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Glial cell line-derived neurotrophic factor (GDNF) enhances sympathetic neurite growth in rat hearts at early developmental stages

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ABSTRACT

Molecular signaling of sympathetic innervation of myocardium is an unresolved issue. The purpose of this study was to investigate the effect of neurotrophic factors on sympathetic neurite growth towards cardiomyocytes. Cardiomyocytes (CMs) and sympathetic neurons (SNs) were isolated from neonatal rat hearts and superior cervical ganglia, and were co-cultured, either in a random or localized way. Neurite growth from SNs toward CMs was assessed by immunohistochemistry for neurofilament M and α-actinin in response to neurotrophic factors—nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and a chemical repellent, semaphorin 3A. As a result, GDNF as well as NGF and BDNF stimulated neurite growth. GDNF enhanced neurite outgrowth even under the NGF-depleted culture condition, excluding an indirect effect of GDNF via NGF. Quantification of mRNA and protein by real-time PCR and immunohistochemistry at different developmental stages revealed that GDNF is abundantly expressed in the hearts of embryos and neonates, but not in adult hearts. GDNF plays an important role in inducing cardiac sympathetic innervation at the early developmental stages. A possible role in (re)innervation of injured or transplanted or cultured and transplanted myocardium may deserve investigation.

Sympathetic nerves regulate cardiac function in balance with the parasympathetic nerves. The mechanism underlying cardiac sympathetic innervation, however, remains an unresolved issue. In general, neurotrophic factors play a critical role in the peripheral organs and the central nervous system. At present, neurotrophic factors have been classified into the following groups: 1) the “neurotrophin family”, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), all of which bind to the receptors Trk (high affinity) and p75 (low affinity); 2) the “GDNF family”, which includes glial cell line-derived neurotrophic factor (GDNF), neurturin and artemin, which bind to the GFRalpha receptors, with RET acting as a key signaling molecule; and 3) the “CNTF family”, which includes ciliary neurotrophic factor (CNTF) with CNTF-R-GP130 as a complex receptor.

Among the neurotrophic factors, NGF has been most extensively studied as the prototype of neurotrophins and is known to facilitate sympathetic axon growth in various organs and tissues including the heart (1, 12, 13). In contrast to the neurotrophin...
members, with regard to cardiac sympathetic innervation, little attention has been paid to the other neurotrophic factors, such as GDNF or CNTF, probably because these non-neurotrophin members are less abundantly expressed in the adult heart (18). Recent studies have reported that GDNF was upregulated after chemical sympathectomy in rats, suggesting a role in sympathetic nerve regeneration (14). Upregulation of both NGF and GDNF was demonstrated in rats with Trypanozooa cruzi infection (Chagas disease) causing sympathetic as well as parasympathetic denervation (15). GDNF was also demonstrated to express in the hearts of murine embryos and neonates by quantitative RT-PCR (11). In addition, artemin, a neurotrophic factor of the GDNF family was shown to express along blood vessels in the early developmental stages and promote the development of sympathetic innervation of blood vessels (3, 8).

In the present study, to elucidate the underlying mechanisms of sympathetic innervation in the heart, we shed light on the effects of the neurotrophic factors on the neurite growth of sympathetic neurons (SNs) for rat neonatal cardiomyocytes (CMs). We also examined the expression of GDNF in the different developmental stages.

Here, we demonstrate that 1) GDNF directly enhances sympathetic neurite growth toward cardiomyocytes, and that 2) GDNF is abundantly expressed in the heart at the early developmental stages but not in adult hearts. These results suggest that GDNF is a key molecule for sympathetic innervation in the heart. A possible role in regenerative innervation after injury or dysfunction deserves future investigation.

MATERIALS AND METHODS

Experiments were performed according to protocols approved by the Animal Experimentation Committee at the Research Institute of Environmental Medicine, Nagoya University.

