Myotrophin in Human Cardiomyopathic Heart

Parames Sil, Kunio Misono, Subha Sen

Earlier, myotrophin, a factor, has been isolated, purified, and partially sequenced from spontaneously hypertensive rat hearts that stimulated myocyte growth. To evaluate the role of myotrophin in the initiation of the human dilated cardiomyopathic heart, we have isolated and purified myotrophin to homogeneity (approximately 50,000-fold) as defined by reverse-phase high-performance liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). During purification, we used a bioassay system in which adult myocardial cells maintained in culture were used to evaluate protein synthesis by the incorporation of [3H]leucine into myocyte protein. Myotrophin purified from human dilated cardiomyopathic hearts is composed of a single polypeptide chain having an apparent molecular mass of 12 kD, determined by SDS-PAGE. The partial internal amino acid sequence of human myotrophin is very similar to that of rat myotrophin peptide T9. Using a rat myotrophin peptide (T26) antibody, we identified human myotrophin on an immunoblot. These results showed that human myotrophin possesses the T9 and T26 regions of rat myotrophin. Human myotrophin stimulated myocardial protein synthesis and cell growth, similar to the way in which rat myotrophin stimulated these factors. Western blot analysis showed the presence of myotrophin in both dilated cardiomyopathic and normal human hearts. In addition, we observed significantly elevated levels of myotrophin in dilated cardiomyopathic human hearts when compared with age- and sex-matched normal control hearts. From these observations, we conclude that myotrophin is present in normal human hearts, is found at higher levels in dilated cardiomyopathic human hearts, and may play a role in the initiation of cardiac hypertrophy as well as in normal growth of cardiac myocytes in humans. (Circulation Research 1993;73:98-108)

KEY WORDS • cardiac hypertrophy • adult myocytes • protein synthesis • stimulatory factors • amino acid sequence

Cardiomyopathy is a heart muscle disease of unknown cause in which significant hypertrophy has been documented.1 The molecular mechanism for development of hypertrophic cardiomyopathy is not well understood. Since dilated cardiomyopathy or hypertrophic cardiomyopathy is of common occurrence in humans and eventually leads to heart failure, the molecular mechanism for the initiation of such hypertrophy must be identified. Clinical studies2-3 and studies in animal models4,5 have demonstrated that the development of hypertrophy and its regression cannot be fully explained by medical treatment and by the control of blood pressure alone. In addition, in the presence of hypertensive hypertrophy in humans and in animal models, a dissociation has been established between blood pressure control and myocardial mass. This suggests that a factor(s) other than blood pressure control plays an important role in altering myocardial structure. Studies from our laboratory6-9 and from other laboratories10-13 have confirmed that antihypertensive therapy can either prevent or reverse cardiac hypertrophy in spontaneously hypertensive rats (SHR) and in humans. These studies suggested that, although all of the antihypertensive drugs studied were able to normalize blood pressure to the same extent, their abilities to reverse cardiac hypertrophy were markedly diverse.5,14-16 This further suggests that factors other than blood pressure control are responsible for modulation of myocardial growth. One can postulate several mechanisms by which protein synthesis of the myocardium stimulates cell growth: (1) mechanical factors, eg, stretch, (2) local growth factor(s), or (3) the existence of some intermediary extracellular signal such as catecholamines. Another possibility is the interaction between all these factors. Our hypothesis is that the mechanism for the initiation of myocardial hypertrophy resides within the cell and that during stress or stretch a factor is released from the myocardium that, in turn, increases the rate of myocyte protein synthesis and stimulates myocardial cell growth. Following this hypothesis, we have identified, isolated, purified, and sequenced a factor, myotrophin, from the myocardium of SHR heart17 that stimulates protein synthesis in vitro in rat myocytes. To evaluate whether myotrophin plays a role in human dilated cardiomyopathy, where significant hypertrophy is noted, we studied the existence of myotrophin in the hypertrophied human heart and its effect on myocardial protein synthesis by use of adult rat cardiac myocytes in vitro. At The Cleveland Clinic Foundation we have the opportunity to obtain explanted cardiomyopathic human hearts from the cardiac

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TABLE 1. Summary of Purification of Human Myotrophin

<table>
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<th>Purification steps</th>
<th>Protein (µg)</th>
<th>Amount of protein required for maximal stimulation (µg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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</table>

*Protein concentration was estimated by the relative intensities of bands in silver-stained sodium dodecyl sulfate gels.

transplant patients. The present study describes the isolation, purification, and partial sequence of myotrophin obtained from cardiomyopathic human hearts. This study also shows that the myotrophin level is significantly elevated in cardiomyopathic human hearts compared with age- and sex-matched normal control hearts.

