Tenascin-C Modulates Adhesion of Cardiomyocytes to Extracellular Matrix during Tissue Remodeling after Myocardial Infarction

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SUMMARY: Tenascin-C (TNC), an extracellular matrix glycoprotein, plays important roles in tissue remodeling. TNC is not normally expressed in adults but reappears under pathologic conditions. The present study was designed to clarify the contribution of TNC to ventricular remodeling after myocardial infarction. We examined the expression of TNC after experimental myocardial infarction in the rat by immunohistochemistry and in situ hybridization. Within 24 hours of permanent coronary ligation, interstitial fibroblasts in the border zone started to express TNC mRNA. The expression of TNC was down-regulated on Day 7 and was no longer apparent by Day 14 after infarction. During the healing process, TNC protein and TNC-producing cells were found at the edges of the residual myocardium. Some of the TNC-producing cells were immunoreactive for α -smooth muscle actin. In culture, TNC increased the number of cardiomyocytes attached to laminin but inhibited the formation of focal contacts at costameres. The results indicate that during the acute phase after myocardial infarction, interstitial cells in the border zone synthesize TNC, which may loosen the strong adhesion of surviving cardiomyocytes to connective tissue and thereby facilitate tissue reorganization. (*Lab Invest* 2001, 81:1015–1024).

Tenascins constitute a family of extracellular matrix glycoproteins (Erickson, 1993). The first member found, tenascin-C (TNC), is highly expressed in embryonic tissue during morphogenesis and sparsely expressed in the adult, but reappears during wound healing, regeneration, or cancer invasion (Chiquet-Ehrismann et al, 1986). TNC is transiently expressed in distinct areas in association with active tissue remodeling. Various in vitro findings have indicated that TNC has multiple functions in the regulation of cell migration, proliferation, and apoptosis (Chung et al, 1996; Jones and Jones, 2000). TNC counterbalances cell adhesion to substrata, correlated with cytokinesis and motility, and prevents cells from adhering too tightly to other extracellular matrix proteins (Chiquet-Ehrismann, 1995; Chiquet-Ehrismann et al, 1988; Chung et al, 1996; Murphy-Ullrich et al, 1991; Prieto et al, 1992).

In the heart, TNC appears only at very early stages of embryonic development and may play important roles in the differentiation of cardiomyocytes, struc-

tural organization of the myocardium, or development of coronary vessels (Imanaka-Yoshida et al, 2000). TNC is not normally expressed in the adult heart but reappears under various pathologic conditions such as dilated cardiomyopathy, myocarditis, or myocardial infarction (Imanaka-Yoshida et al, 1998, 2000; Tamura et al, 1996; Willems et al, 1996).

To understand its roles in tissue remodeling in the diseased myocardium, we examined the expression of TNC during tissue repair after experimental myocardial infarction in rats. Sequential changes in the localization of the molecule were analyzed by immunohistochemistry, and TNC-producing cells were identified by in situ hybridization (ISH) combined with immunohistochemistry. Additionally, to study the biologic functions of TNC in cardiomyocytes, we isolated the cells from an adult rat heart and examined the effects of addition of exogenous TNC on cell attachment. Cardiomyocytes have a special cell-extracellular matrix attachment system, the costamere (reviewed in Imanaka-Yoshida, 2000), which was originally described as a striated distribution of vinculin colocalized with the Z-lines of myofibrils (Craig and Pardo, 1983; Pardo et al, 1983). Costameric attachment prevents slippage of cardiomyocytes during contraction and transmits contraction forces from myofibrils to surrounding connective tissue (Danowski et al, 1992; Imanaka-Yoshida et al, 1996, 1999). In culture, costameric attachment can be identified as a characteristic

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banded distribution of focal contacts by interference reflection microscopy (IRM) (Danowski et al, 1992; Imanaka-Yoshida et al, 1996, 1999). We investigated the effects of TNC on this adhesion.

Our results suggest that during the acute phase after myocardial infarction interstitial cells in the border zone synthesize TNC, which may loosen strong adhesion to connective tissue to help in the rearrangement of surviving cardiomyocytes.

Results

Localization and Expression of TNC Protein and mRNA after Myocardial Infarction

Twenty-four hours after coronary ligation, positive interstitial TNC immunoreactivity was found in the border zone between infarcted and intact areas (Fig. 1a). TNC mRNA was observed in interstitial cells but not in cardiomyocytes (Fig. 1b). Neither immunoreac-

Figure 1.

