Involvement of Cardiotrophin-1 in Cardiac Myocyte-Nonmyocyte Interactions During Hypertrophy of Rat Cardiac Myocytes In Vitro

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Background—The mechanism responsible for cardiac hypertrophy is currently conceptualized as having 2 components, mediated by cardiac myocytes and nonmyocytes, respectively. The interaction between myocytes and nonmyocytes via growth factors and/or cytokines plays an important role in the development of cardiac hypertrophy. We found that cardiac myocytes showed hypertrophic changes when cocultured with cardiac nonmyocytes. Cardiotrophin-1 (CT-1), a new member of the interleukin-6 family of cytokines, was identified by its ability to induce hypertrophic response in cardiac myocytes. In this study, we used the in vitro coculture system to examine how CT-1 is involved in the interaction between cardiac myocytes and nonmyocytes during the hypertrophy process.

Methods and Results—RNase protection assay revealed that CT-1 mRNA levels were 3.5 times higher in cultured cardiac nonmyocytes than in cultured cardiac myocytes. We developed anti–CT-1 antibodies and found that they significantly inhibited the increased atrial and brain natriuretic peptide secretion and protein synthesis characteristic of hypertrophic changes of myocytes in the coculture. In addition, non–myocyte-conditioned medium rapidly elicited tyrosine phosphorylation of STAT3 and induced an increase in natriuretic peptide secretion and protein synthesis in cultured cardiac myocytes; these effects were partially suppressed by anti–CT-1 antibodies. Finally, the hypertrophic effects of CT-1 and endothelin-1, which we had previously implicated in the hypertrophic activity in the coculture, were additive in cardiac myocytes.

Conclusions—These results show that CT-1 secreted from cardiac nonmyocytes is significantly involved in the hypertrophic changes of cardiac myocytes in the coculture and suggest that CT-1 is an important local regulator in the process of cardiac hypertrophy. (Circulation. 1999;100:1116-1124.)

Key Words: hypertrophy ■ natriuretic peptides ■ cells ■ antibodies ■ interleukin

Cardiac hypertrophy ultimately leads to heart failure, although initially it is an adaptive process that occurs in response to mechanical load or tissue injury. The mechanism responsible for cardiac hypertrophy is complex, and our understanding of its details remains incomplete. It is characterized by an increase in cardiac myocyte (MC) cell size, proliferation of cardiac fibroblasts, and progression of interstitial and perivascular fibrosis. Therefore, the interaction between MCs and the surrounding nonmyocytes (NMCs) is likely to be an important component of the hypertrophy process.1 Consistent with this notion, many growth factors and cytokines, acting as autocrine/paracrine factors, are involved in the hypertrophic response,2–13 and “cross talk” between MCs and NMCs via such humoral factors appears to play an important role in the pathophysiology of cardiac hypertrophy. In an earlier study, we used an in vitro, MC/NMC coculture model to show that hypertrophic responses in MCs, including increased cell size and secretion of natriuretic peptides, occur in coculture and that endothelin-1 (ET-1) secreted from NMCs (fibroblasts or myofibroblasts but not endothelial cells) acts as a paracrine factor during the process.14 We also concluded that other, as yet unidentified, humoral factors secreted from NMCs are involved in the hypertrophic response.14

Recently, gp130-dependent signaling pathways were implicated in the heart development and the progression of cardiac hypertrophy.15,16 Cardiotrophin-1 (CT-1) is a novel member of the interleukin-6 (IL-6) family of cytokines identified by expression cloning based on its ability to induce cardiac MC hypertrophy.17 CT-1 binds to the leukemia inhibitory factor (LIF) receptor/gp130 heterodimer and activates both mitogen-activated protein

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kinase and Janus kinase signal transducers and activators of transcription (STAT) signaling pathways. Through these signaling pathways, CT-1 induces MC hypertrophy and prolongs the survival of both embryonic and neonatal rat MCs. Indeed, the restricted expression of CT-1 mRNA in the primitive heart tube and the continued myocardial expression during embryogenesis suggest that CT-1 may activate gp130-dependent signaling pathways during cardiac development. Because the heart continues to be a prominent site of CT-1 expression throughout adulthood, it may also mediate hypertrophic responses in MCs in vivo through the same gp130-dependent signaling pathways. Using an adult animal model of cardiac hypertrophy (stroke-prone spontaneously hypertensive rats/Lzm), we previously showed that CT-1 mRNA expression is specifically augmented in the heart. This suggests a possible role of CT-1 in vivo cardiac hypertrophy. Nevertheless, despite the growing evidence concerning the pharmacological actions and the unique expression profile of CT-1, little is known about the function of endogenous CT-1 in the development of cardiac hypertrophy.

