whereas the cytokine-specific receptors show a somewhat restricted distribution. Since the functions of gp130 stimulatory cytokines have been studied in greater detail *in vitro*, physiological functions of gp130 are not considered to have been fully elucidated. To investigate detailed functions of gp130 *in vivo* and to determine the pathological consequence of abnormal activation of gp130, transgenic mice with continuously activated gp130 protein have been made. Two transgenic lines were separately prepared by introducing minigenes of human (h) IL-6 and membrane-anchored hIL-6R under promoters constitutively switched on in immune (murine major histocompatibility complex class I H-2L^d promoter) (22) and ubiquitous cell systems (chicken β-actin promoter) (23), respectively. These two transgenic lines were mated to obtain "double-transgenic" mice expressing both hIL-6 and hIL-6R proteins, in which gp130 protein is continuously activated. We here describe the physiological and pathological roles of gp130 in the double-transgenic mice by focusing our attention on the myocardial system.

MATERIALS AND METHODS

Transgene Construction and Production of Transgenic Mice. The IL-6R transgene was prepared by inserting an 1.7-kb fragment of hIL-6R cDNA (24) into a unique *Xho* I site of pCAGGS (23), which carries the chicken β-actin gene promoter. This vector was then digested with *Sac* I and *Hin*dIII to generate a linear fragment and microinjected into the pronuclei of fertilized BDF$_1$ mouse eggs. Mice were obtained as described (25). The generation of IL-6 transgenic mice has been described elsewhere (22).

Determination of shIL-6R. Serum levels of shIL-6R were measured by ELISA (26) with anti-hIL-6R monoclonal antibody (Ab) MT18 (27).

Immunoblot Analysis. Heart cells were solubilized with Nonidet P-40 lysis buffer, and clear lysates obtained by centrifugation were incubated with anti-gp130 monoclonal Ab and anti-APRF/STAT3 polyclonal Ab (12). Immunoprecipitates using protein A-Sepharose (Pharmacia) were analyzed by SDS/PAGE and subsequent immunoblotting with anti-gp130 polyclonal Ab (19), anti-APRF/STAT3 polyclonal Ab, and anti-phosphotyrosine Ab (4G10; Upstate Biotechnology, Lake Placid, NY) using an enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's procedures.

Histology. Samples were fixed in 3.7% (wt/vol) formaldehyde in phosphate-buffered saline and processed for paraffin

Abbreviations: IL-6, interleukin 6; IL-6R, IL-6 receptor; s, soluble; h, human; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; APRF/STAT3, acute phase response factor/signal transducer and activator of transcription 3; Ab, antibody.

embedding. Sections (6 μm) were stained with hematoxylin/eosin.

Culture of Heart Cell. Cells were prepared from hearts of 1-day-old ICR pups and cultured as described (28). The morphological change was detected 3 days after the simultaneous addition of hIL-6 (2 μg/ml) and shIL-6R (0.5 μg/ml) to the culture [1 ml per well at 2.6×10^5 cells per ml in culture medium (Eagle's minimal essential medium)]. In the heart muscle cell preparation, ≈80% of the cells were initially beating, indicating a relatively high purity of cardiomyocytes.

RESULTS

Generation of IL-6 and IL-6R Double-Transgenic Mice. Offspring from eggs microinjected with the hIL-6R DNA construct were selected by Southern blot analysis, and three founders containing the transgene were identified. To obtain mice overexpressing both IL-6 and IL-6R, double-transgenic mice were generated by mating animals from IL-6 [B6L^{d}46 (22)] and IL-6R transgenic lines. The offspring were divided into four types by transmitted genes: 6^+6R^+TG, 6^+6R^-TG, 6^-6R^+TG, and 6^-6R^+TG, where 6^+ and $6R^+$ represent the presence of IL-6 and IL-6R transgenes, respectively, and TG indicates transgenic mouse. Adult mice at ≈5 months of age were used for biochemical and histological studies. shIL-6R concentrations in the sera from these four types of transgenic mice were assessed by ELISA. As shown in Fig. 1, serum levels of shIL-6R in 6^+6R^+TG and 6^-6R^+TG at 1 and 5 months of age were considerably higher than those of 6^+6R^-TG and 6^-6R^-TG, which were below the detection limit. It should be noted that mouse IL-6 does not bind to hIL-6R and that normal C57BL/6 serum contains mouse sIL-6R at a concentration <5% of that of shIL-6R in 6^+6R^+ or 6^-6R^+TGs (29). hIL-6 at a concentration of 0.1–5 ng/ml was detected in the sera of IL-6 transgenic mice at the age of 5 months, whereas normal controls have mouse IL-6 in the sera at a concentration of <0.02 ng/ml (22). Thus, in 6^+6R^+TG with high concentrations of sIL-6R, the gp130 protein was supposed to be continuously and systemically activated by hIL-6 complexed with shIL-6R.