**Materials and Methods**

**Human Hearts**

All human cardiomyopathic hearts were obtained from the transplantation program at The Cleveland Clinic Foundation. As soon as the heart was explanted, it was immersed in a solution containing 20 mL cardioplegic concentrate and 10 mL sodium bicarbonate in 1 L lactated Ringer’s solution. In a procedure lasting approximately 30 minutes, the left ventricle, right ventricle, septum, and other parts of the heart were cut into pieces, frozen in liquid nitrogen, and kept in a freezer at −80°C. In the present study, myotrophin was isolated and purified from the left ventricles of four patients with dilated cardiomyopathy. We have also isolated and purified myotrophin from a single dilated cardiomyopathic left ventricle obtained from a 23-year-old woman, as shown in Table 1.

**Animals**

Sprague-Dawley rats weighing 250 to 300 g used in the preparation of adult myocytes were purchased from Harlan Sprague Dawley, Inc, Indianapolis, Ind.

**Reagents**

All chemicals used in this study were American Chemical Society–certified analytical reagents. The solvents used were of high-performance liquid chromatography (HPLC) grade. Medium 199 used for adult myocytes culture was obtained from Gibco Biresearch Laboratories, Grand Island, NY. All other media and reagents used for adult myocyte preparation and culture, including fetal bovine serum albumin, insulin, and laminin, were purchased from Sigma Chemical Co, St Louis, Mo. Collagenase type II and N-acetyl-L-lysine chloromethyl ketone (TLCK)–treated trypsin were purchased from Worthington Biochemicals, Freehold, NJ. [³H]Leucine was obtained from Amersham Corp, Arlington Heights, Ill. Heparin and pentobarbital were purchased from Lyphomed, Inc, Rosemont, Ill, and from Abbott Laboratories, North Chicago, Ill, respectively.

**Development of the Bioassay System**

We used adult myocytes maintained in culture for the bioassay system, which we developed in our laboratory to evaluate the effect of human myotrophin and to follow its activity during purification.

**Isolation, purification, and maintenance of adult myocytes in culture.** Calcium-tolerant adult myocytes were isolated, purified, and maintained in culture after a combination of perfusion techniques and an attachment procedure as described by Borg et al and Bugaisky and Zak, with slight modifications in both the isolation procedure and the maintenance in culture. Adult Sprague-Dawley rats (13 to 16 weeks old) were injected intraperitoneally with heparin (100 U/100 g body wt). Fifteen minutes later, they were injected intraperitoneally again with pentobarbital (50 mg/100 g body wt). When the animal was fully anesthetized, its thorax was opened with a midline incision, the heart was exposed, and the aorta was separated from surrounding adjacent tissues and cut with fine sterile scissors. A syringe of “Joklik’s medium” containing Joklik’s minimal essential medium (nominally calcium free), 25 mM glutamic acid, 30 mM taurine, and 1 mM adenosine was attached to a cannula that was introduced into the lumen of the aorta. A knot was tied using a piece of silk thread, thus fixing the cannula into the aorta. The heart was then aseptically taken out, and the blood was removed through the Joklik’s medium with a syringe. By use of Joklik’s medium at 37°C, the heart was then retrogradely perfused without recirculation on a modified Langendorff apparatus for approximately 10 minutes. The same procedure was continued in the same medium containing collagenase (100 U/mL); the recirculation lasted for 30 minutes at the same temperature. After perfusion, the heart was removed from the apparatus, the atria and vessels were removed, and the ventricles were cut into
small pieces. Those pieces were placed into a 25-mL Erlenmeyer flask. Five milliliters of fresh Joklik's medium containing collagenase (100 U/mL) was added, and the flask was kept in a water bath at 37°C for 5 minutes with occasional shaking. After that, the tissue was broken by trituration with a disposable transfer pipette, and the released cells were removed by filtration through a piece of sterile nylon net into a 15-mL sterile modified polystyrene tube. Five milliliters of Joklik's medium containing 5% fetal bovine serum was added. The cells were then allowed to settle by gravity (10 to 15 minutes). Fresh collagenase solution in Joklik's medium was added, and the process was repeated two more times. The fractions obtained were combined and washed twice with Joklik's medium containing 5% fetal bovine serum. After the last wash, the cells were suspended in medium 199 containing 5% fetal bovine serum and antibiotics.

For the culture of the cells, a 35-mm six-well plate was precoated with laminin (20 μg per well) and kept at 37°C overnight. Cells from one heart were usually split equally into those six wells so that each well contained approximately 1×10⁶ cells. Most of the cells began to attach on the laminin almost immediately. To ensure a good attachment, they were left undisturbed overnight at 37°C in 4 mL medium 199 containing 5% fetal bovine serum. Approximately 60% of the cells adhered to the substrate.

