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IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A HEART-MUSCLE CELL TRANSFORMING GROWTH FACTOR β-1 RECEPTOR

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Abstract—Binding of human-recombinant transforming growth factor-β1 (TGF-β1) to the neonatal rat heart-muscle cell (cardiomyocyte) was characterized as a potential element in the cardioprotective pharmacology of this growth factor. The cardiomyocytes were found to express a single class of specific, high-affinity TGF-β1 binding sites. Ligand binding to these sites was rapid, saturable, selective, and reversible, characteristics of a receptor-mediated process. Scatchard and iterative non-linear least-squares regression analyses demonstrated that the cardiomyocyte TGF-β1 receptor had a $K_d \leq 40$ pM, a B_{max} of ~3.4 fmol/10^6 cells, and a density of ~2000 binding sites/cell. Binding was selective for TGF-β1 as compared with other TGF-β isoforms (i.e. TGF-β2 and -β3) and nonrelated cytokines (e.g. acidic fibroblast growth factor). Affinity-binding experiments to probe the molecular nature of the specific binding revealed three types of cardiomyocyte TGF-β1 binding proteins, the most prominent of which corresponded to the high-molecular-mass proteoglycan observed in nonmuscle cell types. These data raise the possibility that the known pharmacological effects of TGF-β1 on heart muscle may be direct actions via specific receptor-mediated events.

The peptide transforming growth factor-β (TGF-β)† exists as a family of three highly-conserved mammalian isoforms (TGF-β1, -β2, and -β3) with significant (~70%) sequence homology and similar, though not always identical, bioactivity [1]. TGF-β is synthesized by many tissues and exerts multiple effects on diverse cell types. In the cardiovascular system, TGF-β is a constituent of blood vessels, circulating blood elements, and heart muscle (myocardium), at least some of which also synthesize TGF-β [2]. Aside from effects on healthy myocardial tissue such as modulation of contractile-protein gene expression, exogenously-supplied TGF-β1 helps preserve heart-muscle function during the disease state of myocardial ischemia [3]. The mechanism of this anti-ischemic cardioprotection is undefined and has been postulated to involve autocrine and/or indirect (i.e. vascular) effects of TGF-β1 on the heart-muscle cell (cardiomyocyte) [4]. The initial interaction of TGF-β with target cells is generally mediated by specific cell-surface receptors [1]. Although information on the cardiomyocyte as a target for peptide growth factors is, as yet, limited [5], direct modulation of cardiomyocyte physiology by TGF-β would likely require expression of a specific sarcolemmal receptor. We have identified and characterized herein a receptor population responsible for human-recombinant TGF-β1 binding to the neonatal rat cardiomyocyte. Our data represent the first biochemical description of a specific cardiomyocyte TGF-β1 receptor.

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† Abbreviations: TGF-β1, transforming growth factor-β; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DSS, disuccinimidyl suberate; aFGF, acidic fibroblast growth factor; BME, Eagle's basal medium without phenol red; SDS, sodium dodecyl sulfate; and PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials. Essentially fatty acid-free bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), Tris, protease inhibitors, and miscellaneous biochemicals were obtained at the highest available grade from Sigma (St. Louis, MO). Protease inhibitor mixture I was an aqueous solution of (final concentrations) 1 mg/mL leupeptin, 1 mg/mL antipain, 5 mg/mL aprotinin, 10 mg/mL soybean trypsin inhibitor, and 10 mg/mL benzamidine hydrochloride. Protease inhibitor mixture II consisted of (final concentrations) 1 mg/mL pepstatin, 1 mg/mL bestatin, and 30 mM phenylmethanesulfonyl fluoride (PMSF) in dimethyl sulfoxide. Disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL). TGF-β1 was from human platelets, and acidic fibroblast growth factor (aFGF) was from bovine brain (R & D Systems, Minneapolis, MN). Human-recombinant TGF-β2 and -β3 were provided by N. Cerletti (Ciba-Geigy, Basel, Switzerland) [6]. Human-recombinant [^{125}I]TGF-β1 (sp. act. 3000–4500 Ci/mmol) was from New England Nuclear (Boston, MA).

