Overexpression of long or short FGFR-1 results in FGF-2-mediated proliferation in neonatal cardiac myocyte cultures

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Abstract

Objective: The type 1 fibroblast growth factor receptor (FGFR-1) is the only high affinity receptor for fibroblast growth factor-2 (FGF-2) in the rat myocardium, and is essential for normal growth and development of the heart. Levels of FGFR-1 are developmentally regulated, being high in embryonic cardiac myocytes. Also, FGFR-1 exists as both ‘long’ and ‘short’ isoforms, and there is a switch from predominant expression of the ‘long’ isoform in the embryo to the ‘short’ isoform in the adult heart. Both the decrease in receptor levels and the isoform switch in postnatal cardiac myocytes correlate with a loss of proliferative potential. We investigated whether an increase in either ‘long’ or ‘short’ FGFR-1 isoforms could stimulate proliferation in postnatal rat cardiac myocyte cultures. Methods and Results: Previously we cloned cDNAs corresponding to ‘long’ (L) and ‘short’ (S) FGFR-1 isoforms from embryonic mouse hearts. Hybrid FGFR-1(L) and (S) genes, directed by a myosin light chain-2 promoter and SV40 enhancer sequences, were generated and used to transiently transfect neonatal rat cardiac myocytes. Overexpression of FGFR-1 mRNA and protein was detected by RNA blotting and immunocytochemistry. Ligand-crosslinking confirmed the presence of specific receptors capable of binding FGF-2 on the cell membrane. Overexpression of either FGFR-1(L) or (S) was associated with stimulation of proliferation as assessed by significant increases in bromodeoxyuridine uptake (DNA synthesis) and cell number. To determine whether this response was FGF-2 specific, the level of FGF-2 was assessed in the culture medium of cardiac myocytes overexpressing FGFR-1 isoforms. A three-fold increase was detected in the media of cardiac myocytes overexpressing either FGFR-1(L) or (S) compared to control levels. Neutralization of this FGF-2 with antibodies inhibited the proliferative response. Conclusion: Overexpression of either FGFR-1(L) or (S) resulted in an increase in FGF-2-mediated proliferation of postnatal rat cardiac myocytes. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Fibroblast growth factor-2 (FGF-2) is a multifunctional protein that plays a vital role in regulating growth and differentiation of various cell types including cardiac myocytes. FGF-2 exerts many of its effects by binding to specific high affinity cell surface receptors (FGFR) of the tyrosine kinase family [1,2]. The FGFR family includes four members (FGFR-1, 2, 3 and 4) [3–10], however, FGFR-1 is the only high affinity receptor detected so far in the rat heart [5,8,10]. FGFR-1 consists of three domains: a ligand binding domain containing immunoglobulin-like (Ig-like) loops I–III, a membrane-spanning domain, and a functional tyrosine kinase domain. There are two prevalent FGFR-1 isoforms, ‘long’ and ‘short’, which occur through alternative splicing of RNA corresponding to a region of the ligand binding domain [9,11]. The ‘long’ and ‘short’ isoforms contain three (I–III) and two (II and III) Ig-like loops, respectively. It has been suggested that binding to low affinity binding sites on the cell surface, consisting of heparin sulfate proteoglycans (HSPG), plays a vital func-
tion in the binding of FGF-2 to its high affinity receptor [12,13]. However, some evidence indicates that FGF can signal via the HSPGs independently of the FGFR [14].

FGFR-1 plays an essential role in the development of the heart [15,16]. Studies show that a mutant form of FGFR-1 in Drosophila (heartless) was unable to induce formation of cardiac muscle from mesoderm [17]. Also, knockout of FGFR-1 function during early avian development results in inhibition of cardiac muscle growth and improper looping of the heart [18]. In the developing heart, embryonic cardiac myocytes are highly proliferative, however, postnatal maturation results in a loss of their proliferative potential and they enter a state of, presumably, terminal differentiation in the adult heart [19]. RNA analysis of embryonic versus postnatal hearts reveals a significant decrease in FGFR-1 levels, as well as a switch in the relative levels of the ‘long’ versus ‘short’ isoform [9,10,20]. The ‘long’ and ‘short’ FGFR-1 mRNAs predominate in the embryonic and adult heart, respectively. Both the decrease in FGFR-1 levels and the isoform switching correlate with the transition of cardiac myocytes from a hyperplastic to a hypertrophic and non-proliferative phenotype. Thus, we investigated the possibility that an increase in FGFR-1 levels, and or changing the relative levels of the ‘long’ versus ‘short’ isoform, might increase the proliferative potential of postnatal cardiac myocytes.

