Syndecan-4 is a unique member of the syndecan gene family that has the ability to bind and activate protein kinase C-α. Whereas increased syndecan-4 levels have been noted in ischemic hearts, little is known regarding the regulation of its expression. To investigate the role of cardiac myocytes in induction of syndecan-4 expression, human endothelial cells (ECV304) were exposed to a medium conditioned by primary mouse cardiac myocytes or H9c2 cells. The medium conditioned by hypoxic but not normal myocytes was able to induce syndecan-4 expression in ECV cells. Western analysis of the conditioned medium demonstrated an increased presence of tumor necrosis factor-α (TNF-α) in the medium conditioned by hypoxic but not normal myoblasts. Primary cardiac myocytes collected from the wild type C57/129 but not the homozygous TNF-α−/− knockout mice were able to induce syndecan-4 expression in ECV cells when cultured under hypoxic conditions. In vitro studies demonstrated that TNF-α induced endothelial cell syndecan-4 expression by both increasing syndecan-4 gene expression in an NF-κB-dependent manner and by prolonging syndecan-4 mRNA half-life. We conclude that TNF-α is the principal factor produced by the ischemic myocytes that is responsible for induction of endothelial cell syndecan-4 expression and that this requires both transcriptional and posttranscriptional mechanisms.

Syndecan-4 is one of the principal heparin sulfate-carrying proteins on the cell surface (1). It is expressed by a number of different cell types including endothelial cells, smooth muscle cells, and cardiac myocytes; although expression in quiescent tissues is fairly low. Syndecans can participate in a variety of biological functions including regulation of blood coagulation, cell adhesion, and low density lipoprotein transport (2, 3). In addition to these activities shared by all syndecans, syndecan-4 cytoplasmic domain can bind to and activate protein kinase C-α with the degree of activation regulated by the extent of syndecan-4 cytoplasmic tail phosphorylation (4–7).

Despite these interesting properties, little is known about the regulation of syndecan-4 gene expression. Its levels are increased after various forms of tissue injury including skin wounds (8), vascular wall injury (9), or myocardial infarction (10). The increase in syndecan-4 expression in skin wounds and in the heart following myocardial infarction has been ascribed to the presence of a proline-arginine-rich peptide, PR39. No other factors responsible for this increase have been identified (8, 10). The present study was designed to study the role of cardiac myocytes in the induction of syndecan-4 expression under normal and ischemic (hypoxic) conditions. To this end, we determined the ability of myocyte- or myoblast-conditioned medium to induce syndecan-4 expression in a human endothelial cell line. We find that TNF-α secreted by hypoxic but not normal myocytes is able to induce syndecan-4 expression and that the mechanism of this induction involves both transcriptional activation of syndecan-4 gene expression and posttranscriptional regulation of syndecan-4 message half-life.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A human umbilical endothelial cell line, ECV304 (ATCC), was cultured in M199 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. A rat cardiac myoblast cell line, H9c2 (ATCC), was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. IC-21 (ATCC), a mouse macrophage cell line, was cultured in RPMI 1640 medium supplemented with 10% FBS. Cardiac myocytes were isolated from day 2 neonatal C57/129 or same background TNF-α−/− mice (11). A total of 24 hearts from each mouse strain was used. At sacrifice the hearts were removed aseptically, cut into 1–2-mm pieces, placed in ice-cold Hank's balanced saline solution without either Ca2+ or Mg2+, and subjected to serial digestions with 0.5 mg/ml collagenase II ( Worthington), 0.1% trypsin, 15 μg/ml DNase 1, and 1% chicken serum at 37 °C with constant rotation. Supernatant was collected every 10–15 min, and digestion was stopped by addition of 10% horse serum. Dissociated cells were collected by centrifugation and resuspended in DMEM/F12 medium supplemented with 5% horse serum, 2 g/liter bovine serum albumin (fraction V), 3 mM pyruvic acid, 10 mM HEPES (pH 7.6), 100 μM ascorbic acid, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite, and 5 μg/ml linoleic acid for 1 h to enrich for myocytes. The suspension was then plated onto 1% gelatin-coated dishes at 2.5 × 106 cells/60-mm dish. Bromodeoxyuridine (0.1 μM) was added to prevent proliferation of non-myocytes. Horse serum and bromodeoxyuridine were removed from the medium after 36 h.