In this report, we examined the involvement of CT-1 in MC/NMC interactions during MC hypertrophy using an in vitro coculture system. We demonstrate dominant expression of CT-1 mRNA in NMCs compared with MCs and show that anti–CT-1 blocking antibodies, developed in our laboratory, suppress hypertrophic responses in MCs. These results suggest that CT-1 functions as a paracrine factor in our in vitro

Figure 1. CT-1 mRNA expression in MCs and NMCs. A, Representative Northern blot analysis from 3 different experiments with identical results (total RNA, 50 μg or 20 μg/lane as indicated). B and C, RNase protection assay for CT-1 mRNA. B, Representative autoradiographs (10 μg/lane). C, Results from 6 independent experiments (n=13). In each experiment, CT-1 mRNA level in MCs was arbitrarily assigned a value of 1. Values are mean±SEM. *P<0.001 vs MC.

Figure 2. Inhibitory effect of anti–CT-1 antibodies on CT-1–induced ANP and BNP secretion and [3H]leucine incorporation in MC cultures. Neonatal MCs were incubated with either 0.1 mg/mL of anti–CT-1 antibodies or control rabbit IgG for 60 minutes before addition of either CT-1 10-8 mol/L, LIF 10-8 mol/L, IL-6 10-9 mol/L, ET-1 10-9 mol/L, or vehicle. A, Concentrations of ANP (left) and BNP (right) in culture medium 48 hours after stimulation. B, Radioactivity of incorporated [3H]leucine (cpm/well). Bars represent mean±SEM. *P<0.05 vs vehicle with control IgG. †P<0.05 vs CT-1 with control IgG.
system and implicates it as a possible local regulator during the processes of cardiac hypertrophy and heart failure.

Methods

Agents
Human ET-1 was purchased from Peptide Institute. Human LIF and rat IL-6 were purchased from Immugenex Corp. BQ123, an ET-A receptor antagonist, was provided by Banyu Pharmaceutical Co Ltd.

Preparation of Recombinant Rat CT-1
The recombinant rat CT-1 was prepared by the GST-fusion system according to the manufacturer’s manual (Pharmacia Biotechnology Inc).

Cell Culture
Neonatal rat ventricular MCs and NMCs were prepared on a Percoll gradient and maintained as previously described.14 After MCs were separately collected, the cells were preplated on noncoated dishes for 1 hour to reduce contamination by NMCs. Nonattached cells were then collected; this cell population consisted of >97% MCs, as assessed by immunofluorescence with anti–rat sarcomeric actin antibodies (DAKO Japan Co, Ltd).

NMCs were plated and maintained for 2 or 3 passages as previously reported.14 The absence of endothelial cell contamination of the NMC cultures was confirmed by the lack of 1,1’dioctadecyl 3,3’,3’-tetramethylindocarbocyanine perchlorate acetyl LDL binding (Biochemical Technologies Inc) to the cells.14 NMC-conditioned medium (NMC-CM) was prepared as described previously.14

Cocultures of MCs and NMCs were prepared as previously described14 (the ratio of the cell numbers of NMCs to MCs in the coculture was 30%).

Northern Blot Hybridization Analysis
A 473-bp EcoRI-Apal fragment of rat CT-1 cDNA was used as a probe.22 Total cellular RNA was extracted from cultured MCs or NMCs with Trizol (Gibco BRL), and Northern blot analysis was performed as previously described.14

RNase Protection Assay
The 473-bp EcoRI-Apal fragment of rat CT-1 cDNA was ligated with pBluescript II SK(−). Radiolabeled RNA transcripts were then synthesized by use of the DNA template linearized by EcoRI digestion according to the technical manual from Promega Co. The RNase protection assay was performed with the RPA Hybspeed kit (Ambion Inc) as instructed by the manufacturer’s manual. All signals were normalized to an internal control RNA (β-actin) (Ambion Inc).
Preparation of Polyclonal Blocking Antibodies to Rat CT-1

Rabbits were injected with 25 mg SC of recombinant rat CT-1 emulsified in complete Freund’s adjuvant (Difco Laboratories) every 2 weeks. Anti–rat CT-1 antibodies (KCT-1) were purified by protein G–sepharose chromatography (Pharmacia Biotech).

