Inhibition of E2F Abrogates the Development of Cardiac Myocyte Hypertrophy*

Received for publication, December 11, 2002, and in revised form, March 18, 2003
Published, JBC Papers in Press, April 6, 2003, DOI 10.1074/jbc.M212612200

Dharmesh Vara, Katrina A. Bicknell, Carmen H. Coxon, and Gavin Brooks‡

From the Cardiovascular Research Group, School of Animal and Microbial Sciences, The University of Reading, Reading, Berkshire RG6 6AJ, United Kingdom

Growth of the post-natal mammalian heart occurs primarily by cardiac myocyte hypertrophy. Previously, we and others have shown that a partial re-activation of the cell cycle machinery occurs in myocytes undergoing hypertrophy such that cells progress through the G1/S transition. In this study, we have examined the regulation of the E2F family of transcription factors that are crucial for the G1/S phase transition during normal cardiac development and the development of myocyte hypertrophy in the rat. Thus, mRNA and protein levels of E2F-1, 3, and 4 and DP-1 and DP-2 were down-regulated during development to undetectable levels in adult myocytes. Interestingly, E2F-5 protein levels were substantially up-regulated during development. In contrast, an induction of E2F-1, 3, and 4 and the DP-1 protein was observed in the development of myocyte hypertrophy in neonatal myocytes treated with serum or phenylephrine, whereas the protein levels of E2F-5 were decreased with serum stimulation. E2F activity, as measured by a cyclin E promoter luciferase assay and E2F-DNA binding activity, increased significantly during the development of hypertrophy with serum and phenylephrine compared with non-stimulated cells. Inhibiting E2F activity with a specific peptide that blocks E2F-DP heterodimerization prevented the induction of hypertrophic markers (atrial natriuretic factor and brain natriuretic peptide) in response to serum and hypertrophic stimuli. Thus, we have shown that the inhibition of E2F function prevents the development of hypertrophy. Targeting E2F function might be a useful approach for treating diseases that cause pathophysiological hypertrophic growth.

Shortly after birth, the majority of rat cardiac myocytes lose the ability to undergo cell division such that post-natal growth of the heart occurs primarily by myocyte hypertrophy (1, 2). This adaptive physiological growth response enables the heart to grow to an adequate size in order to maintain sufficient cardiac output. However, myocardial injury, e.g. myocardial infarction and/or prolonged periods of chronic stress on the heart (e.g. hypertension), leads to excessive hypertrophic growth, commonly referred to as “decompensated hypertrophy,” which adversely affects cardiac function and accelerates heart failure (3). Clinically, there is a need to develop new therapeutic entities that target specific molecules involved in this detrimental growth, which will ultimately improve the prognosis for patients.

Previously, we have reported that a partial re-activation of specific components of the cell cycle machinery occurs during the development of cardiac hypertrophy, leading to transition of myocytes through the G1/S cell cycle checkpoint (2, 4). E2F is a family of transcription factors that are known to play a pivotal role in the G1/S phase transition of the cell cycle (5, 6); however, very little is known about the potential role(s) of these transcription factors in cardiac hypertrophy.

Structurally and functionally, the E2F transcription factors can be divided into three main categories as follows: (i) E2Fs 1–3, which are involved in proliferation; (ii) E2F-4 and E2F-5 that are thought to play a role in differentiation; and (iii) E2F-6, which is described as a transcriptional repressor (7). For full transcriptional activity, the E2F transcription factors heterodimerize with a DP partner protein, of which two mammalian forms have been described, namely DP-1 and DP-2 (8). The transcriptional activity of E2F-6 members is sterically regulated by the pRb family of pocket proteins that consists of pRb, p107, and p130 (5, 7). E2F activation occurs when cyclin D-cyclin-dependent kinase (CDK) 4/6 complexes in the G1 phase of the cell cycle phosphorylate the pRb pocket proteins, which then dissociate from the E2F-DP complex, resulting in E2F-mediated gene transcription and progression to the S phase of the cell cycle (5, 6). Indeed, various studies have shown that E2F is instrumental in cardiac myocyte cell cycle progression. Thus, Flink et al. (11) showed that a switch occurs from the E2F-p107 complex in proliferating fetal myocytes to E2F-p130 in 2-day-old neonatal cells. Also, von Harsdorff et al. (13) showed that adenoviral delivery of E2F-1 drove a significant number of adult cardiac myocytes into the S phase of the cell cycle (5, 6).