**Growth Factor Studies**—For determination of growth factor responsiveness of syndecan-4 expression, ECV304 cells were cultured in 10% FBS-DMEM to 80% confluence. At that point the cells were washed with PBS, the medium was replaced with serum-free DMEM for 6 h, and then one of the following growth factors was added: hrVEGf-1α (25 ng/ml, Genentech, Inc.), hrhFGF (25 ng/ml, Chiron Corp.), hrPDGF-AA (20 ng/ml, Sigma) or hrPDGF-AB (20 ng/ml, Sigma), hrTGF-β (20 ng/ml).

* This paper was supported in part by National Institutes of Health grants R01 HL53793 and P50 HL56993 (to M. S.) and by a grant from Chiron Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Cardiovascular Division, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-5364; Fax: 617-975-5201; E-mail: msimons@bidmc.harvard.edu.

‡ The abbreviations used are: TNF-α, tumor necrosis factor-α; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; NF-κB, nuclear factor-κB.
TNF-α Regulation of Syndecan-4 Expression

ng/ml, R&D Systems), hrTNF-α (20 ng/ml, Sigma), or mouse epidermal growth factor (20 ng/ml, Becton Dickinson Labware). Cells were harvested and total RNA was extracted after 24 h and subjected to Northern blot analysis.

Preparation of Myoblast- or Myocyte-conditioned Medium—H9c2 cells were cultured in DMEM supplemented with 10% FBS in 100-mm dishes to 90% confluence. At that point, the cells were washed three times with PBS and the medium was changed to serum-free DMEM. Following 24 h of culture under hypoxic (0–2% O₂) or normal oxygen conditions, the medium was collected, centrifuged for 10 min at 2000 × g to remove debris, and then stored at −80 °C for further use. Similar procedures were used for collection of myocyte-conditioned medium.

Analysis of Syndecan-4 Expression—For Northern analysis, total RNA extracted from cultured cells with Tri Reagent (Sigma) was fractionated on 1.2% formaldehyde-agarose gel and transferred onto GeneScreen Plus membrane. cDNA probes for human syndecan-4 or glyceraldehyde-3-phosphate dehydrogenase were labeled with α-[32P]dCTP using a random priming kit (Roche Molecular Biochemicals), and hybridization was carried out for 3 h at 65 °C in Quickhyb solution (Strategene), followed by serial washes with 0.1× SSC, 0.1% SDS at room temperature for 15 min, 45°C for 15 min, and 65°C for 15 min. The quantitative analysis of hybridization signal was carried out using PhosphorImager (Molecular Dynamics) and ImageQuant software. RNA loading was adjusted using ethidium bromide gel 18 S RNA signal.

For determination of syndecan-4 protein levels, ECV cells were washed with PBS (137 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KCl, 1.8 mM KH₂PO₄, pH 7.4), dissociated by 0.05% trypsin, 0.5 mM EDTA (Life Technologies, Inc.) in PBS for 10 min at 37 °C, and sedimented by 200 × g centrifugation at 4°C for 5 min. Western blotting was carried out with anti-syndecan-4 cytoplasmic domain antibody following SDS-polyacrylamide gel electrophoresis of the total cell protein extract as described previously (6).

Western Blot Analysis of TNF-α in Myoblast-conditioned Medium—Conditioned medium collected from five 100-mm Petri dishes of confluent H9c2 cells cultured for 48 h under hypoxic or normal conditions was concentrated using Centriprep followed by Centricon columns (Amicon, Inc.) to a final volume of 80 μl. Aliquots of the concentrated material (20 μl) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon™-P membrane (Millipore), and the blot was then incubated with a polyclonal goat anti-rat TNF-α antibody (1:200 dilution, Santa Cruz Biotechnology) in PBS supplemented with 5% nonfat milk overnight at 4 °C. An ECL system (Amersham Pharmacia Biotech) was used to detect the signal.