Cell counts per well were determined by counting the cells of 16 random fields of known area on several wells, and the average value obtained was then extrapolated for the entire well. The viability and the integrity of the cells were examined under a phase-contrast microscope.

**Description of the assay.** The next day, medium 199 was aspirated, and 2 mL fresh medium 199 without fetal bovine serum was added per well. An appropriate amount of either buffer or the factor to be assayed was then added to each well. We used six-well plates for all bioassays. The first and sixth wells were used as controls in which only buffer was added. Insulin was added in the fifth well, and the factors were added in the second, third, and fourth wells. After 3 hours of incubation at 37°C, the medium was aspirated, and 1 mL fresh medium 199 was added per well. Then an appropriate amount of eithor the factor or buffer was added to each well. [3H]Leucine (10 μCi) diluted in medium 199 was added per well and incubated for 2 hours at 37°C. At the end of this incubation time, the medium was aspirated, and 1 mL of 0.1% sodium dodecyl sulfate (SDS) was added to each well. The plate was kept at room temperature for some time, with occasional shaking to lyse the cells. Cells were monitored under a light microscope to ensure complete lysis. After lysis, 50 μL lysis was removed from each well for use in determining the concentration of DNA. Known quantities of calf thymus DNA (1 μg/mL) were also dissolved in 0.1% SDS solution and used for establishing the standard curve. The rest of the lystate was adjusted to 1N NaOH and kept for 30 minutes. Then 1 mL bovine serum albumin solution (0.5 mg/mL) was added per well. Finally, 1 mL of 20% trichloroacetic acid solution was added to precipitate the protein, and the mixture was kept at 4°C for 30 minutes. The protein from each individual well was filtered and collected using a cell harvester on individual filter paper, and 5% trichloroacetic acid was used to wash the wells exhaustively until they were free from radioactivity. The filter papers were air-dried for 30 minutes and then counted in a beta scintillation counter after the addition of 5 mL scintillation fluid. Data were expressed as disintegrations per minute per microgram DNA. Insulin was used as the external standard for the validation of each assay.

**Purification of Human Myothropin**

Initially, the purification procedure developed for rat myothropin was adapted to purify human myothropin, but in ion-exchange chromatography using a mono-Q column, human myothropin was identified in protein fractions (No. 6), which were eluted in the high concentration of NaCl. Hence, in the subsequent step of purification using a second mono-Q column, we had to use a higher linear gradient of NaCl (0.3 M) in 20 mM l-histidine buffer. Briefly, left ventricles obtained from the hypertrophied human hearts of patients (23- to 35-year-old men and women) all diagnosed as having dilated cardiomyopathy were minced and then homogenized in 50 mM sodium phosphate buffer, pH 7.6. All the steps followed were up to the first ion-exchange chromatography using the first mono-Q column as described previously. Fractions exhibiting major stimulatory activity of adult myocytes protein synthesis were pooled. The buffer in this fraction was changed to 20 mM l-histidine buffer, pH 5.6, by gel filtration using a desalting column (Econo Pac 10 DG, Bio-Rad Laboratories, Richmond, Calif). The material was then chromatographed on the same column in 20 mM l-histidine buffer, pH 5.6. Elution was carried out using a gradient of NaCl from 0 to 0.3 M in a total gradient volume of 30 mL. The material of the active part was pooled and followed the subsequent purification steps as described in Reference 17 using a linear gradient of acetonitrile from 0% to 60% over a period of 40 minutes in 0.1% trifluoroacetic acid in water at a flow rate of 1 mL/min. The final active eluate was dried using a Speed Vac concentrator and used for bioassay, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), amino acid sequence determination, and other biochemical studies.

**Tests for Homogeneity**

The homogeneity of human myothropin in the final preparation was confirmed by two means: (1) the re-injection of the material of the active peak on a Vydac C18 column by reverse-phase HPLC using various elution conditions and (2) SDS-PAGE.