Cardiomyocyte culture. Cardiomyocytes were isolated from left-ventricular myocardium of 3-day-old Sprague–Dawley rats [7]. The isolated cardiomyocytes were seeded in complete Eagle's basal medium without phenol red (BME) at

5×10^5 cells/35-mm well and incubated at 37° under 95% air:5% CO_2. By 72-hr post-plating, a confluent, synchronously-beating cardiomyocyte monolayer had formed which contained 110 ± 6.5 μg cell protein/well (mean ± SD; N = 10). These experimental cultures consisted of ≥95% ventricular cardiomyocytes [8].

Binding assays. Cardiomyocyte monolayers were preincubated in serum-free BME for 18 hr. The cells were then washed with 6 mL of ice-cold binding buffer [serum-free BME, 0.1% (w/v) BSA, 25 mM HEPES, pH 7.4] and incubated with gentle agitation at 4° in 1.0 mL of binding buffer/well containing a known concentration of [^{125}I]TGF-β1. At 4°, potential complications due to TGF-β1 internalization during the binding assay were obviated (below). The [^{125}I]-TGF-β1 concentrations used and the incubation times are specified in the text and figure legends. The binding assay was terminated by removing the [^{125}I]TGF-β1-containing binding buffer and washing each labeled monolayer with 6 mL of ice-cold binding buffer. The cells were then dissolved in 0.6 mL of solubilization buffer [20 mM HEPES, pH 7.4; 1.0% (v/v) Triton X-100; 10% (v/v) glycerol; 0.01% (w/v) BSA], and triplicate aliquots were counted for ^{125}I label. Nonspecific binding was taken as the cell-associated [^{125}I]TGF-β1 in the presence of a 100-fold molar excess of unlabeled ligand. Competition studies were performed by incubating cells with 40 pM [^{125}I]TGF-β1 in the presence of various concentrations of unlabeled TGF-β1, -β2, -β3, or aFGF. Displacement studies were conducted after an equilibrium binding period with 4, 20, or 40 pM [^{125}I]TGF-β1 by incubating the labeled cardiomyocytes for various time periods with a 100-fold molar excess of unlabeled TGF-β1 under binding assay conditions.

TGF-β1 internalization assay. Cardiomyocytes were incubated with 40 pM [^{125}I]TGF-β1 for 60 min at 4° and then washed free of unassociated radioactivity (above). The labeled cells were lysed hypotonically to prepare soluble ("cytoplasmic") and particulate ("membrane") fractions which were counted for ^{125}I label [8].

Affinity-labeling of cardiomyocyte TGF-β1 binding proteins. Confluent cardiomyocyte monolayers were incubated with [^{125}I]TGF-β1 under equilibrium-binding conditions (above) to generate cells with ligand-occupied TGF-β1 receptors. Each monolayer was then washed with ice-cold binding buffer and incubated at 4° for 15 min with 1.0 mL of BSA-free binding buffer containing 135 μM DSS. After the cross-linking reaction, the cells were harvested into ice-cold detachment buffer (10 mM Tris, pH 7.4; 0.25 M sucrose; 1 mM EDTA; 0.3 mM PMSF) and lysed with hypotonic buffer [8] containing 10 μL/mL of each protease inhibitor mixture in order to prepare a membrane fraction [8]. The isolated membranes were solubilized in 10 mM Tris, pH 7.0, containing 125 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, and 10 μL/mL of each protease inhibitor mixture. The samples were then prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 7% gels according to Laemmli [9], and the gels were analyzed by autoradiography [10].

Data analysis. The "ReceptorFit" program (Lundon Software, Chagrin Falls, OH) [11] was used for equilibrium-binding data analysis by weighted non-linear least-squares regression and for generation of Klotz [12] plots. The program featured iterative, progressive-complexity analysis with one- and multi-site binding models. Selection of the model of best-fit was based on the F test for the extra sum of squares principle at a statistical significance level of $P < 0.01$. Scatchard [13] analysis of the binding data was carried out with computer-assisted linear regression (Cricket Software, Malvern, PA). All data were obtained from replicate (≥3) samples in a given culture over N independent preparations.