Tyrosine Phosphorylation of gp130 and APRF/STAT3. We examined the activation status of gp130 and APRF/STAT3 proteins in the transgenic mice by looking at tyrosine phosphorylation of these proteins. The 5-month-old adult heart, which was shown to express abundant gp130 protein (21), was used. As shown in Fig. 2*A*, the extent of tyrosine phosphorylation of gp130 in 6^+6R^+TG was more greater than that of the others. The expression of gp130 protein was comparable in the four types of transgenic mice (Fig. 2*B*). The downstream signaling molecule, APRF/STAT3, occurred in this type of transgenic mice. As shown in Fig. 2*C*, the extent of tyrosine phosphorylation of APRF/STAT3 in 6^+6R^+TG was more prominent than that of the others, whereas the expression of APRF/STAT3 protein was comparable (Fig. 2*D*). Thus, the gp130 signaling cascade, at least the gp130–JAK–STAT pathway, was considered to be continuously activated in double-transgenic mice.

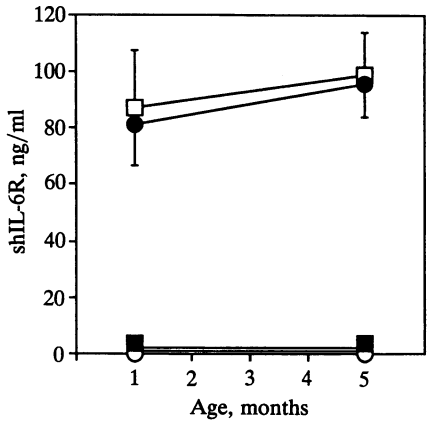

FIG. 1. Serum levels of shIL-6R in transgenic mice. shIL-6R levels in the sera of 6^+6R^+TG (□; $n = 3$), 6^+6R^-TG (○; $n = 3$), 6^-6R^+TG (●; $n = 3$), and 6^-6R^-TG (■; $n = 3$) at 1 and 5 months of age were measured by ELISA. Averages of triplicate measurements are shown. Vertical bars indicate SD.

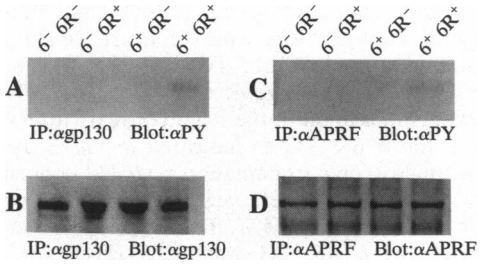

FIG. 2. Tyrosine phosphorylation of gp130 and APRF/STAT3 in 5-month-old transgenic mice. (*A* and *C*) Tyrosine phosphorylation of gp130 and APRF/STAT3, respectively, in transgenic mice. Nonidet P-40 lysates from the heart of each mouse were subjected to immunoprecipitation (IP) with anti-gp130 (αgp130) monoclonal Ab or anti-APRF/STAT3 (αAPRF) polyclonal Ab. Precipitates were analyzed by SDS/PAGE and subsequent immunoblotting with anti-phosphotyrosine Ab (αPY). (*B* and *D*) Detection of gp130 and APRF/STAT3 proteins, respectively. Immunoprecipitates prepared as in *A* or *C* were analyzed by SDS/PAGE and immunoblotting with antibodies to respective proteins.