Clinically, E2F has proved to be a viable target in the treatment of certain vasculoproliferative diseases. Thus, Mann et al. (10) showed that blocking E2F-1 function in human vein grafts ex vivo, using a decoy oligonucleotide strategy, reduced the incidence of neointimal hyperplasia and subsequent graft failure. The importance of E2F in the heart was shown recently by Cloud et al. (14), who reported that E2F-3a and E2F-3b null mice die from symptoms of congestive heart failure during development, indicating a crucial role for E2F3 in normal cardiac growth. Although much is known about E2F function in a variety of diseases, currently no study has characterized expression and/or inhibited E2F function during the development of cardiac myocyte hypertrophy.

In the present study, we have determined the expressions of E2F and DP family members at the mRNA and protein levels.

* This work was supported by British Heart Foundation Ph.D. Studentship Grant FS/99029 (to G. B.). 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 Research Group, School of Animal and Microbial Sciences, The University of Reading, P. O. Box 226, Whiteknights, Reading, Berkshire RG6 6AJ, UK. Tel.: 44-118-931-6363; Fax: 44-118-931-6562; E-mail: g.brooks@reading.ac.uk.
in myocytes obtained from the developing rat heart. In addition, we have determined the expressions and activities of these molecules during the development of myocardy hypertrophy in vitro and shown that blocking E2F function with a peptide that abrogates E2F-DP heterodimerization (15) inhibits the development of hypertrophy. Our results suggest that E2F plays an important role in cardiac myocyte growth and that targeting these transcription factors could provide us with an effective therapy for treating detrimental left ventricular hypertrophy (LVH) leading to heart failure.

EXPERIMENTAL PROCEDURES

Myocyte Isolation—Fetal Wistar rat cardiac myocytes were isolated from 70 fetuses at 18 days of gestation (E18), and neonatal rat myocytes were isolated from 30 Wistar pups (2–3 days postnatal (P2)), according to the method described by Poolman and Brooks (1) with minor modifications, including enzymatic digestions for 10 min, preplating for 1 h, and centrifugation and re-suspension of cells in myocyte media (one part M199, four parts Dulbecco’s modified Eagle’s medium, and 5% fetal calf serum (FCS)1 with 100 IU of penicillin/streptomycin) containing 500 µM 5-bromo-3-deoxyuridine (BrdUrd) prior to culture on Primaria plates (BD Bioscience). Myocytes were isolated as described by Brooks et al. (16), except that 5 µg of protease (Sigma) and 50 µg of bovine serum albumin (Sigma) were added to the digestion mixture.

Peptide Studies—E2F blocking and control peptides were used at concentrations of 30 nM in myocyte media as described by Bandara et al. (15). Peptide sequences were as follows: AH2, RQIKIWFQNRRMKWKMKGKKKKKKK; AH2R, RQIKIWFQNRRMKWKMKGKKKKKKK; Ant, RQIKIWFQNRRMKWKK; DRKVKAVRNLIMAYSRMLNI; and AN, RQIKIWFQNRRMKWKK.

Flow Cytometric Analysis—Two-color flow cytometric analysis was performed as described by Poolman and Brooks (1), except that BrdUrd was added directly to myocyte media at a final concentration of 10 µM. Also, pepsin was omitted when myocytes were exposed to 0.1M HCl and cells were incubated for 10 min. Finally, the staining solution was modified by replacing Isoton with phosphate buffered saline.

Isolation of Total RNA, Semi-quantitative, and Quantitative RT-PCR Analyses—Fetal, neonatal, and adult myocytes were homogenized in TRI Reagent (1 ml per 50–100 mg of tissue). Total RNA was then prepared from the sample in accordance with the manufacturer’s instructions (Sigma). RT-PCR was performed on 5 µg of DNase-treated total RNA using AMV Reverse Transcriptase (Promega) and oligo(dT).