Previously, we cloned FGFR-1 cDNAs from embryonic mouse heart corresponding to both ‘long’ and ‘short’ isoforms [9]. Here we report the effect of FGFR-1 isoform expression, directed by a myosin light chain-2 promoter and SV40 enhancer sequences, on DNA synthesis and cell number in transiently transfected neonatal rat ventricular cardiac myocytes. We show that both ‘long’ and ‘short’ FGFR-1 cDNAs are expressed in cardiac myocytes, resulting in increased specific binding of FGF-2 on the cell surface, and stimulation of proliferation in a ligand-dependent manner.

2. Methods

2.1. Cell culture

Postnatal ventricular cardiac myocytes were isolated from 1–2 day old Sprague Dawley rats by enzymatic digestion with 0.1% (wt/vol) trypsin (Sigma, St. Louis, MO) using a temperature regulated (35°C) spinner flask, followed by fractionation on a Percoll gradient [21]. Cells were counted using a hemocytometer and plated on collagen-coated dishes in Ham’s F10 medium containing 10% (v/v) fetal bovine serum (FBS), 10% (v/v) horse serum, 140 mg/ml (w/v) calcium chloride and antibiotics. Plates were coated using 0.1% (w/v) type I collagen stock solution (UBI, Lake Placid, NY). The investigation conforms with the current Guide To The Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

2.2. Cell transfections

For gene transfer, cardiac myocytes were plated at 1.3×10⁶ cells per 60-mm dish or 0.7–0.9×10⁶ cells per 35-mm dish and were maintained in serum containing F12-DMEM nutrient mixture. Transient transfection of cardiac myocytes was done using the calcium phosphate/DNA precipitation method [22]. Briefly, cells were transfected 24 h after plating with 10 μg of test plasmid DNA. After 24 h, cells were refed with growth medium and maintained for a further 48 to 72 h before processing. Hybrid genes for transfection included: full-length cDNAs for both long and short mouse FGFR-1 fused to a cardiac specific myosin light chain-2 (MLC-2) promoter with simian virus 40 (SV40) enhancer sequences to generate SVenhMLCp.FGFR(L) and SVenhMLCp.FGFR(S), respectively, as previously described [23]. The expression vector containing the promoter, but with the fire fly luciferase gene instead of any FGFR-1 sequences was used as a control (SVenhMLCp.luc). Generation and characterization of these hybrid genes were described previously [23,24].

Transfection efficiency was assessed using the β-galactosidase assay [25]. Cardiac myocytes, co-transfected with RSVβgal as described above, were rinsed with calcium and magnesium-free PBS, fixed (1.8% formaldehyde, 0.2% glutaraldehyde, 0.002 mol/l magnesium chloride, 50 mM sodium phosphate, pH 7.4) and incubated in an X-gal solution containing 1 mM magnesium chloride, 0.0033 mol/l potassium ferrocyanide, 0.0033 mol/l potassium ferricyanide, 0.15 mol/l sodium chloride, 0.01 mol/l sodium phosphate buffer pH 7.4 and 0.2% (w/v) X-gal for 18 h at 37°C. For quantitative analysis, about 12 000 cardiac myocytes were assessed by counting from 15 randomly selected fields on three separate coverslips, representing four independent transfection experiments. The percentage of X-gal stained cells was determined.

2.3. RNA blotting

Total RNA was isolated from transfected cultures which had been maintained for 48 h in 10% FBS F12-DMEM, using the Trizol-extraction method (Gibco-BRL, Burlington, ON). Total RNA was denatured with formaldehyde and resolved by electrophoresis through a 1.0% agarose gel [26]. The RNA was blotted to nitrocellulose, probed with either radiolabelled mouse FGFR-1 (Xba I/Sal I) or rat FGF-2 (Sma I/Xho I) cDNA fragments and assessed by autoradiography as previously described [9]. The FGFR-1 cDNA fragment represents a region common to both long and short FGFR-1 transcripts [20], and the rat FGF-2 fragment corresponds to the full length FGF-2 cDNA deleted of those sequences peculiar to the high molecular
weight isoforms [27]. Hybridization with the GAPDH cDNA (Pst I/Bgl I) was used to assess loading and standardise FGFR-1 and FGF-2 mRNA levels. Relative messenger RNA levels were assessed from autoradiographs by scanning densitometry.