Hypertrophic Myocardium in Double-Transgenic Mice. The distinguishing feature of the 6^+6R^+ double-transgenic mice was hypertrophy in the adult left ventricular myocardium. As shown in Fig. 3*A*, the heart from a 5-month-old 6^+6R^+TG was apparently bigger than that from a 6^-6R^- control of the same age. The increase in heart size was observed in almost all 5-month-old or older double-transgenic mice when compared

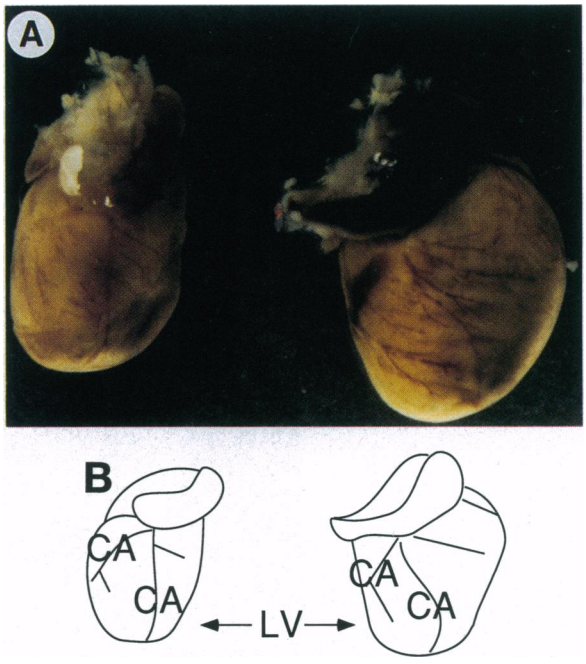

FIG. 3. Pathology of the heart from a 5-month-old 6^+6R^+TG compared with that from an age-matched 6^-6R^-TG. A wide view (*A*) and schematic drawing (*B*) of hearts, placed in similar orientations, from 6^+6R^+TG (*Right*) and 6^-6R^-TG (*Left*) are shown. CA, coronary artery; LV, left ventricle.

to age-matched single transgenic (6^+6R^- and 6^-6R^+) or nontransgenic controls. The ratio of heart weight to body weight was higher in the 6^+6R^+TG (0.66% ± 0.07%; $n = 3$) than that in the other transgenic mice (0.51% ± 0.04%; $n = 3$; $P < 0.05$). A dilatation of the main coronary artery usually occurred in the 6^+6R^+TG as indicated in Fig. 3. As shown in Fig. 4*A*, microscopic examination revealed concentric hypertrophy of the heart. Left ventricular thickness was increased in 6^+6R^+TG by 44%. The inner diameter of the left ventricle of 6^+6R^+TG was 32% less than that of 6^-6R^-TG. Higher magnification (Fig. 4*B*) indicated an increase in cell volume in 6^+6R^+TG (the width of the cells were increased by 48%). No evidence for disarray, ischemic necrosis, fibrous scar formation, or calcification was observed in any region of the left ventricle or papillary muscle. Medial thickness was not increased in myocardial arterioles and coronary arteries of double-transgenic mice (data not shown), suggesting that smooth muscle cells were not affected. In our studies, at 1 or 2 months after birth, hypertrophy in the heart was not detectable.

Hypertrophic Response of Cultured Heart Muscle Cells to the IL-6–sIL-6R Complex. To ascertain that the ventricular hypertrophic phenotype of the double-transgenic mice directly involves the activation of gp130 signaling processes, we examined the effect of the gp130 stimulation, which is triggered by a combination of IL-6 and sIL-6R, on heart muscle cells *in vitro*. Heart muscle cells from normal newborn mice were cultured with or without the IL-6–sIL-6R complex. As shown in Fig. 5, a morphological change was detected 3 days after the addition of IL-6–sIL-6R complex to the culture, in which the size of cardiomyocytes was dramatically increased. No obvious effect of IL-6 or sIL-6R alone was detectable. The viability, beating rate, and number of cultured cells were not apparently affected by the IL-6–sIL-6R complex.