Cultures of cardiomyocytes and sympathetic neurons. Ventricular cardiomyocytes (CMs) and sympathetic neurons (SNs) of the superior cervical ganglion were obtained from wild-type or GFP-transgenic 1-day-old neonatal Wistar rats through enzymatic digestion (4, 7). The SNs and CMs dissociated were co-cultured in two different modes:

1) Random co-culture: SNs and CMs were randomly mixed and seeded on a gelatin-coated cover slip to examine the effects of four neurotrophic factors and a neuronal repellent (Fig. 1), and the concentration of NGF in the culture medium (Fig. 2).

2) Proximity co-culture: SNs (4 x 10^5/mL) and CMs (2 x 10^6/mL) were seeded separately on a gelatin-coated cover slip to observe the outgrowth of neurites toward CMs (Fig. 3). A glass ring frame (outer diameter 6 mm, inner diameter 4 mm) was placed between CMs and SNs (15 h) for their initial separation (CMs outside and SNs inside).

In both modes, SNs and CMs were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 10% (vol/vol) FBS (Gibco BRL, Gaithersburg, MD), ITS liquid media supplement (Sigma-Aldrich) and cytosine arabinofuranoside (1 μmol/L; Sigma-Aldrich) at 37°C in an incubator with 5% CO_2 and 98% humidity. The culture medium was supplemented either with NGF (50 ng/mL; Sigma-Aldrich), BDNF (50 ng/mL; R&D systems, Minneapolis, MN), GDNF (10 or 50 ng/mL; R&D systems), CNTF (50 ng/mL; R&D systems) or human semaphorin 3A (Sema3A, 50 ng/mL; R&D systems). Cell cultures were continued for 5 days to observe the neurite outgrowth of SNs among or toward the CMs.

Immunofluorescence staining of cultured cells. Cultured cells were fixed with phosphate-buffered saline (PBS) containing 2% paraformaldehyde (PFA) for 15 min at 4°C and permeabilized with 0.05% Triton X-100, then washed twice with PBS and blocked for 1 h at room temperature (RT) in PBS containing 10% BSA. Cells were incubated overnight at 4°C with primary antibodies: anti-α-actinin antibody (mouse monoclonal; 1 : 200; Sigma-Aldrich) and anti-neurofilament M (NFM) (rabbit polyclonal or mouse monoclonal; 1 : 200; Chemicon International, Temecula, CA). The samples were then incubated for 1 h at RT with a 1 : 200 (v/v) dilution of appropriate secondary antibodies: Alexa Fluor 488-conjugated goat anti-mouse IgG (1 : 200; Invitrogen, Carlsbad, CA), Alexa Fluor 568-conjugated goat anti-rabbit IgG (1 : 200; Invitrogen). Immunofluorescence images were acquired using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc., Jena, Germany). The fractions of NFM-positive neurite were calculated using Image-Pro Plus software (MediaCybernetics, Inc., Bethesda, MD).

Immunohistochemistry for detection of GDNF in developing hearts. For whole-mount immunostaining, rat embryo (E14.5) and neonatal (P1) hearts were prefixed with microwave irradiation, and fixed with PBS containing 2% PFA and microwave irradiation.
for 20 s. The specimens were dehydrated in 50, 75 and 100% methanol. To block endogenous peroxidase, the fixed specimens were bleached (methanol; 0.3% H$_2$O$_2$) for 30 min at 4°C, then hydrated in 100, 75, 50 and 25% methanol and PBS, then permeabilized and blocked by incubating twice in PBSMT (2% skim milk and 0.1% Triton X-100 in PBS and 0.2% BSA) for 1 h at RT. They were incubated overnight with PBSMT containing goat anti-GDNF antibody at 4°C, then washed five times each in PBSMT for 1 h at 4°C. The primary antibody was developed by incubating 1 μg/mL horseradish peroxidase-conjugated anti-goat IgG antibody (Biosource, Camarillo, CA) overnight at 4°C. After extensive washing with more than five exchanges of PBSMT, including the final 20 min wash in PBST (0.1% Triton X-100 in PBS) at RT, the specimens were soaked in PBST containing 0.05% NiCl$_2$ and 250 mg/mL diamobenzidine (Chemical Dojin, Kumamoto, Japan) for 10–30 min, and hydrogen peroxide was added to 0.01%. The enzymatic reaction was allowed to proceed until the desired color intensity was reached, and the specimens were rinsed three or four times in PBST. The images of stained embryos and tissues were taken using a transmitted light microscope (Nikon, Tokyo, Japan).