RIAs for Atrial and Brain Natriuretic Peptides

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) concentrations were measured with specific radioimmunoassays as previously reported.23

Analysis of Protein Synthesis in Cultured Cells

Protein synthesis in cultured cells was evaluated by analysis of [3H]leucine incorporation. Cells were cultured under various experimental conditions for 48 hours. At that time, 3 μCi of [3H]leucine (Amersham Life Science) was added, and the cells were cultured for an additional 24 hours. After being washed twice with ice-cold PBS, the cells were then incubated with 10% trichloroacetic acid for 30 minutes at 4°C. Cell precipitants were then solubilized in 0.2N NaOH for >4 hours. Radioactivity was measured in a liquid scintillation counter.

Western Blot Analysis for STAT3

After stimulation, whole-cell protein extracts were obtained and applied to a 10% SDS-PAGE; the resolved proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were then blocked with 5% skim milk (Difco Laboratories) and probed with anti-STAT3 antibodies (Santa Cruz Biotechnology, Inc) or phosphospecific STAT3 antibodies that recognize only activated STAT3 (New England Biolabs, Inc). The antibodies were applied at a 1:1000 dilution for 1 hour to detect STAT3 or tyrosine-phosphorylated STAT3, respectively. Immune complexes were visualized on Konica Medical film (Konica Co) with an enhanced chemiluminescence system according to the manufacturer’s instructions (NEN Life Science Products).

Western Blot Analysis for CT-1 Purified From NMC-CM

We coupled anti–CT-1 antibodies to a HiTrap NHS-activated column (Pharmacia Biotech) as instructed by the manufacturer’s manual. Ten milliliters of the NMC-CM was applied to the column. The eluate was lyophilized and applied to an SDS-PAGE. Western blot analysis was performed as indicated above using anti–CT-1 antibodies raised by the synthetic 21 peptides corresponding to rat CT-1 (183 to 203) (OCT-6).

Statistical Analysis

Data are presented as mean±SEM. Unpaired Student’s t tests were used to determine significant differences between 2 groups, and ANOVA with subsequent Fisher’s test was used to determine significant differences among 3 or 4 groups. A value of \( P<0.05 \) was considered significant.

Figure 4. A through C, Suppression of natriuretic peptide secretion and [3H]leucine incorporation in MC/NMC cocultures by anti–CT-1 blocking antibodies. A, ANP and B, BNP concentrations in CM after 48 hours of incubation, and C, radioactivity of incorporated [3H]leucine (cpm/well). Values obtained from 3 independent experiments in quadruplicate are shown as mean±SEM. *\( P<0.0001 \) vs pure MCs with control IgG. †\( P<0.001 \) vs coculture with control IgG. D through F, Anti–CT-1 blocking antibodies suppressed NMC-CM-induced increase in natriuretic peptide secretion and [3H]leucine incorporation in MC cultures. D, ANP and E, BNP concentration in cultured medium after 48 hours of stimulation, and F, radioactivity of incorporated [3H]leucine (cpm/well). Values obtained from at least 2 independent experiments in quadruplicate are shown as mean±SEM. *\( P<0.05 \) vs vehicle with control IgG. †\( P<0.05 \) vs NMC-CM with control IgG.
Results

CT-1 mRNA Expression in MCs and NMCs

To determine which cell types synthesize CT-1 in the heart, we analyzed CT-1 mRNA expression in cultured MCs and NMCs by use of Northern blots. The 1.4-kb CT-1 mRNA signal was clearly detected in NMCs; on the contrary, little signal was detected in MCs (Figure 1A). We also evaluated CT-1 mRNA expression using RNase protection assay, which is a much more sensitive procedure. CT-1 mRNA protected bands were detected in both MCs and NMCs (Figure 1B). However, CT-1 mRNA levels in NMCs were 3.5 times higher than those in MCs (Figure 1C).

Effects of Anti–CT-1 Antibodies in MC/NMC Interactions

To investigate the function of CT-1, we developed anti–CT-1 antibodies. Initially, the capacity of purified anti–CT-1 antibodies to block the effects of CT-1 was tested. MCs were stimulated with 10−8 mol/L CT-1, which is a dose for maximal hypertrophic activity,17 in the presence of 0.1 mg/mL of anti–CT-1 antibodies. As shown in Figure 2, whereas CT-1 significantly increased ANP and BNP secretion (Figure 2A) and [3H]leucine incorporation (Figure 2B) in MCs, the augmentation was completely suppressed in the presence of anti–CT-1 antibodies. To elucidate the specificity of the antibodies for CT-1, we also examined whether the antibodies attenuate the effects of other IL-6 related cytokines, IL-6 and LIF, or a known trophic factor, ET-1. The antibodies did not affect ANP and BNP secretion (Figure 2A) and [3H]leucine incorporation (Figure 2B) in MCs induced by LIF or ET-1. IL-6 had little effect on MCs, as previously reported by others.17 In addition, the antibodies completely blocked both morphological changes and STAT3 tyrosine phosphorylation induced by CT-1 (Figure 3, A and B).