DISCUSSION

In regards to the signaling processes of IL-6-related cytokines, it has been demonstrated that IL-6 stimulation induces homodimerization of gp130, whereas stimulation by, for example,

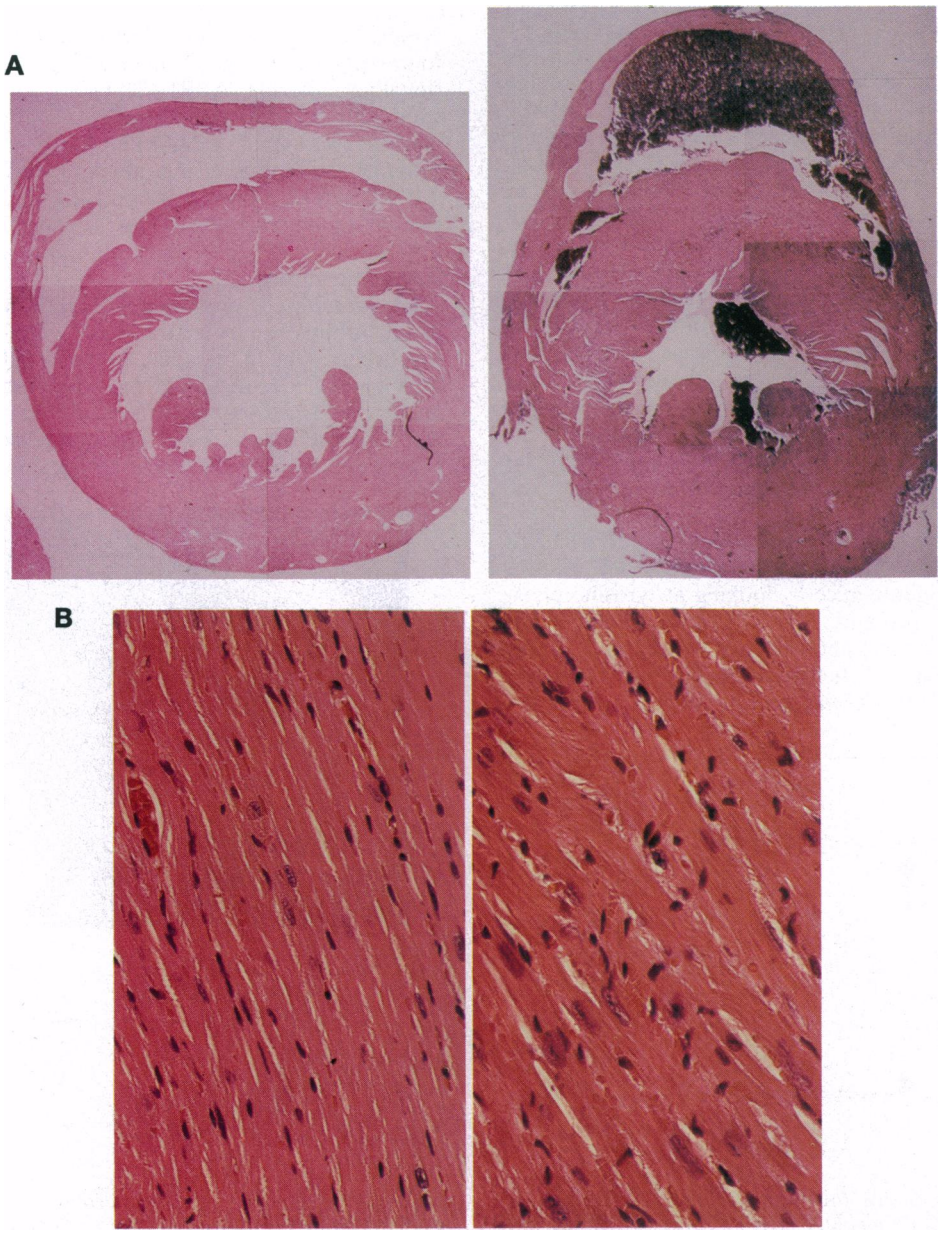

FIG. 4. Histology of transgenic hearts. (*A*) Coronal sections of the heart in Fig. 3 (*Right*, 6^+6R^+TG; *Left*, 6^-6R^-TG) at the level of the papillary muscles (×30), stained with hematoxylin/eosin. (*B*) Higher magnification (×210) of the left ventricular region in *A* (*Right*, 6^+6R^+TG; *Left*, 6^-6R^-TG).