Immunoreactivity (a, c, e, and g) for tenascin-C (TNC) and in situ hybridization (ISH) for TNC mRNA (b, d, f, h, and i) and the TNC alternative splicing region (j) in rat myocardial tissue sections 24 hours (a and b), 5 days (c, d, e, and f), 7 days (g and h), and 14 days (i and j) after coronary ligation. Deposition of TNC protein and mRNA for TNC is apparent in the border zone between infarcted (*inf*) and intact (*int*) areas within 24 hours (a and b). On Day 5, immunoreactivity and mRNA for TNC are localized in the border zone between the intact (*int*) myocardium and granulation tissue (*gr*) (c and d). Enlarged images of serial sections of a border zone (i and j). Interstitial cells express TNC mRNA (i) with alternative splicing sites (j). Protein and mRNA levels are low at Day 7 (e and f) and are almost lacking by Day 14 (g and h). Bars: a, 50 μ m for panels a to h; j, 50 μ m for panels i and j.

tivity nor mRNA was detected within the infarcted area or in the remote areas.

Approximately 3 days after infarction, the necrotic myocardium began to be replaced by granulation tissue. Deposits of TNC-immunoreactive and TNC mRNA-positive cells were observed in the marginal zone (Fig. 1, c and d) and at developing fronts of granulation tissue around necrotic cardiomyocytes (Fig. 2, f and g). No TNC immunoreactivity or mRNA was detected in the granulation tissue itself (Fig. 1, c and d). Around Day 7 after infarction, both the expression of TNC mRNA and the level of TNC protein in the border zone seemed to be down-regulated (Fig. 1, e and f), and they had almost disappeared by Day 14 (Fig. 1, g and h). No discrepancy between mRNA and protein levels was evident. The duration of expression of TNC in the infarcted areas depended on the size of the infarct. The expression continued as long as the necrotic masses remained.

Assessment of the expressed isoforms of TNC by a cRNA probe for the alternative splicing region of fibronectin type III-like repeats of TNC (see "Materials and Methods") revealed that cells expressed the larger isoform, which contains the alternative splicing sites (Fig. 1, i and j).

Characterization of TNC-Expressing Cells

Signals for TNC mRNA were detected only in interstitial cells, not in cardiomyocytes (Fig. 1, b, d, f, i, and j, and Fig. 2b). However, not all interstitial cells were positive for TNC mRNA. To characterize the TNC-producing cells, we examined the distribution of myofibroblasts using antibodies to α -smooth muscle actin (SMA). After 24 hours, although TNC mRNA-positive interstitial cells and deposition of TNC molecules were detected (Fig. 2, a and b), only vascular cells were immunoreactive for SMA (Fig. 2a). Other SMA-immunoreactive cells started to appear around Day 3, consistent with a previous report (Vracho and Thornington, 1991). Double immunoreactivity for TNC and SMA in the same tissue section showed that SMA-immunoreactive cells appeared in the area where TNC immunoreactivity was present (Fig. 2, c and d).

However, double-labeling for SMA and TNC mRNA in the same tissue section showed that most TNC-producing cells were negative for SMA, and most SMA-immunoreactive cells were negative for TNC mRNA at this stage (Fig. 2e). At Day 5, TNC-synthesizing cells were localized at the border zone and at the periphery of the necrotic masses (Fig. 2g) where TNC molecules were deposited (Fig. 2f). In contrast, SMA-immunoreactive cells were distributed over a broader area within granulation tissue (Fig. 2, f and h). Careful comparison of serial sections (Fig. 2, i and j) showed that some cells expressing TNC mRNA were also immunoreactive for SMA, which indicated that some myofibroblasts were expressing TNC.

Western Blotting

By Western blot analysis, two isoforms of TNC, of approximately 230 and 220 kd, were identified in Day

7 culture medium (Fig. 3). On Day 3, a faint band for the larger isoform was detected. No bands were detected on Day 0, the day the cells were plated.

Immunofluorescence

Cardiomyocytes plated on laminin-coated coverglasses gradually spread, and reorganized myofibrils with regular striation of I-bands by Day 7 (Fig. 4a). In the same culture, contaminating fibroblasts started to proliferate at approximately Day 3. After treating the cells with monensin to prevent intracellular vesicular transport, immunofluorescent reactivity for TNC revealed accumulation of TNC-positive granules in the perinuclear region of fibroblasts but not in cardiomyocytes (Fig. 4b), which confirmed the observations obtained by ISH in the tissue sections.