Syndecan-4 mRNA Stability Assay—100 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was added to ECV304 cells following overnight culture in 0.5% FBS-M199. The cells were then harvested at the indicated time points and total RNA was extracted as described above. 10 μg of total RNA for each time point were subjected to Northern blot analysis with a syndecan-4 cDNA probe. Signal was quantified using PhosphorImager with RNA loading adjusted by glyceraldehyde-3-phosphate dehydrogenase expression levels. The mRNA half-life was calculated using the formula t₁/₂ = ln2/(mRNA decay rate constant) with the decay rate constant derived from the slope of the decay curve.

Syndecan-4 Transcription Studies—A 715-nucleotide fragment (~690 to +25 nucleotide of the murine sequence) encompassing the basal elements of mouse syndecan-4 promoter (courtesy of Dr. T. Kojima, Nagoya University) was cloned into a pGL-3 vector (Promega) containing the luciferase reporter construct. The syndecan-4 promoter construct and a pGRE-Neo (U. S. Biochemical Corp.) construct were linearized and, following phenol/chloroform purification, used for electroporation. To this end, ECV304 cells were trypsinized and washed twice with a fresh medium; 6 × 10⁶ of cells in 0.75 ml of medium were incubated with 30 μg of syndecan-4 promoter-containing pG3-3 and 3 μg of pGRE-Neo linearized plasmids for 5 min on ice in a 0.4-cm cuvette. Electroporation was carried out at 290 V, 600 microfarads using Gene Pulser II (Bio-Rad). Following electroporation, the cells were quickly diluted with the fresh M199 medium containing 10% FBS supplemented with 5 mM sodium butyrate and plated onto a 100-mm Petri dish. Sodium butyrate was removed after overnight exposure, and the culture was continued for another 36 h in 10% FBS-M199. The cells were then passaged into two dishes and maintained in 1 μg/ml G418 (Life Technologies, Inc.). All colonies were pooled together after 10 days and maintained in the selection medium.

For promoter studies, cells stably expressing syndecan-4 promoter construct were plated onto 12-well plates and allowed to come to 80% confluence in 10% FBS-M199 medium. The medium was then changed to 0.5% FBS-M199 overnight. TNF-α was added at the indicated concentration. After 6 h of exposure, cells were lysed, and luciferase activity was determined using the luciferase assay system (Promega).

To assess the role of NF-κB in the TNF-α-dependent stimulation of syndecan-4 gene expression, ECV304 cells plated on 60-mm dishes were cultured until 90% confluence and subjected to serum starvation overnight. Serum-free cell culture medium (Cultilene) was then added to medium at a concentration of 10 μM. 1 h later, TNF-α (20 ng/ml) was added. Syndecan-4 mRNA levels were determined using Northern blot analysis 4 h after exposure of lactacystin-treated or control cells to TNF-α (20 ng/ml).

RESULTS

Induction of Syndecan-4 Expression in Endothelial Cells by Hypoxic Myoblast Cell-conditioned Medium—To investigate the role of myocyte-endothelial cell interaction in the regulation of syndecan-4 expression in endothelial cells, we studied the ability of myoblast- or cardiac myocyte-conditioned medium to induce syndecan-4 expression in ECV304 cells. Exposure of ECV304 cells to the medium conditioned by the H9c2 cells cultured under normal conditions did not affect syndecan-4 expression. However, medium conditioned by H9c2 cells cultured under hypoxic conditions produced a nearly 2-fold increase in syndecan-4 mRNA expression in ECV304 cells and a 2.5-fold increase in syndecan protein (Fig. 1). These results suggested that myoblasts exposed to hypoxia secreted a factor that was responsible for induction of syndecan-4 expression. To determine whether any of the known myoblast-secreted growth factors were responsible for this event, ECV304 cells were exposed to a panel of cytokines. Of the growth factors tested, only TNF-α was able to induce syndecan-4 expression in ECV304 cells (Fig. 2) in a dose-dependent manner. The induction of syndecan-4 mRNA expression by TNF-α was prompt, beginning at 1 h, reaching maximal effect at 3 h (Fig. 2), and lasting for at least 24 h (data not shown).