2.4. Immunofluorescence microscopy

Cardiac myocytes were fixed 72 h after transfection, using 1% paraformaldehyde for 15 min at 4°C. Coverslips were first incubated with affinity purified rabbit FGFR-1 (flg) antibodies (1:200; Santa-Cruz Biotechnology, Santa Cruz, CA) or nonimmune serum at the same dilution in 1% (w/v) BSA in PBS for 16 h at 4°C, then with biotinylated anti-rabbit immunoglobulins (Ig, 1:20; Amersham, Arlington Heights, IL) for 1 h at room temperature, followed by incubation with fluorescein conjugated to streptavidin (Strep-FITC, 1:20, Amersham) for 1 h at room temperature. The rabbit FGFR-1 antibodies were raised against the carboxyl terminal residues 802–822 of the human flg receptor and, thus, detect both the ‘long’ and ‘short’ FGFR-1 isoforms. These antibodies are highly specific and do not crossreact with FGF-2, FGFR-3 and FGFR-4 [28]. Labelling for myosin, to identify myocytes, was performed using monoclonal antibodies specific for striated muscle myosin (1:50, MF20) in 1% BSA in PBS followed by visualization with Texas Red conjugated anti-mouse Ig (1:20, Amersham). The MF20 hybridoma was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. When identification of nuclei was necessary, cellular DNA was stained with 0.0125% Hoechst dye 33342 (Calbiochem-Behring, San Diego, CA). Coverslips were mounted using mounting media for fluorescence (Vector Vectashield; Burlingame, CA), examined and photographed with a Nikon Diaphot microscope equipped with epifluorescence optics.

2.5. FGFR-1 crosslinking

For FGFR-1 crosslinking studies, rat ventricular cardiac myocytes were plated on collagen-coated dishes, transfectioned for 24 h with either SVenhMLCP.FGFR(L) or SVenhMLCP.FGFR(S) as well as control DNA, maintained for 48 h, and incubated with 125I-FGF-2 (2.5 ng/sample; Dupont Canada Inc., Mississauga, ON) for 90 min at 4°C in the absence or presence of 0.1 μg unlabelled FGF-2 (Upstate Biotechnology, Lake Placid, NY). The crosslinking reaction was initiated by adding 0.15 mol/l disuccinimidyl suberate (DSS; Pierce Chemical, Rockford, IL) to transfected cultures for 15 min at room temperature. The reaction was terminated by adding 10 μl of 0.5 mol/l Tris–hydrochloric acid, pH 7.4. Cells were subsequently scraped off, followed by centrifugation (10 min, 10 000×g) and resuspension of the pellet in 30 μl of homogenizing buffer consisting of 0.02 mol/l HEPES pH 7.4, 0.25 mol/l sucrose, 0.001 mol/l ethylenediaminetetraacetic acid, 1 mg/ml aprotinin, 1 mg/ml leupeptin and 1% Triton X-100. Insoluble residue was removed by centrifugation (10 min, 16 000×g) and the supernatant analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in a 7.5% gel, and visualized by autoradiography. Levels of labelled FGF-2 complexes were assessed from autoradiograms by scanning densitometry.

2.6. FGF-2 assay

Conditioned media was collected after 48 h from plates containing cardiac myocytes following transfection with SVenhMLCP.luc (control), SVenhMLCP.FGFR(L) and SVenhMLCP.FGFR(S). Quantitative determination of FGF-2 in media was assessed using a Quantikine HS Human FGF basic Immunoassay (R & D Systems, Minneapolis, MN), as directed by the manufacturer’s instructions. Cardiac myocytes transfected with the control gene were used to determine basal levels of FGF-2 48 h after transfection.