Next we examined the effects of the blocking antibodies in MC/NMC cocultures. As we have previously reported,14 MCs showed hypertrophic responses when cocultured with NMCs. The presence of 0.1 mg/mL of purified anti–CT-1 blocking antibodies partially, but significantly, inhibited the increased secretion of natriuretic peptides and [3H]leucine incorporation normally seen in MC/NMC cocultures (Figure 4, A, B, and C). By contrast, when the antibodies were added to pure MC cultures, they had no effect at all (Figure 4, A, B, and C). These results indicate that CT-1 contributes significantly to the hypertrophic activity seen in MC/NMC cocultures.

To test whether CT-1 secreted into the culture medium by NMCs mediates the hypertrophic effects, MCs were incubated with NMC-CM containing anti–CT-1 blocking antibodies. It was observed that the antibodies significantly suppressed the increase in secretion of natriuretic peptides and [3H]leucine incorporation normally seen in MC/NMC cocultures (Figure 4, A, B, and C). Furthermore, NMC-CM induced tyrosine phosphorylation of STAT3 in MCs (Figure 5A), and the time course of tyrosine phosphorylation of STAT3 was similar to that of CT-1–induced phosphorylation (Figure 5B), although the time of peak phosphorylation of STAT3 was not completely identical to that of CT-1. The anti–CT-1 blocking antibodies partially inhibited the tyrosine phosphorylation of STAT3 elicited by NMC-CM (Figure 5C). Thus, the tyrosine phosphorylation of STAT3 induced by NMC-CM appears to be mediated, at least in part, by CT-1. The partial inhibitory effect of anti–CT-1 antibodies suggests that other humoral factors present in NMC-CM also stimulate tyrosine phosphorylation of STAT3.

We also performed Western blotting analysis on samples of NMC-CM using anti–CT-1 antibodies (OCT-6). As shown in Figure 5D, the band responsible for CT-1 was detected in NMC-CM. We estimated that the NMC-CM contains 0.5 μg/L (3.3 × 10−11 to 2 × 10−11 mol/L) of CT-1 by comparing the intensity of the band with that obtained with different quantities of recombinant rat CT-1, as indicated. The concen-
tration of CT-1 is high enough to induce hypertrophic responses, because we and others have confirmed that CT-1 induced MC hypertrophy at concentrations as low as $10^{-11}$ mol/L.

Additive Hypertrophic Effect of CT-1 and ET-1 in MCs

In an earlier report, we showed that ET-1 plays a role in the MC hypertrophic response in MC/NMC cocultures and that NMC-CM contains ET-1. Then we investigated whether the hypertrophic effects of CT-1 and ET-1 were additive. Simultaneous administration of CT-1 and ET-1 augmented secretion of ANP and BNP (Figure 6A) and $[^3H]$leucine incorporation into cells (Figure 6B) to a significantly greater degree than either CT-1 or ET-1 alone. Addition of anti–CT-1 blocking antibodies suppressed the responses to the levels induced by ET-1 alone (Figure 6C). Moreover, the coadministration of the anti–CT-1 antibodies and BQ123 inhibited the NMC-CM–induced increase in MC size to a greater degree than either the antibodies or BQ123 alone (Figure 8, B through G). These results indicate that CT-1 and ET-1 secreted by NMCs elicit the hypertrophic response in MCs in an additive fashion.

Discussion

The mechanism responsible for cardiac remodeling is currently considered to have 2 components mediated by MCs and NMCs, respectively. To investigate the interaction between MCs and NMCs, we established an in vitro MC/NMC coculture system. Using this system, we found that ET-1 secreted from NMCs is implicated in the hypertrophic response observed in MC/NMC coculture. Because ET-1 blockade did not inhibit the response completely, however, it was concluded that other as yet unidentified factors might also be involved. Thus, we hypothesized that CT-1 secreted by NMCs also contributes to the development of MC hypertrophy.