**Characterization of Human Myothropin**

**Perimic acid oxidation, tryptic digestion, and sequence determination.** Perimic acid was prepared by mixing 900 μL of 90% formic acid and 100 μL of 30% hydrogen peroxide and allowing them to react for 1 hour at room temperature. To 10 μg of dried pure protein, 50 μL of the perimic acid was added, and the mixture was kept at room temperature for 15 minutes. The material was cooled in a dry ice-acetone bath and evaporated to dryness in a Speed Vac concentrator. Fifty microliters of 0.1 M ammonium bicarbonate was added, vortexed, and evaporated again to remove the acid. The material was dissolved in 0.1 M ammonium bicarbonate containing 0.1 mM CaCl₂ at a concentration of 1 μg/μL. Tryptic digestion was carried out by adding
trypsin at a protein to enzyme ratio of 50 to 1 and incubating at 37°C for 4 hours. Digestion was terminated by adding acetic acid to a final concentration of 5%. The tryptic digest was chromatographed on a Vydac C₄₅ column using a linear gradient of acetonitrile from 0% to 60% in water containing 0.1% trifluoroacetic acid. The various peptide fragments obtained were dried and sequenced using a protein sequencer (model 470A, Applied Biosystems, Inc, Foster City, Calif) attached to an on-line model 120A PTH analyzer.²⁰

SDS-PAGE. SDS-PAGE was carried out in 8% to 25% gradient gels using a Phast System at pH 8.0. For the determination of the molecular weight, standard protein markers from molecular masses of 6.5 to 205 kD were used. Gels were stained by silver nitrate.²¹

Western blot analysis. Western blot analysis was performed with the normal and dilated cardiomyopathic human heart proteins according to the procedure as described by Towbin et al²² and Tsang et al²³ with modifications. This procedure includes the electrophoretic transfer of different proteins from SDS-polyacrylamide gels to GenScreen and identification of different protein bands using specific antibodies raised against myotrophin in rabbits, followed by a second reagent step using ¹²⁵I-labeled protein A. SDS-PAGE was performed in 4% to 20% acrylamide gels for 40 minutes at a constant voltage of 200 V at room temperature according to the method of Laemmli²⁴ with modifications. Electrophoretic transfer was continued for 5 hours at 4°C at a constant voltage of 40 V. After transfer, the GenScreen membrane was rinsed with 100 mM sodium phosphate buffer, pH 7.2, containing 1% sodium chloride and 1% Tween 20 and dried in air for 30 minutes. The dried blot was incubated with an excess of 10% Carnation instant nonfat dry milk (milk solution) in the same buffer without Tween 20 for 90 minutes. Later, the solution was removed, and an excess of diluted (1:500 dilution) rat myotrophin (diluted in milk solution with 1% Tween 20) was added and incubated for 90 minutes. Afterwards, the blot was rinsed three times with an excess of 10 mM sodium phosphate buffer containing 1% NaCl and 1% Tween 20. The blot was then immersed in milk solution containing ¹²⁵I-labeled protein A (1 to 2×10⁵ cpm/mL) and incubated for 4 hours at room temperature. The blot was then rinsed with excess of phosphate-buffered saline containing 1% Tween 20 until the unbound radioactivity was removed. Finally, the membrane was air-dried at room temperature and autoradiographed. A set of standard molecular weight marker proteins was applied to the SDS-polyacrylamide gel and stained by silver nitrate.²¹

Determination of Myotrophin Levels in Normal and Cardiomyopathic Human Hearts

Myotrophin levels in six normal human hearts (four female and two male; age, 19 to 44 years) and six cardiomyopathic human hearts (four female and two male; age, 23 to 51 years) were determined by dot blot analysis using myotrophin-specific antibodies. The myotrophin-specific antibodies were raised against a multiple-antigen peptide system as described by Tam.²⁵ This myotrophin–multiple-antigen peptide system represents a partial amino acid sequence of a segment of rat myotrophin (T26, tryptic fragment) consisting of 17 amino acid residues, namely, Gly-Pro-Asp-Gly-Leu-Thr-Ala-Leu-Glu-Ala-Thr-Asp-Asn-Gln-Ala-Ile-Asp. These antibodies have been characterized by solid-phase radioimmunoassays using both rat and human myotrophin. Specificity of these antibodies was established by solid-phase radioimmunoassays when they did not cross-react with a number of other factors (namely, acidic and basic fibroblast growth factor [FGF], nerve growth factor, insulin-like growth factor, norepinephrine, and insulin) known to stimulate myocyte growth (manuscript in preparation). The antibodies formed against the multiple-antigen peptide system cross-reacted only with human and rat myotrophin. These antibodies completely blocked the myotrophin-induced stimulatory effect on protein synthesis in myocytes (manuscript in preparation).

The dot blot analysis was carried out by applying 2-μL samples on GenScreen paper. The protein samples were prepared from individual normal and cardiomyopathic human hearts by homogenizing the tissues in homogenizing buffer as previously mentioned.²¹ The total protein samples were applied to a piece of GeneScreen paper, and dot blot analysis was carried out.²²,²³ Antibodies were allowed to react with myotrophin, and bound antibodies were detected using ¹²⁵I-labeled protein A. The quantification of autoradiographs (determination of the intensities of the various dots) was achieved using an image analyzer in which the photographic image from a video camera was transferred to a Macintosh II computer, and the accurate quantification of each dot was performed after subtracting the background.