Adhesion Assay

Cardiomyocytes attached to the coverglasses maintained their original rod shape and the striated structure of myofibrils at 24 hours after plating (Fig. 5b). With IRM, approximately 60 percent of cardiomyocytes attached to laminin showed a characteristic striated distribution of dark contact, whose periodicity resembled that of Z-lines of myofibrils (Fig. 5b), which we refer to as costameric attachment, as previously reported (Imanaka-Yoshida et al, 1996). The other cells did not show costameric attachment but attached to the substratum with gray or white contacts (Fig. 5b). The total number of attached cells was higher on the mixture of laminin and TNC than on laminin alone. Addition of TNC to laminin significantly reduced the adhesion at costameric attachments on laminin, but promoted noncostameric attachment. Addition of fibronectin did not affect the number of the cells, regardless of whether there was costameric attachment (Fig. 5a).

Discussion

TNC Appears Transiently in the Acute Phase after Myocardial Infarction

Our present study demonstrated that both TNC mRNA and TNC protein deposition appear in the border zone as early as 24 hours after coronary ligation and that they are both down-regulated approximately 7 days after infarction. Such transient up-regulation of TNC is consistent with the previous immunohistochemical findings for human infarcted myocardium (Willems et al, 1996). Early expression of TNC is also reported after various tissue injuries, and is related to cell migration or proliferation (Fassler et al, 1996; Imanaka-Yoshida et al, 2001; Latijnhewers et al, 1996; Whitby and Ferguson, 1991; Yoshimura et al, 1999).

TNC Inhibits Stable Adhesion, but Supports the Transient Attachment of Cardiomyocytes

The most striking feature of TNC expression after myocardial infarction is that its distribution is restricted to the border zone. This limited distribution

Figure 2.

The distribution of α -smooth muscle actin (SMA)-immunoreactive cells, TNC molecule, and TNC-expressing cells at Day 1 (a and b), Day 3 (c, d, and e), and Day 5 (f, g, and h). ISH for TNC (b, g, and i), immunoreactivity for SMA (h), double labeling for immunoreactivity of SMA and ISH for TNC mRNA (e), double immunoreactivity for TNC and SMA in the same tissue section (a, c, d, and f). At Day 1 (a), deposition of TNC can be detected (brown), but only vascular cells are immunoreactive for SMA (blue), although several interstitial cells express TNC mRNA (b). At Day 3 (c), SMA-immunoreactive interstitial cells (blue) appear in the TNC-immunoreactive area (brown) at the border zone. d, High-power view of c. Double labeling for SMA immunoreactivity and TNC mRNA (e) shows that most TNC-producing cells (purple) are negative for SMA (brown) and most SMA-immunoreactive cells are also not expressing TNC. At Day 5, TNC immunoreactivity (brown in f) and cells positive for TNC mRNA (g) are seen at the periphery of the necrotic mass in the granulation tissue. Some SMA-immunoreactive cells (blue in f) are seen in the TNC-positive area but distributed over a broader area (brown in h; compare with g). High-power view of ISH for TNC (i) and immunoreactivity for SMA (j) of serial sections shows that the same cells are positive for both SMA and TNC signals (arrows). Bars: a, 50 μ m for panels b and c; e, 50 μ m for panels d, e, i, and j; g, 50 μ m for panels f, g, and h). Abbreviations: *inf*, infarcted area; *int*, intact area; *nec*, necrotic mass; *gr*, granulation tissue.

Figure 3.

Western blot analysis of culture medium of adult rat cardiomyocytes with fibroblasts on Days 0, 3, and 7. Two isoforms of TNC are evident at approximately 220 kD and 230 kD on Day 7. A faint band at 230 kD is seen on Day 3. No bands are evident on Day 0.

Figure 4.

Adult rat cardiomyocytes cultured for 7 days, doubly stained for rhodamine-phalloidin (a) and anti-TNC (b), 2 hours after monensin treatment. A cardiomyocyte showing striated myofibrils and fibroblasts with nonstriated stress fibers is present in the same culture. Numerous TNC-immunoreactive granules are evident in the perinuclear region only in fibroblasts. Bar: 10 μ m.

suggests special roles of TNC at the edges of the residual myocardium during tissue remodeling after infarction.