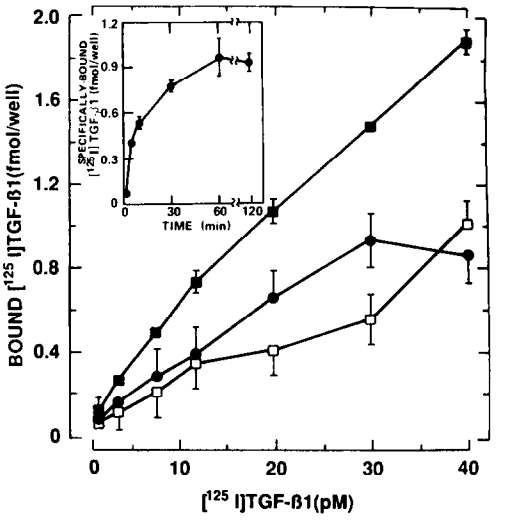

Fig. 1. Concentration- and time-dependent cardiomyocyte TGF-β1 binding. Monolayers (5×10^5 cells/well) were incubated for 60 min at 4° in binding buffer containing 2–40 pM [^{125}I]TGF-β1 or for up to 120 min with binding buffer containing 40 pM [^{125}I]TGF-β1 (inset). Parallel incubations were conducted in the presence of a 100-fold molar excess of unlabeled TGF-β1 for determination of nonspecific binding. Following the binding assay, the cardiomyocyte-associated TGF-β1 was quantified as detailed in the text. Specific binding (●) represents the difference between total (■) and nonspecific (□) binding. Data are means ± SEM (N ≥ 6).

RESULTS

Incubations with [^{125}I]TGF-β1 were performed using cardiomyocyte monolayers under serum-free conditions to evaluate the potential for receptor-mediated TGF-β1 interaction. Cardiomyocyte [^{125}I]-TGF-β1 binding was rapid and concentration- and time-dependent (Fig. 1). All of the cell-associated [^{125}I]TGF-β1 was recovered in a particulate membrane fraction prepared from the cells by hypotonic lysis; the soluble cytoplasm contained a negligible (<2%) proportion of the total cellular ^{125}I label at any [^{125}I]TGF-β1 concentration utilized. A saturable, specific-binding component was identified which reached apparent equilibrium by 60 min of incubation with 40 pM [^{125}I]TGF-β1. The specific-binding plateau did not reflect ligand depletion, since the

Table 1. Displacement of receptor-bound [^{125}I]TGF-β1 from cardiomyocytes

[^{125}I]TGF-β1 (pM)	Displacement (% total specifically bound)				
	0 min	20 min	60 min	120 min	240 min
4	0	51.0	60.2	78.0	89.0
20	0	76.1	81.5	87.5	100.0
40	0	72.0	84.2	84.2	100.0

Data are mean-% loss of specifically-bound [^{125}I]TGF-β1 after the labeled cells were incubated with a 100-fold molar excess of nonradioactive TGF-β1 for the indicated time periods. At 0 min, ~0.85 fmol [^{125}I]TGF-β1 was specifically bound/well (cf. Fig. 1). Values are averages of three independent experiments; the range was <10% about the means given.

40 pM [^{125}I]TGF-β1 supplied was in ~40-fold excess of that specifically bound, and nonspecific binding continued to increase thereafter. At equilibrium (i.e. 30–45 pM [^{125}I]TGF-β1), nonspecific [^{125}I]TGF-β1 binding averaged ~40% of total, a proportion observed by others in nonmuscle cells as a likely reflection of the adhesive properties of this growth factor [14]. Specific binding was >90% reversible within 2 hr after addition of excess TGF-β1 (Table 1).