Measurement of Protein

Protein was measured by the Bradford²⁶ protein microassay procedure using bovine serum albumin as standard.

Determination of DNA

DNA determination was performed by following the fluorometric method of Labarca and Paigen.²⁷ The standard curve was drawn using calf thymus DNA. Bis-benzimidide solutions (Hoechst 33258) were used as fluorescence dyes. Samples were read on a luminescence spectrometer (model LS-5, The Perkin-Elmer Corp, Norwalk, Conn) with excitation at 356 nm and emission at 458 nm.

Statistical Analysis

For the statistical analysis, we used Student’s t test and analysis of variance when appropriate. For the bioassay procedures, adult myocytes were cultured in six-well plates, and the first and the sixth wells of each plate were used as controls. If the numbers of disintegrations per minute per microgram DNA were different in two control wells, an average was taken. The amount that one control value differed from the average was subtracted from the disintegrations per minute per microgram DNA in the other wells (wells 2, 3, 4, and 5). Finally, the corrected number of disintegrations per minute per microgram DNA in each of the wells 2, 3, 4, and 5 was expressed as percent stimulation over control. These guidelines were followed for all the bioassays used during the purification procedure.
Results

Adult Myocyte Culture and Bioassay System

We used calcium-tolerant adult myocytes for all bioassays to monitor the biological activity of human myotrophin during the purification procedure because adult myocytes responded to very low levels of myotrophin compared with neonatal myocytes. After isolation and washing with medium 199, 10% or less of the total number of myocytes demonstrated sporadic beating; most of them attached to laminin were cylindrical. A linear rate of incorporation of [3H]leucine into adult myocyte protein was observed during 1, 2, and 4 hours of incubation. On this basis, a 2-hour incubation time was used for the routine assay. Insulin was used as a standard for the routine assays because it stimulated [3H]leucine incorporation into adult myocyte protein in a dose-dependent fashion from 0.25 to 1.0 μg per well. Data were expressed as disintegrations per minute per microgram DNA. The criteria mentioned above established the healthy structural, biochemical, and functional integrity of the adult myocytes and, hence, the validity of the assay system.

Purification of Human Myotrophin

Fig 1 shows the Sephadex G-75 chromatogram of the crude protein from the human dilated cardiomyopathic hearts that resulted after the ammonium sulfate precipitation procedure and the dialysis of the proteins against 20 mM Tris-HCl buffer, pH 7.6. Among the three peaks, the material in the region of peak 2 showed major stimulatory activity, as determined by the incorporation of [3H]leucine into adult myocyte protein (42%). That material was pooled and applied to a mono-Q column attached to a fast protein liquid chromatogram for anion-exchange chromatography. Among the six different fractions, the sixth fraction showed the major stimulatory activity (36%) (Fig 2). Here, we would like to point out that human myotrophin, compared with rat myotrophin, was identified in protein fractions that were eluted using a high salt gradient. A 5-mL protein solution (1 mL per tube) of this active fraction was desalted and applied to the same column for rechromatography at a lower pH (5.6) using a linear gradient of 0.5 M NaCl (rat myotrophin was eluted using 0.2 M NaCl). The fourth fraction showed the major stimulatory activity (35%), as shown in Fig 3. Four milliliters of material (1 mL per tube) from that fraction was dried, dissolved in water containing 0.1% trifluoroacetic acid, and applied to a reverse-phase column by HPLC. Among the different protein peaks, the most prominent one showed major stimulatory activity (30%) (Fig 4). The material of this peak showing stimulatory activity (30%) (Fig 4) was collected and rechromatographed under the same condition. A sharp symmetrical peak with potent biological activity was obtained, indicating that, as shown in Fig 5, the homogeneous preparation of human myotrophin had been accomplished. No stimulatory activity was found in the other areas.

Table 1 summarizes the purification data for human myotrophin. In a typical purification, 100 g of left ventricle containing approximately 6 g protein was obtained from the heart of a 23-year-old woman with dilated cardiomyopathy. At the final step of purification, 5 μg myotrophin was obtained. The seven-step purification procedure resulted in an apparent 50,000-fold purification of human myotrophin, with a 4% recovery of activity. The data are summarized in Table 1.

Characterization of Human Myotrophin

SDS-PAGE. When human myotrophin was subjected to SDS-PAGE under reducing conditions, only a single sharp protein band was detected, as shown in Fig 6. The molecular mass was estimated to be approximately 12 kD. This result shows that human myotrophin was homogeneous and consisted of a single polypeptide chain.