In normal myocardium, cardiomyocytes are not only joined together at intercalated disks but also firmly anchored to connective tissue with costamere complexes containing vinculin, talin, integrin $\alpha 6 \beta 1$, and laminin (Danowski et al, 1992; Imanaka-Yoshida, 2000; Imanaka-Yoshida et al, 1996, 1999). Costameric adhesion prevents cardiomyocytes from slipping during contraction, and transmits the contraction force of myofibrils to interstitial collagen fibrils surrounding individual myocytes, allowing the normal heart to function as a single pump (Danowski et al, 1992; Imanaka-Yoshida et al, 1999). However, during tissue remodeling after myocardial infarction, the surviving cardiomyocytes at the border zone need to loosen this attachment to reorganize the cell shape or arrangement.

In fact, although adult rat cardiomyocytes can form costameric focal contacts upon plating on laminin (Imanaka-Yoshida et al, 1996, 1999), the cells transiently lose these strong attachments during the changing of cell shape and reorganization of the cytoskeleton to adapt to the new environment in culture (Imanaka-Yoshida et al, 1996, 1999; LoRusso et al, 1992). Instead, cells maintain their adherence to substrates via noncostameric white or gray contacts during the extensive remodeling (Imanaka-Yoshida et al, 1996). When cytoskeletal reorganization is completed, costameric attachment is regained and becomes strong enough to link myofibrils to the substratum and transmit contraction forces (Danowski et al, 1992; Imanaka-Yoshida et al, 1996, 1999).

As we showed in this study, TNC inhibits cardiomyocytes from forming costameric focal contacts but increases the number of cells with noncostameric attachment. TNC is generally thought to be a counter-adhesion molecule, but its effects on cell adhesion are complex. Several lines of evidence indicate that TNC has both adhesive and counter-adhesive domains, and that its binding to cells is mediated by various receptors (Fischer et al, 1997a, 1997b; Gotz et al, 1996; Prieto et al, 1992, 1993). Thus, TNC can inhibit adhesion (Chiquet-Ehrismann et al, 1988; Lightner and Erickson, 1990) or support weak and transient attachment (Lotz et al, 1989) depending on the cell type. TNC may mediate de-adhesion by shifting cells from strong adhesion to temporary attachment (Greenwood and Murphy-Ullrich, 1998). As we found here, TNC may inhibit strong adhesion at costameres but promote temporary noncostameric attachment of cardiomyocytes. This de-adhesion effect would be helpful for dynamic tissue remodeling at the edges of residual myocardium. Furthermore, TNC has the ability to induce production of matrix metalloproteinase (MMP) in certain types of cells (Tremble et al, 1994). The degradation of connective tissue should also put the cells into an adaptable state for active tissue reorganization. These alterations would be useful for an early adaptive reaction but might also cause cell slippage,

Figure 5.

The effects of TNC on cardiomyocyte attachment (a). Adult rat cardiomyocytes were plated on cover glasses coated with laminin (LN), TNC plus laminin (LN+TNC), fibronectin plus laminin (LN+FN), or TNC plus fibronectin and laminin (LN+FN+TNC). The total number of attached cells and costameric attachment-positive and -negative cells within 10 fields per cover glass were counted and averaged. Error bars represent the standard deviation for triplicate samples. The number of costameric attachment-positive cells on TNC plus laminin was significantly lower than the numbers on laminin or laminin plus fibronectin. Asterisks denote significant differences (* $p < 0.05$ or ** $p < 0.01$) according to Students' t test. Similar results were obtained in three separate experiments. b, Interference reflection microscopy (IRM) and phase-contrast photomicrographs of cardiomyocytes after 24 hours of incubation. The cells are rod shaped and maintain their original myofibrils under phase contrast microscopy. The attachment pattern observed by IRM was classified into two groups: costameric adhesion, showing striated dark contacts whose periodicity resembled that of Z-lines of myofibrils (arrows), and noncostameric adhesion, with gray and white contacts.

leading to ventricular dilatation after myocardial infarction. In fact, side-to-side slippage of cardiomyocytes is greater in the border zone (Anversa et al, 1993).

Are Myofibroblasts a Major Source of TNC?

During cancer invasion (Hanamura et al, 1997; Yoshida et al, 1997) or embryonic development of mammary glands (Kalembey et al, 1997), both mesenchymal and

epithelial/parenchymal cells synthesize TNC. In contrast, our present study showed that TNC production after myocardial infarction was limited to interstitial cells. Injured cardiomyocytes may send signals to neighboring fibroblasts to synthesize TNC, which regulates myocyte adhesion in a paracrine fashion.