Concentration-dependent binding data from experiments of the type depicted in Fig. 1 were analyzed according to Scatchard [13]. The Scatchard transforms indicated that [^{125}I]TGF-β1 labeled a single class of high-affinity (K_d = 37 pM) specific binding sites with a B_{max} = 3.2 fmol/10^6 cells and, maximally, 1947 binding sites/cardiomyocyte (Fig. 2A, Table 2). Inherent limitations of Scatchard analysis, which have led to gross quantitative errors in biochemical receptor characterization [12], prompted us to re-evaluate our binding data by a rigorous iterative non-linear least-squares regression program [11]. The observed [^{125}I]TGF-β1 concentration-dependent binding profile was incompatible with any multi-site model in this program. However, the data did fit with statistical significance (F test, $P < 0.01$) into a one-site model (Fig. 2B) to define a high-affinity cardiomyocyte TGF-β1 receptor population with characteristics similar to those

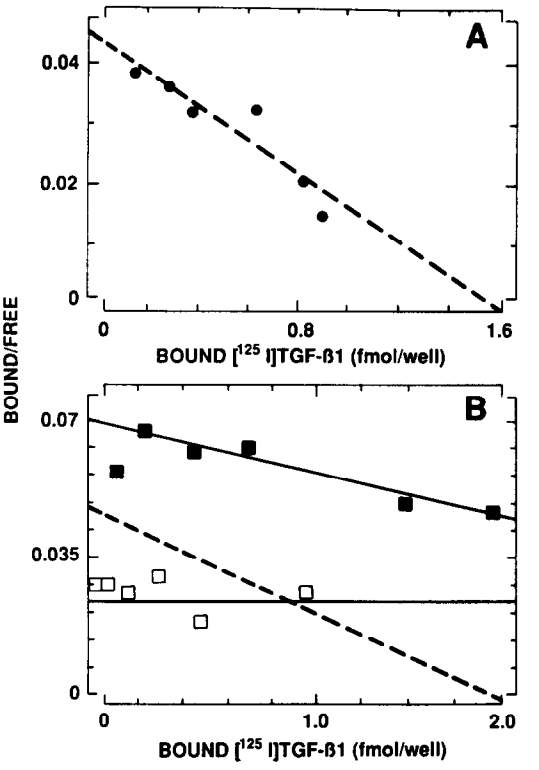

Fig. 2. Scatchard (panel A, ------) and non-linear least-squares regression (panel B, ------) analyses of concentration-dependent cardiomyocyte TGF-β1 binding data obtained from experiments of the type depicted in Fig. 1. For panel B, total (■) and measured nonspecific (□) binding data incorporated into the model are also displayed. The data are mean values from a representative experiment for 4 culture dishes per point; replicates varied <10% about each mean.

derived from the Scatchard analysis, regardless of whether measured (Table 2) or computed theoretical (data not shown) nonspecific binding data were used in the iterative fitting. Klotz transformations [12] of the concentration-dependent binding data also supported the existence of a single class of specific, high-affinity cardiomyocyte TGF-β1 receptors having

Table 2. Cardiomyocyte TGF-β1 receptor-binding parameters

Analysis	Best-fit model	Receptor parameter		
		K_d (pM)	B_{max} (fmol/10^6 cells)	Receptor density (number/cardiomyocyte)
Scatchard	One-site	37.2	3.2	1947
Non-linear least-squares regression	One-site	39.4 ± 8.5	3.6 ± 0.4	2239 ± 365

Characterization of the cardiomyocyte TGF-β1 receptor population was based upon analyses (Fig. 2) of concentration-dependent binding data obtained from experiments of the type depicted in Fig. 1. Data are means for a typical experiment involving 4 replicate cultures (Scatchard analysis) or means ± SEM (N = 6) for the least-squares regression analysis.

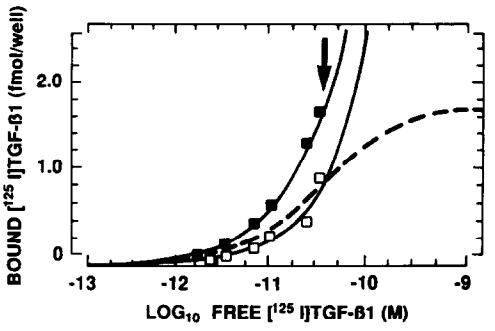

Fig. 3. Klotz analysis of cardiomyocyte TGF-β1 binding. Computer-assisted non-linear least-squares fitting was applied to the total (■) and measured non-specific (□) binding data shown (from Fig. 2) to generate a semi-logarithmic Klotz transform of cardiomyocyte [^{125}I]TGF-β1 binding (------). The *arrow* represents the calculated inflection point of the Klotz plot, yielding an apparent K_d value of ~40 pM.