To examine this hypothesis, we first studied the expression profile of CT-1 mRNA in cultured cardiocytes. Northern blot analysis and RNase protection assays revealed that CT-1

Figure 6. Additive hypertrophic effects of CT-1 and ET-1 on MCs. A and B, MCs were incubated with either anti–CT-1 antibodies or control IgG for 1 hour before addition of either $10^{-9}$ mol/L ET-1 or $10^{-8}$ mol/L CT-1 alone or both. ANP (A, left) and BNP (A, right) concentrations in CM 48 hours after stimulation by agents, as indicated (+), and radioactivity of incorporated $[^3H]$leucine (cpm/well) (B). Values obtained from 2 independent experiments in triplicate are shown as mean±SEM. *$P<0.05$ vs ET-1 alone. †$P<0.05$ vs CT-1 alone. ‡$P<0.05$ vs ET-1 $+$ CT-1 with control IgG. Additive effects of CT-1 and ET-1 on ANP secretion were observed in both maximal and submaximal conditions. MCs were incubated for 48 hours with either CT-1 or ET-1 alone or with both CT-1 and ET-1 at indicated doses. Values obtained from 2 independent experiments in triplicate are shown as mean±SEM. *$P<0.05$ vs vehicle. †$P<0.05$ vs $10^{-9}$ mol/L ET-1. ‡$P<0.05$ vs $10^{-8}$ mol/L CT-1. §$P<0.05$ vs $10^{-10}$ mol/L ET-1. ||$P<0.05$ vs $10^{-10}$ mol/L CT-1. ¶$P<0.05$ vs $10^{-11}$ mol/L ET-1. #$P<0.05$ vs $10^{-11}$ mol/L CT-1.
mRNA was expressed to a greater degree in NMCs than in MCs. To determine whether CT-1 acts as an NMC-derived trophic factor, we next studied the effect of anti–CT-1 blocking antibodies in pure MC cultures and in MC/NMC cocultures. Addition of the antibodies to the pure MC cultures had no affect on either secretion of ANP and BNP or protein synthesis assessed by \[^{[3}H]\]leucine incorporation. However, when added to the cocultures, CT-1 blocking antibodies significantly suppressed the hypertrophic response. NMC-CM induced rapid (within 5 minutes) tyrosine phosphorylation of STAT3 in MCs, as is the case for exogenously administered CT-1. Anti–CT-1 blocking antibodies partially inhibited NMC-CM–induced STAT3 tyrosine phosphorylation and significantly suppressed the NMC-CM–induced hypertrophic response in MCs. These lines of evidence all indicate that, like ET-1, CT-1 acts as a local trophic factor and contributes to the process of MC hypertrophy in this in vitro model of cardiac hypertrophy.

CT-1 is reported to share its receptors with LIF,\(^9\) and LIF also can be a candidate for an NMC-derived hypertrophic factor.\(^{24}\) In our in vitro model, LIF gene expression in serum-starved NMCs was not detected by Northern blot analysis, although CT-1 gene expression was detected in this condition. In addition, in vivo, in both humans and rats, the LIF gene expression was lower than that of CT-1 in the heart (LIF gene expression cannot be detected by Northern blot and can only be found by reverse transcription–polymerase chain reaction; unpublished data, 1998). Furthermore, CT-1 gene expression appears to be increased both in the ventricles of a rat model of cardiac hypertrophy (stroke-prone spontaneously
In addition to its hypertrophic effect, CT-1 is also reported to inhibit MC apoptosis. Ciliary neurotrophic factor and LIF, 2 other members of the IL-6 superfamily of cytokines, share the receptor components LIF receptor/gp130 with CT-1 and have a survival effect on motor neurons in vitro. Interestingly, ciliary neurotrophic factor is expressed in Schwann cells in the adult rat sciatic nerve, and LIF is expressed in nonneuronal cells, including fibroblasts and Schwann cells, in the rat superior cervical ganglia. Thus, under certain circumstances, interaction between neurons and nonneuronal cells via cytokines expressed in the surrounding nonneuronal cells may contribute to the survival of differentiated neurons. That CT-1 secreted from surrounding NMCs is involved in the maintenance of differentiated MCs in healthy or injured hearts is an attractive hypothesis. However, whether CT-1 promotes survival of MCs in vivo remains to be determined.

In conclusion, we show that NMCs are the principal source of CT-1 in culture and that when secreted, CT-1 contributes significantly to the MC hypertrophic response in vitro. These results suggest a possible role of CT-1 as a local regulator during the processes of cardiac hypertrophy and heart failure in vivo.

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