In cancer stroma, myofibroblasts are a major cellular source of TNC (Hanamura et al, 1997). Myofibroblasts

share characteristics with both fibroblasts and smooth muscle cells expressing proteins such as SMA (Desmouliere and Gabbiani, 1994), and appear in various tissues during wound healing or cancer stroma. In normal tissue, repertoires of myofibroblasts such as pericyptal cells of the colonic mucosa or stellate cells of the liver synthesize TNC (Hanamura et al, 1997; Murakami et al, 1995).

As we demonstrated in the present study, some myofibroblasts contribute to production of TNC in myocardial scar tissue. However, on Day 1 after infarction, TNC-producing cells were negative for SMA. SMA-immunoreactive cells appeared at approximately Day 3, in the area where the TNC molecules were deposited. TNC might be related to the differentiation of interstitial resident fibroblasts into myofibroblasts or to the activation of the locomotion of myofibroblasts. In the heart, myofibroblasts are thought to provide the contractile force to prevent ventricular dilatation after infarction (Li et al, 1999; Vracho and Thorning, 1991; Vracho et al, 1989; Willems et al, 1994). TNC may not only modulate cardiomyocyte-extracellular matrix attachment, but may also play multiple roles in myocardial tissue remodeling to control interstitial cell behavior during repair of tissue damage.

Materials and Methods

Antibodies and Reagents

TNC was purified from the culture supernatant of U251 MG human glioma cells, as previously described (Yoshida et al, 1999). Laminin (EHS tumor derived) and human fibronectin were purchased from GIBCO BRL (Gaithersburg, Maryland). Rabbit polyclonal antibodies to TNC were raised against human melanoma TNC (Hasegawa et al, 1997). Peroxidase-conjugated anti-SMA (EPOS) was obtained from Dako Japan (Kyoto, Japan).

Myocardial Infarction Model

Adult male Wistar rats weighing 250 to 300 g were used. The animals were anesthetized with pentobarbital (60 mg/kg ip) and ventilated by positive pressure through endotracheal tubes attached to a Harvard small animal respirator. The left descending coronary artery was ligated. At different times (1, 3, 5, 7, 14, and 21 days) after surgery, a total of 18 rats ($n = 3$ for each group) were anesthetized with pentobarbital (60 mg/kg ip) and their hearts were removed and fixed overnight in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at 4° C. After the hearts were rinsed in the same buffer, they were dehydrated through a graded ethanol series and xylene, embedded in paraffin, sectioned at 4- μ m thick, and placed on silane-coated slide glasses (Dako Japan).

Immunohistochemistry

For TNC immunoreactivity, the sections were equilibrated in distilled water, and incubated in 0.4% pepsin (1:60,000; Sigma Chemical Corporation, St. Louis, Missouri) in 0.01 N HCl for 10 minutes at 37° C to retrieve the antigens. The sections were then exposed to 0.3% H₂O₂ in methanol for 15 minutes to block endogenous peroxidase activity, and treated with superbloc solution (ScyTek Laboratories, Logan, Utah) before incubation with primary antibodies (1 μ g/ml) overnight at 4° C. After extensive washing, the sections were incubated with peroxidase-conjugated anti-rabbit IgG (1:500; MBL, Nagoya, Japan) for 1 hour. After the sections were washed, a diaminobenzidine and H₂O₂ solution was used to demonstrate antibody binding. For anti-SMA, a prediluted solution was used. For double immunohistochemistry for TNC and SMA, the sections were first processed for TNC immunoreactivity. After the first coloring reaction, the slides were fixed with 4% paraformaldehyde in PBS for 30 minutes, exposed to 0.3% H₂O₂ in methanol for 15 minutes, treated with superbloc solution, and incubated with peroxidase-conjugated anti-SMA. To detect the second signal, a diaminobenzidine and NiCl₂ solution was used. Therefore, the TNC immunoreactivity was brown in color, whereas the SMA immunoreactivity was blue. For double-labeling with SMA immunoreactivity and ISH for TNC, the sections were first processed for SMA immunoreactivity, labeled with ISH, then lightly counterstained with methylgreen solution.