2.7. Bromodeoxyuridine (BrdU) labelling

Rat ventricular cardiac myocytes were plated onto collagen-coated dishes (containing three coverslips per 60-mm dish or one coverslip per 35-mm dish), transfectioned for 24 h, maintained for a further 24 h and then incubated in the presence of 3 mg/ml (w/v) BrdU (Sigma, St.Louis, MO) for a further 24 h in the presence of 10% FBS-F12-DMEM. To assess for the specific effect of FGF-2 via cell surface receptors, cardiac myocyte cultures were exposed to neutralizing antibodies to FGF-2 using conditions previously described [22]. Briefly, myocytes were maintained for 24 h in either the presence of (i) normal mouse Ig (10 μg/ml; Sigma, St.Louis, MO); or (ii) anti-bovine FGF-2, type I, monoclonal neutralizing antibodies (10 μg/ml; Upstate Biotechnology), followed by incubation in the presence of 3 mg/ml (w/v) BrdU. Myocyte cultures were subsequently fixed with 1% paraformaldehyde for 15 min at 4°C and then with 70% ethanol for 30 min at room temperature, followed by treatment with 70 mmol/l sodium hydroxide for 2 min at room temperature.

Simultaneous labelling for myosin (to identify myocytes) and/or BrdU in ventricular myocytes was done using monoclonal antibodies against striated myosin (1:50, MF20) and BrdU (1:2, Amersham; or 1:7, Becton Dickinson, Cockeysville, MD) in 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS). Both myosin and BrdU were visualized with Texas red-conjugated anti-mouse Ig (1:20; Amersham). For quantitative analysis, approximately 2000 cardiac myocytes were assessed from cultures transfected...
with SVenhMLCp.luc, SVenhMLCp.FGFR(L) or SVenhMLCp.FGFR(S) genes (total ~6000), from 15–20 randomly selected fields on three separate coverslips, representing three or four independent transfection experiments. For DNA synthesis, a BrdU labelling index (LI) was obtained by expressing the number of cardiac myocyte nuclei staining positively for BrdU as a percentage of the total number (~2000 per construct) of cardiac myocyte nuclei assessed.

2.8. Cell number

Cardiac myocytes were plated and transfected as described for the `Bromodeoxyuridine Labelling' experiments above. After 48 h, myocytes were rinsed with PBS, and fixed with 1% paraformaldehyde for 15 min at 4°C. Cardiac myocytes were labelled for myosin using monoclonal antibodies against striated myosin (1:50, MF20) and visualized with Texas red-conjugated anti-mouse Ig (1:20; Amersham). Cell number was assessed by counting the number of myocytes from 15 randomly selected fields on at least three coverslips representing two independent experiments. Cell number was expressed as the fold difference relative to control, which was arbitrarily set to 1.0.

2.9. Statistical analysis

Data presented in the text and figures are represented as mean plus or minus standard error from the mean from at least two independent experiments each done in triplicate, unless stated otherwise. Statistical analysis of the results was done using the non-parametric Mann–Whitney test and ANOVA with Dunn’s multiple-comparison post hoc test. Results were considered significant if \( P \) was determined to be <0.05.

3. Results

3.1. FGFR-1 mRNA levels are increased in neonatal cardiac myocytes transiently transfected with FGFR-1(L) and (S) cDNAs.

Neonatal (1–2 days) rat ventricular myocyte cultures were used to test the expression of the FGFR-1 cDNA constructs coding for either 'long' (L) or 'short' (S) isoforms after gene transfer and RNA blotting. Transfection efficiency for the myocyte cultures was determined initially by expression and detection of \( \beta \)-galactosidase following co-transfection with RSVp.\( \beta \)-gal. Staining was detected in 9.2±0.3% (\( n=4 \)) cells of transfected cultures. As a negative control, ventricular cardiac myocytes were transfected with a hybrid gene using the same promoter used to direct the FGFR-1 cDNAs, but fused to the firefly luciferase gene. Total RNA was isolated from cardiac myocytes 48 h after transfection with SVenhMLCp.FGFR(L) and SVenhMLCp.FGFR(S), as well as SVenhMLCp.luc, as a negative control. Samples were transferred to nitrocellulose and probed for FGFR-1 using a fragment of the cDNA capable of detecting both FGFR-1(L) and FGFR-1(S) transcripts (Fig. 1A). Subsequently, the blot was reprobed with GAPDH to allow a standardization of RNA levels after densitometry of autoradiographs (Fig. 1B). A transcript of about 4.3 kb, reflecting endogenous FGFR-1 mRNA and consistent with the expected size for FGFR-1(L) \([9,20]\), was detected in the control lane using the FGFR-1 probe (lane a). Transfection with the FGFR-1(L) cDNA resulted in about a ten-fold increase in the intensity of the 4.3-kb transcript (lane b). In contrast to the low level of endogenous FGFR-1(L) RNA observed, no endogenous FGFR-1(S) RNA was detected. As a result, transfection with the FGFR-1(S) cDNA resulted in the induction of a 4.1 kb transcript consistent with the predicted size of FGFR-1(S) RNA (lane c). This estimate was based on the size
determined for FGFR-1(L) and subtraction of the 267 bp associated with the first Ig loop-like domain [9,20]. Minor bands were also detected at 4.3 and 4.0 kb, which likely reflect endogenous FGFR-1(L) RNA and an alternative FGFR-1(S) transcript, respectively. A further minor transcript of 1.6 kb was also detected that was common to cardiac myocytes transfected with either FGFR-1(L) or (S) cDNAs (lanes b and c). This transcript initiates, presumably, from an internal start site downstream of the first Ig loop-like domain, since this loop sequence would be absent from the FGFR-1(S) cDNA [9]. It is possible that this transcript is also generated from the endogenous FGFR-1 gene, but is not detected because of the relatively lower levels of expression.