Dose-response stimulatory effect of myotrophin. When myotrophin was added to adult myocytes maintained in culture in three different doses, a dose-dependent increase in [3H]leucine incorporation was found. The data clearly show that human myotrophin stimulated [3H]leucine incorporation into adult myocyte protein in a dose-dependent fashion (Fig 7).

Internal amino acid sequence of human myotrophin. Edman degradation (Edman and Begg) of intact human myotrophin yielded no detectable phenylthiohydantoin amino acid, suggesting that the NH2 terminus of human myotrophin was blocked. To determine the
internal amino acid sequence of human myotrophin, the purified protein was oxidized by performic acid and then digested with TLCK-treated trypsin. The digest was separated by reverse-phase chromatography on a Vydac C18 column attached by HPLC. Edman degradation of a tryptic peptide designated as T9 yielded a 13-amino acid residue sequence as shown in Table 2.

A sequence homology search was carried out on protein identification resources that contain 6330 entries and on Swiss-Port protein sequence data bases that contain 10 008 entries. The sequences in the above data bases contained no segment that was identical to the test segment of myotrophin. The sequence alignment score was calculated by the method of Lipman and Pearson28 after the initial screening using the PROTSOC computer program.

Table 3 shows the results of the comparison of the internal amino acid sequences of human and rat myotrophin. The partial internal amino acid sequence we obtained from the tryptic peptide fraction of human myotrophin showed very close structural similarity with the internal amino acid sequence of one of the tryptic peptide fractions of rat myotrophin.

Western blot analysis. Fig 8 shows the result of Western blot analysis performed with normal and dilated cardiomyopathic human heart homogenates. Approximately 20 µg crude protein was used for this experiment. A number of different standard molecular weight marker proteins were used to find out the molecular weight of the protein that specifically cross-reacted with the antibodies raised against myotrophin. The autoradiograph clearly shows the presence of only one protein band in both normal and dilated cardiomyopathic human hearts in the region of 12 kD. Previously, we have shown (Fig 6) by SDS-PAGE that myotrophin consisted of a single polypeptide chain of approximately 12 kD. Therefore, these results confirm the presence of myotrophin both in normal and dilated cardiomyopathic human hearts.

Myotrophin levels in normal and cardiomyopathic human hearts. Myotrophin levels in normal (four female and two male; age range, 19 to 48 years) and cardiomyopathic (four female and two male; age range, 23 to 51 years) human hearts are shown in Fig 9. The amount of myotrophin present in each dilated cardiomyopathic human heart (expressed as the arbitrary density unit of the image analyzer per microgram protein) was signi-
FIG 4. A typical reverse-phase chromatographic pattern of the material of the fourth fraction obtained from ion-exchange rechromatography (for experimental details, see “Materials and Methods”). CN, control. A Vydac C18 column and high-performance liquid chromatography (HPLC) were used; the column was eluted using a linear gradient of acetonitrile. Top panel: Time course showing absorbance at 214 nm. Bottom panel: Bar graph showing results of adult cell assay. Protein (0.025 μg) was used for the bioassay.

FIG 5. Reverse-phase rechromatography at the final step of purification of human myotrophin. Active peak from the reverse-phase chromatography was collected and applied on a Vydac C18 column attached to high-performance liquid chromatography (HPLC). The column was eluted under the same conditions as those described in the legend of Fig 6. Top panel: Time course showing absorbance at 214 nm. Bottom panel: Bar graph showing results of the bioassay. Pure myotrophin (0.02 μg) was used to evaluate stimulatory activity. Data are expressed as percent stimulation over control (CN) for four separate sets of experiments (mean±SEM).

Discussion

The molecular mechanism for the initiation of heart growth during dilated cardiomyopathy is not known. It is of utmost importance that, if one can identify the mechanism for the initiation or development of such a hypertrophic process, then the proper treatment for such a disease can be determined. There is substantial evidence suggesting that, in humans, factors other than blood pressure control contribute to the initiation and regression of myocardial hypertrophy. Studies from our laboratory have shown that under a condition of stress, such as high blood pressure, a mechanical and humoral signal to the myocardium triggers the myocardium to produce a factor that initiates myocardial protein synthesis in the SHR heart.