TNF-α is a Primary Factor Responsible for Induction of Syndecan-4 Expression by Myoblasts—To demonstrate that TNF-α is produced by hypoxic myoblasts, cell culture medium conditioned by H9c2 cells under normal and hypoxic conditions was subjected to Western analysis with anti-TNF-α antibody. Cell lysate of IC-21 cells, a macrophage cell line known to produce large amounts of TNF-α under normal culture conditions, was used as a control. The presence of secreted TNF-α was detected in IC-21 cell lysate and in H9c2 cells cultured under hypoxic conditions. Compared with the hypoxic H9c2 cell-conditioned medium, only a trace amount of TNF-α was detected in the medium conditioned by the normal H9c2 cells (Fig. 3A). To confirm that TNF-α is the primary factor responsible for myoblast-dependent induction of syndecan-4 expression, we used primary myocyte cultures from homozygous TNF-α knockout mice and age-matched controls to condition serum-free medium in the manner similar to the H9c2 experiments. Cell culture media conditioned by primary myocytes derived from either the TNF-α−/− or wild type C57/129 mice cultured under normal conditions did not induce endothelial cell syndecan-4 mRNA expression. However, medium conditioned by hypoxic myocytes from the C57/129 but not TNF-α−/− mice induced syndecan-4 expression to the extent similar to that seen with hypoxic H9c2 cells (Fig. 3B).

Mechanism of TNF-α-mediated Induction of Syndecan-4 Expression—To determine whether TNF-α regulates syndecan-4 gene expression by transcriptional or posttranscriptional mechanism, we measured syndecan-4 mRNA half-life in ECV304 cells in the presence or absence of TNF-α treatment. Exposure to TNF-α at 20 ng/ml increased syndecan-4 mRNA half-life from 16 to 24 h (Fig. 4A). To assess the effect of TNF-α on syndecan-4 gene transcription, we measured activity of a luciferase construct under control of the mouse syndecan-4 promoter. Exposure of the pooled population of ECV304 cells stably transfected with this construct to TNF-α at 20 ng/ml led
to a 2-fold increase in luciferase activity (Fig. 4B). Because TNF-α is known to activate gene expression in an NF-κB-dependent manner and given that syndecan-4 promoter contains an NF-κB-binding site, we examined the effect of inhibition of NF-κB-dependent transcription on the ability of TNF-α to induce syndecan-4 expression. Pretreatment with lactacystin, an inhibitor of proteasome-dependent degradation of IκBα expression, completely blocked TNF-α-induced activation of syndecan-4 expression. Pretreatment with lactacystin, an inhibitor of proteasome-dependent degradation of IκBα expression, completely blocked TNF-α-induced activation of syndecan-4 expression. Pretreatment with lactacystin, an inhibitor of proteasome-dependent degradation of IκBα expression, completely blocked TNF-α-induced activation of syndecan-4 expression. Pretreatment with lactacystin, an inhibitor of proteasome-dependent degradation of IκBα expression, completely blocked TNF-α-induced activation of syndecan-4 expression. Pretreatment with lactacystin, an inhibitor of proteasome-dependent degradation of IκBα expression, completely blocked TNF-α-induced activation of syndecan-4 expression.
can-4 expression (Fig. 5). These results indicate that TNF-α regulates syndecan-4 expression at both transcriptional and posttranscriptional levels.

DISCUSSION

The principal finding of this study is that TNF-α, secreted by hypoxic but not normal myocytes, is able to increase syndecan-4 expression in endothelial cells. A number of findings point to TNF-α as the main factor responsible for the ability of hypoxic myocyte-conditioned medium to induce syndecan-4 expression. First, the cytokine is capable of increasing syndecan-4 mRNA levels in a dose-dependent manner and with an appropriate time course. Second, TNF-α is present in medium conditioned by hypoxic but not normal myoblasts. Finally, medium conditioned by myocytes cultured under either normal or hypoxic conditions derived from the TNF-α knockout mice fails to induce syndecan-4 expression.