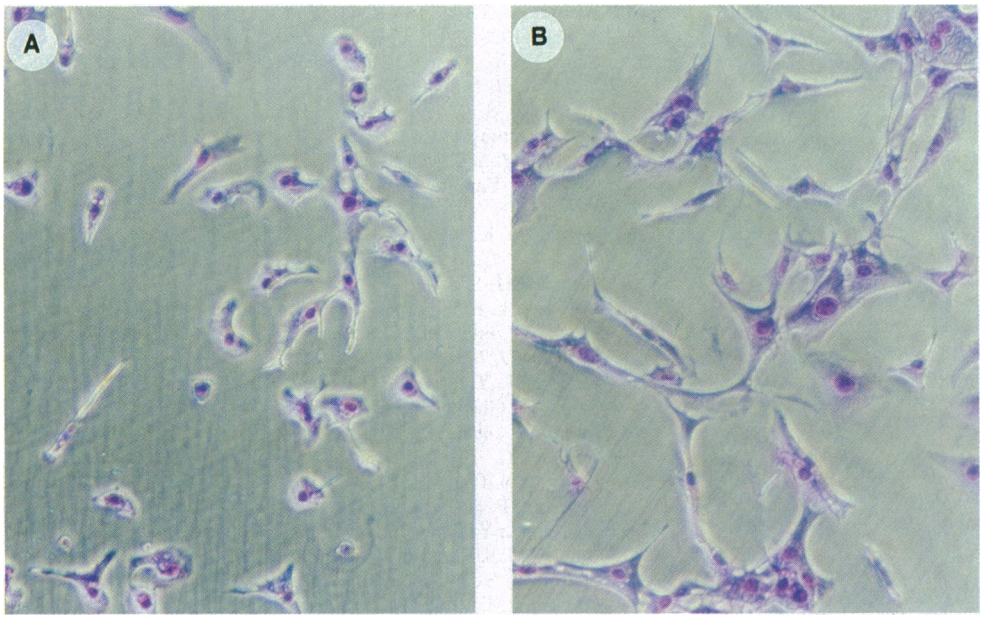

FIG. 5. Morphological appearance of cultured heart muscle cells. The morphological appearance was examined after a 72-hr culture in the absence (*A*) or presence (*B*) of a complex of hIL-6 (2 μg/ml) and shIL-6R (0.5 μg/ml) at 37°C. Heart cells were subjected to May/Giemsa staining and observed under a light microscope. (×900.)

LIF and CNTF leads to the formation of heterodimers composed of gp130 and the LIF receptor (10). It is noteworthy that these two different types of gp130 dimers initiate almost identical cytoplasmic biochemical events, leading to similar biological responses. Furthermore, the IL-6–sIL-6R complex, which induces gp130 homodimerization, mimics biological actions of LIF and CNTF on cells that are normally nonresponsive to IL-6. For instance, the IL-6–sIL-6R complex can sustain self-renewal of pluripotential embryonic stem cells (19) and induce osteoclast formation in a coculture system using mouse osteoblasts and bone marrow cells (20). In double-transgenic mice, the overexpressed IL-6–IL-6R and IL-6–sIL-6R complexes are thus expected to possibly mimic *in vivo* biological actions of all the gp130 stimulatory cytokines, including so far unidentified ones, if any. Cells in the double-transgenic mice are demonstrated here to possess continuously and extensively activated gp130 and APRF/STAT3 proteins and therefore have provided us information about the "gain of function" effect of gp130.