For tissue section immunostaining, hearts obtained from wild-type rats (E14.5) were fixed with PBS containing 2% PFA at 4°C for overnight, and then embedded in OCT compound and frozen on dry ice. Hearts were cryosectioned and stained with anti-GDNF (rabbit polyclonal; 1:200; Chemicon International, Temecula, CA) and anti-α-actinin antibody (mouse monoclonal; 1:200; Sigma-Aldrich) to label GDNF protein and cytoplasm of cardiomyocytes respectively. The sections were incubated with secondary antibodies conjugated with Alexa 488, 568 (Molecular Probes, Carlsbad, CA). All confocal microscopic images were obtained using LSM 510 microscope (Carl Zeiss).

**Measurement of NGF protein.** NGF protein in the culture medium was quantified using ELISA in an antibody sandwich format (NGF Emax Immunoassay System; Promega, USA) (9). The kit displays cross-reactivity in several species, including rats and humans. In brief, a 96-well plate was coated with a rabbit polyclonal antibody to NGF (1:1,000). After blocking, standard (human NGF; 0.24–125 pg/mL) and diluted samples were added. Then, the plate was extensively washed and incubated with a rat monoclonal antibody for NGF (1:4,000). The amount of specifically bound antibody was detected using a goat anti-rat IgG conjugated to horseradish peroxidase (1:100) as a tertiary reactant. The detection limit was 1–2 pg/mL.

**Quantification of GDNF mRNA expression by real-time PCR.** To quantify mRNA expression of GDNF (Assay ID: Rn00569510/mL) (Accession number ID: NC_005101) in the ventricular tissues of wild-type rats, in 14.5-day-old embryos (E14.5), 1-day-old neonates (P1), and 49-day-old adults (P49), quantitative PCR was carried out using real-time Taqman™ technology (6, 17). The results were analyzed with a model 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). 18S ribosomal RNA was used as an internal control.

**Statistics.** Values are presented as mean ± SD. Differences between the experimental groups were evaluated by ANOVA with the Fisher protected least significant difference test. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Effects of neurotrophic factors on neurite growth among CMs**

The effect of four different neurotrophic factors from the neurotrophins (NGF, BDNF), and other families (GDNF, CNTF) on sympathetic neurite growth in randomly mixed co-cultures of SNs and CMs, were examined as well as a neuronal repellent (semaphorin 3A). As a result, NGF (50 ng/mL), BDNF (50 ng/mL) and GDNF (50 ng/mL) significantly increased the density of neurites immunolabeled by anti-neurofilament M (NFM) antibody (red). By far, the largest increase compared to control was found for GDNF (6-fold, $P < 0.05$ vs. CONT, n = 5). Significant increases were also observed for NGF (2.6-fold, $P < 0.05$ vs. CONT, n = 5) and BDNF (2.3-fold, $P < 0.05$ vs. CONT, n = 5). Fig. 1A shows typical examples of these immunolabeling data and Fig. 1B shows the summarized data.

**GDNF directly enhances neurite outgrowth among or toward CMs**

It is, in principle, possible that the potent neurotrophic effect of GDNF is mediated by secondary production and excretion of NGF. Therefore, we quantified the NGF protein level in the medium of CM/SN cultures without neurotrophic factors indicated (CM/SN) and in cultures with either the addition of GDNF (CM/SN + GDNF) or NGF (CM/SN + NGF). Obviously, there was no significant differ-
protein was observed in the embryonic heart, comparable to those in the central nervous system. GDNF-mRNA was expressed abundantly in the embryonic period (E14.5), but the expression level decreased markedly after birth (from P1 to P49) (Fig. 4A). In immunostaining for whole embryo (E14.5), abundant expression of GDNF was shown in the heart as well as central nervous system (midbrain, diencephalon, pons, medulla oblongata), liver and midgut (Fig. 4B). Substantial expression was also observed in the neonatal heart (P1). At this stage, GDNF was expressed in the entire heart although its extent was more remarkable in the atrium and the interventricular septum compared to the ventricle (Fig. 4C).