3.2. Subcellular localization of FGFR-1 isofoms in neonatal cardiac myocytes

Immunofluorescence microscopy was used to visualize FGFR-1 protein in neonatal rat cardiac myocytes transfected with control plasmid (SVenhMLCp.luc) as well as FGFR-1(L) and (S) cDNAs (Fig. 2). Cells were double-labelled for myosin and FGFR-1 to specifically identify myocytes expressing FGFR-1. The endogenous pattern of FGFR-1 localization was assessed in cultures transfected with control plasmid. The overall FGFR-1 specific staining was relatively weak. FGFR-1 was localized to the perinuclear region and a speckled pattern of staining was present in some nuclei. In cultures transfected with FGFR-1 (L) or (S) cDNAs (but not control), intense and specific staining for FGFR-1 was observed. This was considered to represent localization of FGFR-1 in overexpressing cardiac myocytes. The majority of these overexpressing cells displayed strong perinuclear staining and or ‘particles’ staining intensely for FGFR-1 extended out along the length of the myocyte (Fig. 2).

3.3. Overexpression of FGFR-1 isofoms increases specific binding of 125I-FGF-2 on neonatal cardiac myocyte membranes

Crosslinking and SDS–PAGE were used to determine whether transfection of neonatal rat cardiac myocytes with FGFR-1 cDNAs increased specific FGF-2 binding on the cell membrane, and, thus, the presence of receptors capable of binding FGF-2. Cardiac myocytes were transfected with expression plasmids containing FGFR-1 cDNAs or control DNA. Following transfection, cardiac myocytes were maintained for 48 h and incubated for 90 min with iodinated FGF-2 in the absence or presence of greater than 40-fold molar excess of non-radiolabelled FGF-2. Membranes were isolated after crosslinking with DSS and analyzed by SDS–PAGE and autoradiography (Fig. 3). In the absence of ‘cold’ FGF-2, samples from cardiac myocytes transfected with control DNA (lane a) or FGFR-1(S) (lanes c–e) and FGFR-1(L) cDNAs (lanes g–i) revealed crosslinked products of about 35, 50 and 155 kDa forming on the membrane. A further band of 135 kDa was only present in the sample resulting from FGFR-1(S) cDNA expression, and gave rise to a four-fold (n = 2, 6 determinations) increase in overall receptor (135+155 kDa) levels (lane c). Overexpression of the FGFR-1(L) cDNA resulted in a seven-fold (n = 2, 6 determinations) increase in the intensity of the 155-kDa sized band compared to control. Both the 155-kDa band in the control and FGFR-1(L) related samples as well as the 135-kDa band in the FGFR-1(S) related samples were competed effectively in the presence of nonradiolabelled FGF-2 (lanes b,f,j). The 155 and 135-kDa values for the FGF-2/FGFR-1(L) and FGF-2/FGFR-1(S) complexes would be consistent with receptor sizes of about 137 and 117 kDa, respectively, after subtracting 18 kDa for FGF-2. The bands with mobilities corresponding to proteins of about 35 and 50 kDa likely reflect crosslinked multimers of radiolabelled FGF-2 as described previously [10].