Preparation of cRNA Probes

Antisense and sense cRNA probes were prepared by in vitro transcription of mouse TNC cDNA (95% homology with rat) (Saga et al, 1991) using a digoxigenin RNA-labeling kit (SP6/T7; Boehringer Mannheim, Mannheim, Germany), as previously reported (Tsukamoto et al, 1991). We used two types of cRNA probes to examine TNC isoforms. The 6th through 11th fibronectin type III-like repeats (A1, A2, A4, B, C, and D) of mouse TNC are alternatively spliced sites. TNC mRNA including these sequences generates TNC isoforms of large molecular weight. In the present study, a probe derived from the cDNA encoding these repeats, designated FNAE by Tsukamoto et al (Tsukamoto et al, 1991), was used alone to detect large isoforms. Probes for other regions were used as a mixture to detect mRNAs for all isoforms of TNC.

ISH

The preparation of tissue sections, ISH method, and color development were as previously described by Ishihara et al (1995). The processing included treatment with protease K for 20 minutes. The slides were lightly counterstained with nuclear fast red or methylgreen solution.

Cell Culture

Adult rat cardiomyocytes were isolated from the hearts of male Wistar rats (200 to 250 g) by retrograde perfusion with collagenase, as previously described (Imanaka-Yoshida et al, 1993; LoRusso et al, 1992), suspended in DMEM supplemented with 10% fetal calf serum and plated on coverglasses coated with laminin (GIBCO BRL).

Western Blotting

Proteins secreted by mixed cultures of cardiomyocytes and fibroblasts were analyzed by immunoblotting, as previously described (Hasegawa et al, 1997; Kalembe et al, 1997). On Days 0, 3, and 7, supernatants of culture medium (15 μ l/lane) were subjected to SDS-PAGE with 2% to 15% gradient polyacrylamide gels and transferred by electrophoresis onto Immobilon membranes (Millipore, Bedford, Massachusetts). The transferred proteins were immunoreacted with the TNC antibody (2.5 μ g/ml) using the indirect immunoperoxidase method. After several washes, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (H+L-chain specific, 500-fold diluted; MBL), and immunoreactivity was detected with the ECL system (Amersham, Arlington Heights, Illinois).

Adhesion Assay

Coverglasses were coated overnight at 4° C with either laminin dissolved in PBS at a concentration of 20 μ g/ml, or a mixture of laminin (20 μ g/ml) and fibronectin (20 μ g/ml). After rinsing with PBS, some coverglasses were incubated with TNC (40 μ g/ml) for at least 1 hour. Coated coverglasses were blocked with 2 mg/ml of BSA in PBS for 30 minutes.

Freshly isolated cardiomyocytes were suspended in DME containing 10% fetal bovine serum. The cell suspension was adjusted to a final concentration of 2×10^4 cells/ml, and 2 ml aliquots were plated on coverglasses (24 $\times$ 18 mm) coated with various substrates in 35-mm tissue culture dishes and incubated at 37° C for 24 hours. Unattached cells were removed by washing the plates with DMEM. Cells on the coverglasses were fixed with 4% paraformaldehyde in PBS for 15 minutes and mounted with 50% glycerol in PBS.

The total number of attached cells was counted in random fields observed under phase optics with a 40 $\times$ objective. Attachment was confirmed by IRM, as previously described (Imanaka-Yoshida et al, 1999). With IRM, black areas of images indicate distances between the ventral surface and the substratum of approximately 10 to 15 nm, whereas gray areas indicate separation of approximately 30 nm. Light or white areas indicate distances of 100 nm or more (Izzard and Lochner, 1980). Cardiomyocytes can form a regularly striated distribution of dark contacts, corresponding to Z-lines, when they are plated on laminin (Imanaka-Yoshida et al, 1996). The attachment sites of each cardiomyocyte were enlarged with a 60 $\times$ objec-

tive to determine whether they had a striated distribution of dark contacts. Within each experiment ($n = 3$), the cells within 10 fields per coverglass were counted and averaged.

Immunofluorescence of Cultured Cells

For immunofluorescent reactivity, the cells were treated with 1 μ M monensin for 2 hours to allow accumulation of secretory proteins in the cytoplasm. After fixation in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 minutes, the cells were permeabilized with 0.1% Triton X-100, incubated with 10% normal goat serum for 30 minutes, and incubated with rabbit anti-TNC antibody (10 μ g/ml) overnight at 4° C. After the cells were washed with PBS, they were incubated with goat FITC-labeled anti-rabbit IgG antibody (MBL) and rhodamine-phalloidin (Molecular Probes, Eugene, Oregon) for 1 hour at room temperature.

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