In the present study, we demonstrate that myotrophin is also present in normal human hearts and, at higher levels, in human dilated cardiomyopathic hearts and that, after purification, partial sequencing demonstrates that structurally human myotrophin is very similar to the myotrophin that was isolated and purified from the SHR heart. Data presented in the present study demonstrate the following: (1) Myotrophin obtained from human cardiomyopathic heart demonstrates a significant increase in protein synthesis in adult rat myocytes. (2) Myotrophin is present in normal hearts and, at higher levels, in human cardiomyopathic hearts. (3) Perhaps the SHR heart would be a good model for the study of cardiac hypertrophy because of its similarity with the human cardiomyopathic heart.

We have previously reported the existence of a factor in the human cardiomyopathic heart that stimulates the incorporation of [3H]leucine and [14C]phenylalanine into myocyte protein in vitro. Subsequent to that, we also reported the complete purification and partial sequencing of that factor from the SHR heart and the partial characterization of its nature. This factor, when added to myocardial cells, resulted in an increase in cell size in a dose-dependent fashion and markedly affected the acceleration of myofibrillar growth and organization of sarcomeres as well as the maturation of mitochondria. We showed that the factor increased the specific activity of leucyl tRNA without significantly changing the intracellular leucine pool, demonstrating that this
factor increases the actual rate of protein synthesis. We have also shown that trypsin digestion (pH 7 to 7.5) destroys the stimulatory effect of that factor.

As described in the present study, our data show that myotrophin obtained from human heart stimulates protein synthesis in adult myocyte protein. Insulin was used as an external standard throughout the experimental procedure. For each assay system, an average of 60,000 adult myocytes was used. The structural integrity of cell myocytes in culture and their suitability for studying protein synthesis were established by morphology and by studying the cAMP production after the cells were stimulated with norepinephrine, as described previously. When examined under phase-contrast microscopy, a three-dimensional cylindrical structure was found for each myocyte maintained in culture. The cylindrical shape of the myocyte did not change during the incubation period, and a linear rate of tritiated leucine incorporation into myocyte protein was obtained during a 4-hour incubation period.

SDS-PAGE demonstrated a single protein band for myotrophin in the area of 12,000 D. The maximum stimulation of myotrophin was obtained at a concentration of 20 nM. This amount is within the same order of magnitude as that reported for platelet-derived growth factor after [3H]thymidine incorporation into endothelial cells but is less potent than that for FGFs after [3H]thymidine incorporation into fibroblasts. It is important to note that the stability of this factor in the

### Table 2. Internal Amino Acid Sequence of Tryptic Peptide Fraction 9 of Human Myotrophin

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<th>Cycle No.</th>
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<tr>
<td>5</td>
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<td>4.80</td>
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<tr>
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</tr>
<tr>
<td>8</td>
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<td>2.24</td>
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<tr>
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<td>Trp</td>
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</tr>
<tr>
<td>13</td>
<td>Ser</td>
<td>1.21</td>
</tr>
</tbody>
</table>

*Phenylthiohydantoin derivative of the amino acid residue is not detectable.

---

**FIG 7.** Bar graph showing results of stimulation of myocardial protein synthesis by human myotrophin. Adult myocytes maintained in culture were used for the bioassay (for experimental details, see "Materials and Methods"). Data are expressed as percent stimulation over control for three separate sets of experiments (mean±SEM). Note that human myotrophin stimulated [3H]leucine incorporation into adult myocyte protein in a dose-dependent fashion.
TABLE 3. Comparison of Internal Amino Acid Sequence of Rat and Human Myotrophin

<table>
<thead>
<tr>
<th>Amino acid sequences</th>
<th>Human myotrophin</th>
<th>Rat myotrophin</th>
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<tr>
<td>Tyr</td>
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<tr>
<td>Gly</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Ser</td>
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<td></td>
</tr>
</tbody>
</table>

*Phenyldihyrdantoin derivative of the amino acid residue is not detectable.

incubation media is not known. Therefore, it is difficult to estimate the exact amount of myotrophin necessary for maximum stimulation. The purification described above resulted in an apparent increase in specific activity by 50 000-fold, with a 4% recovery of growth-promoting activity (Table 1).

Myotrophin appears to differ from all other growth factors reported to date. One of its most important properties is its lack of mitogenic activity, as evidenced by the absence of any change in tritiated thymidine uptake in neonatal myocytes when compared with that in untreated control myocytes, which is shown to be the case with rat myotrophin. At the end of the incubation period, the total DNA content also remains unchanged compared with that in untreated control myocytes. Our preliminary data suggest that myotrophin is specific for myocytes because it has no effect on fibroblasts, endothelial cells, or aortic smooth muscle cells. The differences between myotrophin and other growth factors can be seen in another way. Some of the growth factors have been reported to be heparin-binding proteins. Acidic and basic FGFs, for example, bind tightly to heparin-Sepharose columns and can be eluted from the column by using 1 and 2 M NaCl, respectively. Furthermore, the acidic FGF can be potentiated by the addition of heparin. In contrast, myotrophin does not bind to heparin-Sepharose. For the purification of FGF, carboxymethyl cellulose is used. Myotrophin does not bind to carboxymethyl cellulose.