3.4. DNA synthesis and cell number are increased significantly in neonatal cardiac myocyte cultures transfected with FGFR-1 cDNAs

The effect of FGFR-1 isoform overexpression on DNA synthesis and cell number were assessed as markers of cardiac myocyte proliferation in cultures maintained in the presence of 10% FBS–DMEM. Immunofluorescence staining with monoclonal antibodies to myosin and BrdU were used to determine a BrdU labelling index (LI), or the proportion of myocytes undergoing DNA synthesis (S-phase nuclei) in cultures transfected with FGFR-1 cDNAs or a control gene. Anti-BrdU staining was confined to the nucleus, whereas anti-myosin staining was exclusively cytoplasmic in the rat cardiac myocytes. The results are shown in Fig. 4A and are presented as the proportion of myocytes staining for BrdU and, thus, showing evidence of DNA synthesis. There was a significant ~three-fold increase in the number of myocyte nuclei staining for BrdU and, thus, showing evidence of DNA synthesis. There was a significant ~three-fold increase in the number of myocyte nuclei staining for BrdU in cultures transfected with SVenhMLCp.FGFR-1(S) (2.6 fold) and SVenhMLCp.FGFR-1(L) (2.8 fold) compared with cells transfected with control plasmid (P<0.001, n=4). There was no significant difference between the results obtained following overexpression of the ‘short’ versus ‘long’ FGFR-1 isofoms.

Myosin positive cells were also scored in 15 random fields per coverslip (n=3) to determine whether the increase in BrdU staining (Fig. 4A) also reflected an increase in cardiac myocyte cell number (Fig. 4B). Myocyte number was significantly increased (~1.7 fold) in cultures transfected with SVenhMLCp.FGFR-1(S) or SVenhMLCp.FGFR-1(L) compared with cells transfected with control SVenhMLCp.luc plasmid (P<0.001, n=3) (Fig. 5B). Myocyte number was not significantly different in cultures overexpressing FGFR-1(S) versus FGFR-1(L) (Fig. 4B).
Fig. 2. Distribution of endogenous FGFR-1 in neonatal rat cardiac myocytes. Cardiac myocyte cultures transfected with control SVenhMLCp.lac plasmid, SVenhMLCp.FGFR-1(S) or SVenhMLCp.FGFR-1(L) cDNA, were stained with FGFR-1 antibodies (a, c, e, i), and co-stained for striated muscle myosin to confirm the identity of myocytes (b, d, f, j). Low levels of endogenous FGFR-1 staining, including the perinuclear region, was seen (a). Cardiac myocytes overexpressing either ‘short’ (c, i) or ‘long’ (e) FGFR-1 show strong perinuclear staining with FGFR-1 antibodies and or an accumulation of particles staining intensely for FGFR-1 around the nucleus and throughout the cytoplasm. The pattern observed with transfected cultures stained with non immune serum is shown for comparison (g). Identification of myocytes was again confirmed by co-staining with an antibody to striated muscle myosin (h). Bar is equivalent to 25 μm in a–h, and to 10 μm in i and j.
3.5. FGF-2 levels are higher in conditioned media from cardiac myocytes overexpressing FGFR-1 cDNAs versus control DNA

A highly sensitive immunoassay specific for quantitating FGF-2 levels (Quantikine HS Human FGF basic Immunoassay) was used to detect FGF-2 in samples of conditioned media from myocyte cultures transfected with FGFR-1(S), FGFR-1(L) cDNAs or control DNA after 48 h. A standard curve for FGF-2 levels in the conditioned media was established using human FGF-2 (0–32 pg/ml). The levels of FGF-2 in the conditioned medium of cultures transfected with SVenhMLCp.FGFR-1(S), SVenhMLCp.FGFR-1(L) or SVenhMLCp.FGFR-1(L) were determined and the results are expressed as mean values (pg/ml) from at least nine determinations (Fig. 5A). A level of 0.12 pg/ml was observed in cultures transfected with control (SVenhMLCp.) plasmid DNA. However, the levels of FGF-2 in the conditioned medium of cultures overexpressing FGFR-1(S) or FGFR-1(L) were 3.2 and 2.9 fold higher, respectively ($P<0.05$, $n=9$) (Fig. 5A). Subsequently, RNA was isolated from these cultures, and assessed for FGF-2 expression by RNA blotting (Fig. 5B). The blot was also probed for GAPDH to allow standardisation of FGF-2 mRNA levels following densitometry of resulting autoradiographs. A 6.0-kb FGF-2 transcript was detected in all samples. After correction for RNA loading, the levels of FGF-2 mRNA were not different between cultures transfected with control, FGFR-1(L) or (S) DNA.