To further observe the localization of GDNF in the developing heart, we conducted immunohistochemical and RT-PCR analyses in embryonic and neonatal rat hearts. As shown in Fig. 4B, expression of GDNF protein was observed in the embryonic heart, comparable to those in the central nervous system. GDNF-mRNA was expressed abundantly in the embryonic period (E14.5), but the expression level decreased markedly after birth (from P1 to P49) (Fig. 4A). In immunostaining for whole embryo (E14.5), abundant expression of GDNF was shown in the heart as well as central nervous system (midbrain, diencephalon, pons, medulla oblongata), liver and midgut (Fig. 4B). Substantial expression was also observed in the neonatal heart (P1). At this stage, GDNF was expressed in the entire heart although its extent was more remarkable in the atrium and the interventricular septum compared to the ventricle (Fig. 4C).
In tissue sections of embryonic hearts (E14.5). Fig. 5 shows representative images of immunofluorescence labeling for GDNF and α-actinin (cytoplasmic marker of cardiomyocytes) in tissue sections of the embryonic heart (E14.5). As a result, GDNF was detected abundantly in the atrium (Fig. 5E), interventricular septum (Fig. 5C), while weak GDNF immunoreactivity was observed in the ventricle (Fig. 5D) and aorta (Fig. 5B). Merged views of GDNF and α-actinin demonstrated that the immunopositive domains for GDNF were detected in the cytoplasm of cardiomyocytes (Fig. 5F right panel).

DISCUSSION

In the present study, we demonstrate that GDNF enhances neurite growth of sympathetic neurons towards CMs. The effect of GDNF was more potent than that of the neurotrophins, such as NGF or BDNF. In addition, we show that GDNF is expressed in the early developmental stages of the heart but not in the adult heart. This study shows, for the first time, that GDNF may be a key molecule in promoting sympathetic innervation of CMs in the developing heart.

It is well known that NGF plays an important role in sympathetic axon growth in the heart, both early development and adult stages (2, 10). In contrast, the roles of other neurotrophic factors such as...
expression of GDNF mRNA close to the rough endoplasmic reticulum of rat cardiomyocytes in response to chemical sympathectomy. They also confirmed GDNF protein expression in granules (atrial myocytes) and cytoplasm (ventricular myocytes), suggesting that GDNF is a cardiomyocyte secretory peptide (14). Consistent with the report by Martinelli et al. (14), our results showed that GDNF was expressed in the cytoplasm of cardiomyocytes (more abundant in atrial myocytes than in ventricular myocytes) (Fig. 5) and suggest that cardiomyocytes may secrete GDNF. Nevertheless, we cannot eliminate other possible cell sources of GDNF secretion from the heart, because artemin, another member of the GDNF family, has been shown to be secreted from vascular smooth muscle (3, 8).

It is worth to note that in the randomly mixed co-cultures of CMs and SNs isolated from neonatal rat hearts and superior cervical ganglia GDNF was up to three times more potent than NGF in promoting neurite growth from SNs to CMs (Fig. 1). The effect of BDNF was comparable to NGF, whereas that of GDNF have not been clarified. Our experiments using exogenously applied GDNF suggest that this molecule potently promotes neurite growth towards CMs. Previously, Martinelli et al. demonstrated that, in a rat model of chemical sympathectomy by 6-hydroxydopamine, the GDNF protein level increased transiently prior to axonal regrowth in a cardiac tissue homogenate (14). The same group reported that, in rats infected with Trypanosoma cruzi (Chagas disease), gold particles showing existence of GDNF in atrial granules increased transiently at the time of maximal autonomic denervation (15).