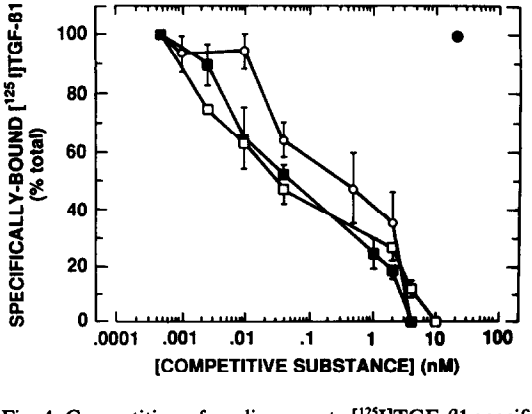

Fig. 4. Competition of cardiomyocyte [^{125}I]TGF-β1 specific binding by unlabeled TGF-β1 (■), TGF-β2 (□), TGF-β3 (○) and aFGF (●). TGF-β1 specific binding is expressed as a percentage of the total binding in contemporary cultures not containing unlabeled test substance. The 100% value represents ~0.85 fmol [^{125}I]TGF-β1 specifically bound/well (cf. Fig. 1). The data are means ± SEM (N = 3).

a K_d ~ 40 pM (Fig. 3), in good agreement with the Scatchard and non-linear least-squares regression analyses (Table 2).

The cardiomyocyte TGF-β1 receptor was ligand-selective (Fig. 4). Unlabeled TGF-β1 and -β2 effectively competed for [^{125}I]TGF-β1 specific binding with an EC_{50} of ~40 pM. TGF-β3 was a significantly less potent competitor (EC_{50} ~ 350 pM), and unrelated growth factors at high concentrations (e.g. 20 nM aFGF) were noncompetitive.

Affinity-binding experiments allowed visualization of discrete cardiomyocyte binding proteins for [^{125}I]-TGF-β1, which were not labeled in the absence of cross-linking reagent. The affinity-labeling pattern obtained on SDS–PAGE (Fig. 5) was similar to that reported for the other cell types [e.g. Ref. 15] in showing three major TGF-β1 binding proteins. Binding of [^{125}I]TGF-β1 to these proteins was concentration-dependent and could be effectively reduced by unlabeled TGF-β1 (Fig. 5), but not by unrelated growth factors such as aFGF (data not shown). Most of the [^{125}I]TGF-β1 was associated with a high-molecular-mass (~280 kDa) protein, likely the type III cell-surface proteoglycan [1, 15]. Two other binding proteins corresponding to ~55 kDa and ~85 kDa likely correspond to the type I and type II binding proteins, respectively [1].

DISCUSSION

The anti-ischemic cardioprotection of exogenously-supplied TGF-β1 has been postulated to reflect direct and indirect effects on cardiac muscle [4]. Although cardiomyocytes contain and secrete TGF-β1 [2], expression of cell-surface TGF-β1 receptors by this cell type as potential signal-transducing elements in its cardioprotective pharmacology has not been rigorously and quantitatively studied. The present work offers several lines of direct biochemical evidence supporting the conclusion that the neonatal rat cardiomyocyte has a population of specific, high-affinity sarcolemmal binding proteins for TGF-β1. The acuteness, saturability, ligand-selectivity, and reversibility of cardiomyocyte TGF-β1 binding are all consistent with a receptor-mediated process. Scatchard [13] and Klotz [12] analyses and iterative non-linear least-squares fitting [11] (Figs. 2 and 3; Table 2) demonstrate with excellent agreement that the cardiomyocyte TGF-β1 binding proteins behave as a single class of high-affinity (apparent $K_d \leq 40$ pM) receptors at ~2000 binding sites/cell and a maximal capacity (apparent B_{max}) of ~3.4 fmol/10^6 cells.