3.6. The increase in DNA synthesis with FGFR-1 overexpression is blocked with neutralising antibodies to FGF-2

We reexamined the BrdU LI in the presence of neutralising antibodies to FGF-2 to determine whether the increased proliferation seen in FGFR-1 overexpressing cultures was FGF-2 dependent (Fig. 6). Cultures were incubated with normal mouse serum (NMS) as a control for the mouse anti-FGF-2 serum. A significant two to three fold increase in BrdU LI was seen with both FGFR-1(S) and (L) transfected versus control cells in the presence of
Fig. 6. The increase in DNA synthesis seen following overexpression of FGFR-1 isoforms in neonatal cardiac myocytes is blocked by neutralizing antibodies to FGF-2. The BrdU LI for cardiac myocytes was determined for cultures transfected with SVenhMLCp.luc, SVenhMLCp.FGFR-1(S) or SVenhMLCp.FGFR-1(L), and maintained in the presence of normal mouse Ig (IgG) or FGF-2 antibodies ($n=3$). A significant increase in BrdU LI was observed in the presence of mouse IgG with either FGFR-1(S) or (L) overexpression ($P<0.001$). However, no significant stimulation was detected in the presence of neutralizing antibodies to FGF-2. Bars represent standard error of the mean.

Fig. 5. Effect of FGFR-1 isoform overexpression on FGF-2 levels in the conditioned medium of neonatal cardiac myocyte cultures. (A) Cardiac myocyte cultures transfected with SVenhMLCp.luc, SVenhMLCp.FGFR-1(S) or SVenhMLCp.FGFR-1(L) were maintained for 48 h before FGF-2 levels in the culture medium were determined. The results are expressed as the mean FGF-2 level plus or minus standard error of the mean ($n=5$). Levels of FGF-2 were significantly higher in the medium of cardiac myocyte cultures overexpressing FGFR-1 versus control DNA. (B) Subsequently, RNA was isolated from each transfected cardiac myocyte culture, electrophoresed, transferred to nitrocellulose and probed sequentially with radiolabelled FGF-2 and GAPDH. Hybridized bands were visualised by autoradiography. No increase in FGF-2 mRNA levels was detected in cultures overexpressing FGFR-1 versus control DNA.

mouse Ig ($P<0.001$, $n=3$). The presence of neutralizing antibodies against FGF-2 (10 μg/ml) blocked this increase so that there was no significant difference between the BrdU LI for cardiac myocytes transfected with FGFR-1 cDNAs versus control DNA ($P>0.05$, $n=3$). Regardless of the presence or absence of antibodies, no significant difference was detected between the effects observed following FGFR-1(S) versus FGFR-1(L) overexpression (Fig. 6).

4. Discussion

With a view to increasing proliferative potential of postnatal cardiac myocytes, we generated expression vectors containing the myosin light chain-2 promoter and SV40 enhancer sequences to overexpress the ‘long’ and ‘short’ FGFR-1 isoforms in neonatal rat cardiac myocytes. Expression of FGFR-1(L) and (S) cDNAs in neonatal rat cardiac myocyte cultures was demonstrated by RNA blotting, through an increase in the levels of the 4.3 and 4.1 kb FGFR-1 transcripts, respectively (Fig. 1). FGFR-1 overexpression in cardiac myocytes was confirmed by immunohistochemistry and co-staining for FGFR-1 and striated muscle myosin (Fig. 2). The FGFR-1 antibodies were raised to the intracellular kinase domain and, thus, recognize an epitope common to both FGFR-1(L) and FGFR-1(S). Finally, as evidence of an increase in FGFR-1 levels, the presence of increased levels of specific plasma membrane FGF-2 binding sites was detected in cultures overexpressing either FGFR-1 isoforms (Fig. 3).