Pathological phenotypes in double-transgenic mice could be divided into two subtypes. The first group includes the phenotypes expected from previous studies on the dysregulation of IL-6 (30, 31). Actually, the double-transgenic mice showed hypergammaglobulinemia, splenomegaly, mesangial proliferative glomerulonephritis, and lymphoid infiltration in the lung (data not shown). These results are considered to be mediated through the enhancement of IL-6 actions by the overexpressed hIL-6 and hIL-6R as expected by *in vitro* studies (1, 17, 18). The second group is not directly related to IL-6 actions and includes an unexpected phenotype—i.e., hypertrophy in adult ventricular myocardium. This may allow us to hypothesize that gp130 signaling may be involved in regulation of cardiomyocytes in a physiological and/or pathological way. This hypothesis is supported by the finding of abnormally thin ventricular walls in gp130 knock-out mouse embryos at late gestational stages (K.Y., T.T., and T.K., unpublished data). Our finding suggests the existence of a so far unidentified member of the IL-6-related cytokines that regulates heart muscle cells. Interestingly, a cytokine called cardiotrophin 1 has recently been cloned, which acts on cardiomyocytes to cause hypertrophy and whose structure is closely related to, for example, IL-6 and LIF (32). An alternative possibility is that a complex of physiologically existing IL-6 and sIL-6R (33) may be involved in the regulation of myocardium or may stimulate the expression of cardiac hypertrophy-inducing factor.

The cardiovascular effects of IL-6 are not fully understood in spite of its wide variety of biological actions. A couple of reports have described a hypothesis that IL-6 may exert a negative inotropic effect and an intracellular Ca^{2+} concentration lowering effect through nitric oxide–cGMP pathways in cultured chicken embryonic ventricular myocytes and isolated hamster papillary muscles (34, 35). This effect might be considered to lead to sustained depression of myocardial contractility in IL-6-overexpressing mice. However, no abnormality in this regard in the cardiovascular system was detected in Eμ–IL-6 transgenic mice (30) and transgenic mice overexpressing another IL-6-related cytokine, LIF (36–39). In transgenic mice with continuously elevated IL-6, a homeostatic pathway to normalize the intracellular Ca^{2+} concentration might be activated to compensate. From our current study, it is obvious that hypertrophic myocardium is specific for 6^+6R^+ double-transgenic mice, indicating that the cardiac abnormalities may be a consequence of simultaneous overexpression of both IL-6 and IL-6R, but not IL-6 alone.

In response to diverse stimuli such as hormones, volume overload, pressure overload, and hypertension, the myocardium becomes adapted to the stimuli-mediated increased workloads through the hypertrophy of muscle cells. In characterized cultured myocardial cell models, several features of hypertrophy can be induced after stimulation with an α-adrenergic agent and endothelin 1 (40–43). To our knowledge, four kinds of animal models for cardiac hypertrophy have been reported: spontaneously hypertensive rat (44), Syrian hamster with cardiomyopathy (45), juvenile visceral steatosis mice (46), and transgenic rat carrying the mouse renin gene (47). The mechanism causing cardiac hypertrophy in the former two animals is suspected to be the abnormality of the cardiac cellular membrane (48, 49) but still remains unclear. Cardiac hypertrophy in juvenile visceral steatosis mice is related to carnitine deficiency because it is curable by carnitine administration (46). In the transgenic rat with overexpressed renin, the heart size increases, and myocardial arterioles and coronary arteries show an increase in their medial thickness (47). Since the renin–angiotensin system is considered to be one of the most important regulatory systems in the development of cardiac hypertrophy, we have examined whether this system is involved in myocardial hypertrophy in double-transgenic mice. There were no significant differences in the serum levels of renin, angiotensin II, and cathecholamines among our transgenic mice of the four genotypes. In the double-transgenic mice, no medial thickness of coronary arteries and arterioles

was found, which was in contrast to the transgenic rats expressing renin. Based on these data, the involvement of renin–angiotensin system in the myocardial hypertrophy occurring in the 6^+6R^+ double-transgenic mice may be negligible.

Taken together with the *in vitro* hypertrophic effect of the IL-6/sIL-6R complex, our findings with the 6^+6R^+TG strongly suggest that the gp130-mediated signals have a physiological role in cardiomyocyte regulation and, when overstimulated, lead to cardiac hypertrophy as a pathological consequence.

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