Although TGF-β1 has been shown to interact with cardiomyocyte membranes [16], this study is the first to identify and characterize biochemically a specific cardiomyocyte TGF-β1 receptor population. Many nonmuscle cell types have high-affinity TGF-β1 receptors with Scatchard-derived K_d values in the picomolar range and receptor densities from 600–80,000 TGF-β1 binding sites/cell [17]. Generally, cells with the highest affinity TGF-β1 receptors have the lowest receptor densities. The data in Table 2 clearly demonstrate that the cardiomyocyte TGF-β1 receptor population is of a capacity and affinity comparable to TGF-β1 receptors in at least some nonmuscle cells. The results of our competition experiments (Fig. 4) suggest that cardiomyocyte TGF-β1 receptors may also bind TGF-β2 and, to a lesser degree, TGF-β3. Since binding characteristics in a given cell type may differ among TGF-β isoforms [1], only direct biochemical analyses of the type performed herein can rigorously characterize potential cardiomyocyte receptors for TGF-β2 and -β3. Likewise, although the neonatal cardiomyocyte cultures used constitute a viable population of beating heart-muscle cells [18], extrapolation of the characteristics of their TGF-β1 binding to adult cardiomyocytes should be made with appropriate caution.

Chemical cross-linking of [^{125}I]TGF-β1 to car-

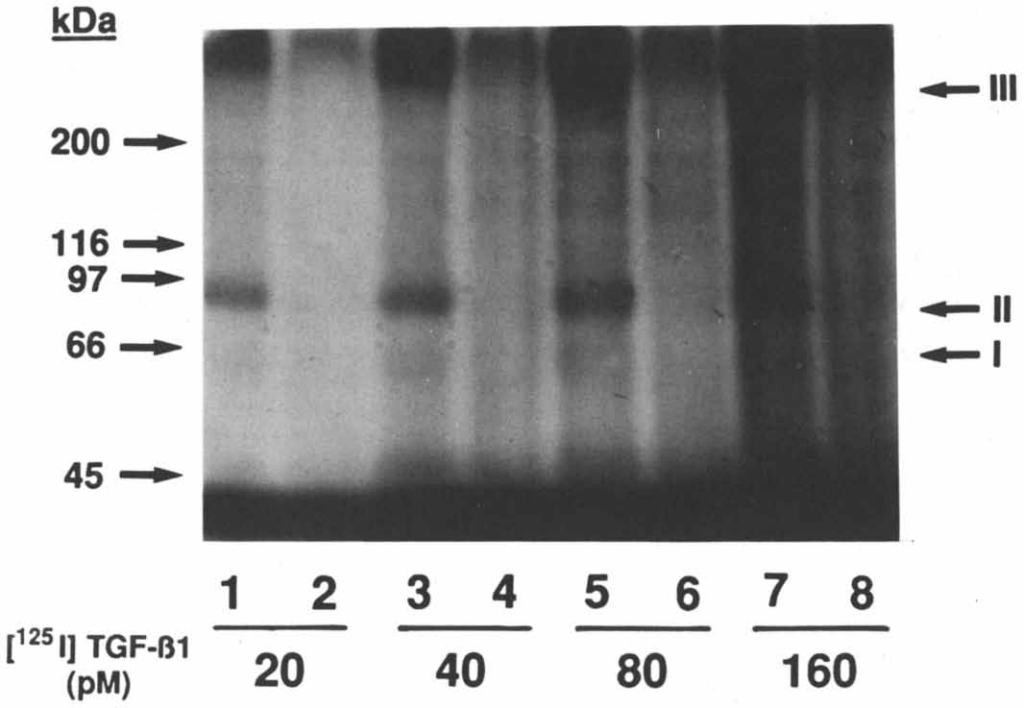

Fig. 5. Affinity labeling of cardiomyocyte TGF-β1 binding proteins. Cardiomyocytes were cross-linked to increasing concentrations of [^{125}I]TGF-β1 in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of a 100-fold molar excess of unlabeled ligand. Extracts were subjected to SDS–PAGE on 7% gels, and the dried gels were analyzed by autoradiography. Molecular mass markers are indicated in kDa to the left of the gel; TGF-β1 binding protein types I, II and III are indicated on the right.