On the other hand, in a mouse model of myocardial infarction and subsequent sympathetic nerve sprouting, myocardial gene expression of GDNF (estimated by DNA microarray) was, unlike NGF, virtually unchanged (16). In humans, cardiac expression of GDNF is limited to the embryonic and early post-natal stage, whereas NGF (estimated by DNA microarray) is abundantly expressed in the adult heart (18). However, Martinelli et al. (14) showed that increased level of GDNF in the heart and expression of GDNF mRNA close to the rough endoplasmic reticulum of rat cardiomyocytes in response to chemical sympathectomy. They also confirmed GDNF protein expression in granules (atrial myocytes) and cytoplasm (ventricular myocytes), suggesting that GDNF is a cardiomyocyte secretory peptide (14). Consistent with the report by Martinelli et al. (14), our results showed that GDNF was expressed in the cytoplasm of cardiomyocytes (more abundant in atrial myocytes than in ventricular myocytes) (Fig. 5) and suggest that cardiomyocytes may secrete GDNF. Nevertheless, we cannot eliminate other possible cell sources of GDNF secretion from the heart, because artemin, another member of the GDNF family, has been shown to be secreted from vascular smooth muscle (3, 8).

Fig. 4 Expression of GDNF in the developing rat heart. (A) Expression of GDNF mRNA in wild-type rat ventricular tissues in a 14.5-day-old embryo (E14.5), a 1-day-old neonate (P1) and a 49-day-old adult (P49). The GDNF mRNA levels were measured by RT-PCR and normalized to the amount of 18S mRNA. The data are from five rats at each stage of development (mean ± SD). *P < 0.05 vs. E14.5. (B) Expression of GDNF protein (purple) was detected in wild-type rat hearts in a 14.5-day-old embryo (E14.5), a 1-day-old neonate (P1) by immunohistochemistry. AO: aorta, IVS: interventricular septum, LA: left atrium, LV: left ventricle, RA: right atrium, RV: right ventricle. Scale bars indicate 1 mm.
Fig. 5  Localization of GDNF protein in the heart. Immunofluorescent images for GDNF (red) and the α-actinin (AA; green) were obtained in the sections across the long axis of the wild-type embryonic rat hearts (E14.5 d.p.c). An upper panel showed the representative image of the entire heart in dark fields (A). Magnified views of box areas indicated in panel A were shown in B–E. Panel F showed magnified view of the box image indicated in E. In B–F, left and middle panels were labeled for AA, GDNF, respectively, and right panels showed merged images. In panel F, arrow heads indicate nucleus of CMs. Scale bars indicate A: 200 μm, B–E: 50 μm, F: 10 μm. AO: aorta, IVS: interventricular septum, LV: left ventricle, RA: right atrium.
CNTF remained insignificant. In addition, we have found by real-time PCR and immunohistochemistry that mRNA and protein of GDNF are expressed in rat hearts from the embryonic to the early postnatal period, although the expression is below a detectable level in adulthood. GDNF may act directly on SNs through c-Ret receptors (5), because it does not increase NGF levels. We also showed in our proximity co-culture experiments using anti-NGF antibody that the sympathtrophic effect of GDNF is preserved under the condition of NGF-depletion (Fig. 3).

Based on our in vitro observations and on the literature, the physiological role of GDNF in cardiac innervation can be summarized as follows: 1) Under physiological conditions, both GDNF and NGF may be required for normal development of sympathetic innervation during the embryonic stage, and 2) in adulthood, NGF may play a more important role than GDNF in survival and maintenance of function in the normal heart. It will be interesting to see whether GDNF is involved in regenerative sympathetic (re)innervation in response to degenerative changes associated with myocardial infarction or heart failure. Also a role in the manipulation of innervation of tissue constructs after transplantation may constitute an interesting avenue of future research.

In conclusions, GDNF is a key molecule for neurite growth in the heart at early developmental stages. Its role in the normal adult heart is small or absent. Its role under pathophysiological conditions remains to be established.

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