Very recently, Henrich and Simpson have reported a heparin-binding hypertrophic growth factor known to be produced by nonmuscle mesenchymal cells. This growth factor increases myocyte size in a dose-dependent fashion without providing a change in DNA synthesis. The growth-promoting activity can be eluted from a heparin-Sepharose bound column using 0.75 M NaCl; since this activity is inhibited by soluble heparin, the factor can be distinguished from other growth factors present in the heart, namely, tumor necrosis factor, platelet-derived growth factor, transforming growth factor-β, and various types of FGFs. However, its complete characterization is yet to be defined.

As reported in the present study, myotrophin from human cardiomypathic heart and that from SHR heart appear to be similar in structure and function. Although the purification steps are similar for the purification of both rat and human myotrophin, the chromatographic separation patterns are not identical. In ion-exchange chromatography using the first mono-Q column, human myotrophin was eluted at a higher salt concentration than that of rat myotrophin, suggesting that human myotrophin is less acidic in nature than rat myotrophin. During the second step of ion-exchange chromatography using the second mono-Q column, we had to use a different gradient (0 to 0.3 M NaCl) for human myotrophin (for rat myotrophin, we used 0.2 M NaCl), which resulted in a rightward shift of the biologically active peak. However, the partial internal amino acid sequence we obtained from the tryptic peptide fragment shows amino acid residues that are very similar to those of rat myotrophin. To more completely compare their amino acid sequences, we are now in the process of analyzing other tryptic fragments obtained from human myotrophin. The presence of myotrophin in SHR heart,
FIG 9. Bar graph showing myotrophin levels in normal and cardiomyopathic human hearts. For experimental details, see "Materials and Methods." Crude homogenates in phosphate-buffered saline from six normal and six cardiomyopathic human hearts containing the same amount of protein were applied to GeneScreen paper. Myotrophin present in those samples was allowed to react with the specific antibodies raised against multiple-antigen peptide system. Bound antibodies were detected using 125I-labeled protein A. Quantification of the intensities of various dots on autoradiographs was done by an image analyzer. Note that each of the six cardiomyopathic human hearts contains significantly more myotrophin than do the six age- and sex-matched normal control hearts. Data are expressed as density per microgram protein for four separate sets of experiments (mean ± SEM).

a genetically hypertensive hypertrophy model, and in human cardiomyopathic heart, in the absence of hypertrophy, may suggest that the mechanism for initiating hypertrophy through myotrophin may be a common pathway of protein synthesis stimulation for both models.

Because our initial observation had shown that normal human heart homogenate contains very little detectable amounts of myotrophin,17 we isolated and purified myotrophin from human dilated cardiomyopathic heart. Using specific immunoblot assays, we have recently shown that myotrophin was increased 10-fold in the SHR heart compared with age- and sex-matched normal control hearts.38 We performed Western blot analysis with the normal and dilated cardiomyopathic human heart homogenate proteins to detect the presence of myotrophin in them. The autoradiograph we obtained from Western blot analysis showed the presence of a single protein band in the region of 12-kD molecular mass in both normal and dilated cardiomyopathic human hearts, which confirms the presence of myotrophin both in normal and cardiomyopathic human hearts.

We also performed dot blot analysis to determine myotrophin levels in normal and dilated cardiomyopathic human hearts. We used four female and two male normal human hearts and four female and two male dilated cardiomyopathic human hearts. The age ranges for both normal and dilated cardiomyopathic human hearts were similar (19 to 48 years for normal hearts versus 23 to 51 years for cardiomyopathic hearts). The results were expressed as density per microgram protein. The amount of myotrophin present in each dilated cardiomyopathic human heart is significantly higher than that present in normal human hearts (Fig 9). It appears that myotrophin does not vary with age and sex in normal control or cardiomyopathic human hearts.

Although the biochemical pathway for the development and/or the regression of myocardial hypertrophy is still unclear, it is obvious that more than one factor leads to the growth of terminally differentiated myocytes and that several factors may ultimately translate the mechanical or humoral stimuli to the message inducing the cell to grow or to atrophy. The myotrophin we purified and partially characterized is one of such factors that may play an important role in the hypertrophic development as well as the normal development of cardiac myocytes. Future studies will elucidate the mechanism by which myotrophin initiates myocardial protein synthesis under physiological and pathological conditions.

Acknowledgments

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P Sil, K Misono and S Sen

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