TNF-α-dependent activation of syndecan-4 expression involves both transcriptional and posttranscriptional events. The study of syndecan-4 transcription was carried out using a mix-
FIG. 5. TNF-α induces syndecan-4 gene expression in an NF-
κB-dependent manner. To study the role of NF-κB-dependent gene transcription in TNF-α-induced activation of syndecan-4 expression, ECV304 cells were exposed to TNF-α (20 ng/ml) in the absence of lactacystin (TNF-α) or following 1 h pretreatment with lactacystin (LC+TNF-α). Note the increased syndecan-4 expression in TNF-α-treated cells that were blocked by pretreatment with lactacystin. Addition of lactacystin by itself (LC) had no effect on base-line syndecan-4 levels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ture of approximately 200 independent stable clones, thus
eliminating artifacts potentially associated with the expression construct integration into hyper- or hypoactive regions of the genome. The promoter fragment used was previously shown to possess basal promoter activity (12), although it is possible that additional control elements were missing. The degree of induction of the luciferase expression was similar to the extent of increase in the endogenous syndecan-4 mRNA level in TNF-α-treated ECV304 cells. Taken together, the extent of transcriptional activation as measured in luciferase assays and prolongation of mRNA half-life closely parallel the increase in endogenous syndecan-4 protein levels in these cells following TNF-α exposure.

TNF-α is thought to regulate gene expression by inducing degradation of a transcriptional inhibitor, IκBα, thus allowing the p50 NF-κB-RelA complex to bind to its nuclear binding sites and activate gene expression (13). Basal syndecan-4 promoter possesses an NF-κB binding site (GGGGGAATT, nucleotides −84 to −76 of the mouse sequence). Therefore, it is possible that syndecan-4 gene expression up-regulation by TNF-α occurs, at least in part, via the NF-κB-dependent pathway. To confirm this, we exposed ECV304 cells to TNF-α following pretreatment with lactacystin. Lactacystin is a specific inhibitor of proteasome function (14) and, in particular, is known to block proteasome-dependent degradation of IκBα. In accordance with these considerations, we found that pretreatment with lactacystin completely blocked the ability of TNF-α to induce syndecan-4 expression.

Recent studies have suggested that syndecan-4 plays an important role in regulation of endothelial cell growth and migration and in basic fibroblast growth factor-dependent signaling (4, 5, 7). Studies in our laboratory have suggested that increased syndecan-4 expression augmented the ability of en-

REFERENCES

1. Kojima, T., Shworak, N. W., and Rosenberg, R. D. (1992) J. Biol. Chem. 267, 4870–4877
2. Carey, D. J. (1997) Biochem. J. 327, 1–16
3. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) J. Clin. Invest. 99, 2062–2070
4. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 8133–8136
5. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 11805–11811
6. Horowitz, A., and Simons, M. (1998) J. Biol. Chem. 273, 10914–10918
7. Horowitz, A., and Simons, M. (1998) J. Biol. Chem. 273, 25548–25551
8. Gallo, R. L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsburn, M., and Bernfield, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11055–11059
9. Nikkari, S. T., Jarvelainen, H. T., Wight, T. N., Ferguson, M., and Clowes, A. W. (1994) Am. J. Pathol. 144, 1538–1556
10. Li, J., Brown, L. F., Laham, R. J., Volk, R., and Simons, M. (1997) Circ. Res. 81, 785–796
11. Pasparakis, M., Alexopoulos, L., Episkopou, V., and Kolihas, G. (1996) J. Exp. Med. 184, 1397–1411
12. Tszuki, S., Kojima, T., Katsumi, A., Yamazaki, T., Sugiyama, I., and Saito, H. (1997) J. Biochem. (Tokyo) 122, 17–24
13. Whiteside, S. T., and Israel, A. (1997) Semin. Cancer Biol. 8, 75–82
14. Fenteany, G., and Schreiber, S. L. (1998) J. Biol. Chem. 273, 8545–8548
15. Kainulainen, V., Nelimanikka, L., Jarvelainen, H., Laito, M., Jalkanen, M., and Elenius, K. (1996) J. Biol. Chem. 271, 18759–18766
16. Mortruche, G., Lupia, E., Battaglia, E., Passerini, G., Bassolino, F., Emanuelli, G., and Camussi, G. (1994) J. Exp. Med. 180, 377–382
17. Norrby, K. (1996) Microvasc. Res. 52, 79–83
18. Chandrasekhar, B., Colston, J. T., and Freeman, G. L. (1997) Clin. Exp. Immunol. 108, 346–351
19. Meldrum, D. R. (1998) Am. J. Physiol. 274, R577–R595
20. Gurevitch, J., Frolikis, I., Yuhas, Y., Paz, Y., Matsu, M., Mohr, R., and Yakirevich, V. (1996) J. Am. Coll. Cardiol. 27, 247–252

2 R. Volk and M. Simons, unpublished observations.