diomyocytes enabled identification of three TGF-β1 binding proteins. These affinity-labeled cardiomyocyte receptor–ligand complexes resemble on SDS–PAGE the type I (~53 kDa), type II (~70–85 kDa), and type III proteoglycan (~250–350 kDa) binding proteins detected on nonmuscle cells [15]. Type I and type II binding proteins act as the signalling receptor when complexed to ligand, whereas the function of the type III binding protein is not clear [15]. Expression of high-affinity TGF-β1 receptors on the mammalian cardiomyocyte provides a means whereby TGF-β1 could act directly on the cell to modulate its physiology and thus potentially exert the anti-ischemic cardioprotection reported elsewhere [3, 4].

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REFERENCES

1. Lin HY and Lodish HF, Receptors for the TGF-β superfamily: Multiple polypeptides and serine/threonine kinases. *Trends Cell Biol* **3**: 14–19, 1993.
2. Wunsch M, Sharma, HS, Markert T, Bernotat-Danielowski S, Schott RJ, Kremer P, Bleese N and Schaper W, *In situ* localization of transforming growth factor-β1 in porcine heart: Enhanced expression after chronic coronary artery constriction. *J Mol Cell Cardiol* **23**: 1051–1062, 1991.
3. Lefer AM, Tsao P, Aoki N and Palladino MA, Mediation of cardioprotection by transforming growth factor-β. *Science* **249**: 61–64, 1990.
4. Lefer AM, Mechanisms of the protective effects of transforming growth factor-β in reperfusion injury. *Biochem Pharmacol* **42**: 1323–1327, 1991.
5. Schneider MD and Parker TG, Cardiac myocytes as targets for the action of peptide growth factors. *Circulation* **80**: 219–233, 1989.
6. Mühl H, Geiger T, Pignat W, Märki F, van den Bosch H, Cerletti N, Cox D, McMaster G, Vosbeck K and Pfeilschifter J, Transforming growth factors type-β and dexamethasone attenuated group II phospholipase A_2 gene expression by interleukin-1 and forskolin in rat mesangial cells. *FEBS Lett* **301**: 190–194, 1992.
7. Janero DR, Burghardt C and Feldman D, Amphiphile-induced heart muscle-cell (myocyte) injury. Effects of intracellular fatty acid overload. *J Cell Physiol* **137**: 1–13, 1988.
8. Janero DR, Hreniuk D and Sharif HM, Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): Lethal peroxidative membrane injury. *J Cell Physiol* **149**: 347–364, 1991.
9. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
10. Thakkar JK, Janero DR, Yarwood C and Sharif HM, Modulation of mammalian cardiac AMP deaminase by protein kinase C-mediated phosphorylation. *Biochem J* **291**: 523–527, 1993.

11. Lundeen JE and Gordon JH, Computer analysis of binding data. In: *Receptor Binding in Drug Research* (Ed. O'Brien RA), pp. 31–49. Marcel Dekker, New York, 1986.
12. Klotz IM, Numbers of receptor sites from Scatchard graphs. *Science* **217**: 1247–1249, 1982.
13. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
14. Massagué J and Like B, Cellular receptors for type β transforming growth factor. *J Biol Chem* **260**: 2636–2645, 1985.
15. Cheifetz S, Weatherbee JA, Tasug ML-S, Anderson JK, Mole JE, Lucas R and Massagué J, The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. *Cell* **48**: 409–415, 1987.
16. Roberts AB, Roche NS, Winokur TS, Burmester JK and Sporn MB, Role of transforming growth factor-β in maintenance of function of cultured neonatal cardiac myocytes. *J Clin Invest* **90**: 2056–2062, 1992.
17. Wakefield LM, Smith DM, Masui T, Harris CC and Sporn MB, Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J Cell Biol* **105**: 965–975, 1987.
18. Janero DR, Oxidative myocardial injury and cardiac-derived experimental systems. In: *Biological Oxidation Systems* (Eds. Reddy CC, Hamilton GA and Madyastha KM), Vol. 2, pp. 977–998. Academic Press, New York, 1990.