A significant, 2.6–2.8 fold increase in DNA synthesis and 1.6–1.8 fold increase in overall cell number (Fig. 4A and B) confirmed stimulation of postnatal cardiac myocyte proliferation in cultures overexpressing FGFR-1(L) and (S). These increases are quite high and disproportionate to the fraction of myocytes (~10%, based on β-galactosidase), expected to be stimulated to divide due to FGFR-1 overexpression, and suggest that all myocytes (not only the overexpressing fraction) may have been subjected to increased mitogenic stimulation. Indeed, overexpression of either FGFR-1 (L) or (S) resulted in a three-fold increase...
in FGF-2 present in the culture medium (Fig. 5A), thus raising the possibility that increased levels of FGF-2 contribute to the overall cardiac myocyte proliferation observed. Exogenous addition of FGF-2 to the culture medium has been previously shown to increase neonatal cardiac myocyte proliferation, to a degree similar to the stimulation observed in our study [29]. Furthermore, the stimulatory effect of FGFR-1 overexpression was fully blocked by neutralising antibodies to FGF-2 (Fig. 6), indicating that the increase in overall myocyte proliferation is FGF-2 dependent and similar for the ‘long’ and ‘short’ FGFR-1 isoforms. The mechanism resulting in increased FGF-2 levels in the medium of FGFR-1 overexpressing myocytes is not as yet known but it does not appear to include stimulation of FGF-2 mRNA expression (Fig. 5B).

Signalling by FGF-2 and FGFR-1 has two major components. The first mode is dependent on ligand–plasma membrane receptor interaction in the extracellular space. The receptor then dimerises, autophosphorylates, and can activate downstream signalling cascades, including the activation of MAPK which has been associated with stimulation of proliferation by FGF-2 [30]. The inhibition of the stimulatory effect of ‘long’ or ‘short’ receptor overexpression on cardiac myocyte proliferation by neutralising antibodies to FGF-2 (Fig. 6) indicate that a ligand-dependent triggering mechanism is operating in our system. Consistent with this observation, no differences have been reported between the intracellular signalling elicited by FGF-2 binding to the ‘long’ versus ‘short’ FGFR-1 isoform. The affinity of FGF-2 for the ‘long’ (50–150 pM) or ‘short’ (100 pM) FGFR-1 is comparable [11]. Although more controversial, this also appears to be the case for FGF-1 and the ‘long’ (20–80 pM) or ‘short’ (50–200 pM) receptor isoform [11,31].

The second mode of signalling appears to involve direct intracellular action of FGF-2 and/or FGFR-1. We showed previously that nuclear localisation of the CUG, but not the AUG-initiated form of FGF-2 exerts specific, and apparently receptor- and/or proliferation-independent, effects on cardiac myocytes [22]. Using antibodies to FGFR-1, staining of the perinuclear region and cytoplasmic ‘particles’ was observed in overexpressing cardiac myocytes (Fig. 2). This distribution of FGFR-1 was similar for the two receptor isoforms, and was consistent with overexpression of FGFR-1 and its presence in the cytoplasm at, presumably, different stages of synthesis and processing. However, mobilization and accumulation of FGFR-1 to a region surrounding the nucleus has been described as nuclear trafficking in several systems, although not cardiac myocytes [32–36]. A translocation of FGFR-1, with its ligand, to the nucleus during the G1 phase of the cell cycle [32] has been associated with a transition from a quiescent to a proliferative cellular state [36]. Thus, the presence of FGFR-1 and its ligand at the nucleus could indicate an increase in proliferative potential, and play a role in signalling this process.

It has been suggested that expression of ‘long’ FGFR-1 isoforms correlates with restriction of cell growth, malignancy and enhanced differentiated function [34]. However, this is not supported by the pattern of FGFR-1 mRNA expression in the developing heart. Previously, we used reverse transcriptase–polymerase chain reaction to assess the relative levels of FGFR-1(S) versus FGFR-1(L) in embryonic (dividing) and adult (non dividing) mouse cardiac myocytes [9]. There appeared to be a switch in the pattern of FGFR-1 expression during development as the ‘long’ and ‘short’ RNAs represented the major transcript detected in embryonic and adult cells, respectively. Thus, there was a correlation between predominantly FGFR-1(L) expression and a proliferative cardiac myocyte phenotype. The presence of endogenous FGFR-1(L) transcript in neonatal rat cardiac myocytes (Fig. 1) would be consistent with this idea, reflecting a limited proliferative capacity at this stage. Although, transfection with the FGFR-1(L) cDNA increased cardiac myocyte growth, no significant difference was detected between the levels of stimulation of proliferation seen with FGFR-1(L) versus FGFR-1(S) overexpression (Fig. 4). Thus, while we have shown that an increase in either ‘long’ or ‘short’ FGFR-1 levels can stimulate postnatal cardiac myocyte proliferation in an FGF-2-dependent manner, there is no evidence that either of the two receptor isoforms stimulated these effects preferentially. The significance, therefore, of the switch to the ‘short’ isoform during cardiac development remains to be established.

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