PCR analysis was carried out using the following primer sequences: E2F-1, sense 5′-TTCTTAGCTGCTGTAAGCC-3′, antisense 5′-TTGGTATGTCATAAGTCG-3′; E2F-2, sense 5′-ATCCAGTGGGATGCGGC-3′, antisense 5′-TGTCAGACTATGGTGTCCTC-3′; E2F-3, sense 5′-TGGCCACAGCTAGTTTCA-3′, antisense 5′-TGTCAGACTATGGTGTCCTC-3′; E2F-4, sense 5′-TGTGGTACCTCAGCAGTG-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-5, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-6, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-7, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-8, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-9, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-10, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-11, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-12, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-13, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-14, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-15, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-16, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-17, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-18, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-19, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′; E2F-20, sense 5′-TGTCAGACTATGGTGTCCTC-3′, antisense 5′-TGGTCCTACCGGAC-3′.

Semi-quantitative RT-PCR and immunoblot analyses showing expressions of E2F and DP family members during cardiac myocyte development. Total cellular RNA and protein were extracted from fetal (E18), neonatal (P2), and adult (Ad) rat myocytes as described under “Experimental Procedures.” Results are representative of at least three separate experiments using mRNAs (panel a) and proteins (panel b) prepared from separate animals.

Brooks and co-workers (16) except that 4:2-aminoethylbenzenesulfonyl fluoride (AEBSF; 0.3 µM), leupeptin (10 µg/ml), and aprotinin (2 µg/ml) were used as protease inhibitors. Following separation in 12% SDS-polyacrylamide gels, proteins were transferred to nitrocellulose membranes (Amersham Biosciences) before incubation with primary antibodies for 2 h at room temperature. Antibodies (Santa Cruz Bio Technology) directed against either E2F-1 (C-20), E2F-2 (C-20), E2F-3a (N-20), E2F-4 (C-20), E2F-5 (M-15), DP-1 (K-20), or DP-2 (C-20 and N-20) were used at a dilution of 1:1000 in Tris-buffered saline (TBS)/0.1% Tween 20 containing 1% milk powder. Membranes were then incubated in goat anti-rabbit- or goat anti-mouse-heralded peroxi- dase-conjugated antibody (1:4000) for 1 h. Finally, all washes were carried out in TBS/0.1% Tween 20 for 10 min, except the final wash, which was TBS alone.

Luciferase Assays—Neonatal myocytes were plated in 6-well tissue culture dishes at 2.5 × 10^5/well and left overnight in 5% FCS myocyte medium containing 500 µg/l BrdUrd. Cells then were co-transfected overnight with either 0.2 µg of a cyclin E promoter-firefly luciferase construct or control vector (lacking the cyclin E promoter) and 0.01 µg of vector encoding the Renilla luciferase enzyme (Promega) using Effectene transfection reagent (Qiagen). Cells were serum-starved for 24 h and then treated with various hypertrophic agonists for 48 h, after which transfected cells were collected and assayed using a Dual-Luciferase™ assay kit (Promega).

Electromobility Shift Assays (EMSA) —EMSAs were performed according to La Thangue et al. (17), except that poly(dI-dC) was used as the non-specific competitor. The E2F binding sequence used was 5′-TAGTTTGCAGCTTAAATTTTG-3′. Myocyte nuclear extracts (20 µg) were used in each assay, and appropriate competition studies were performed.

Statistical Analysis— Autoradiographs and photographs were scanned and analyzed using the Quantity One® densitometric computer analysis program (Bio-Rad). Data was subjected to one-way analysis of variance (ANOVA), and statistical significance was assessed using the Bonferroni t test. p values of <0.05 were considered to be significant.

RESULTS

Developmental Expression of E2F and DP in Rat Cardiac Myocytes—Fig. 1 shows mRNA (Fig. 1a) and protein (Fig. 1b) expressions of E2F and DP family members during development in fetal (E18), neonatal (P2), and adult rat cardiac myocytes. The mRNA expressions of all E2F family members were down-regulated progressively during normal cardiac myocyte development, although E2F-5 levels remained detectable in adult cells compared with other family members (Fig. 1a). A similar pattern of regulation was demonstrated at the protein level, such that E2F-1, -3a, and -4 and DP-1 and DP-2 were down-regulated progressively in both ventricular tissues (data not shown) and in isolated cardiac myocytes (Fig. 1b). Interestingly, levels of E2F-5 protein were seen to increase during development (Fig. 1b). Protein levels of E2F-2 in both whole ventricular tissues and cardiac myocytes were consistently

1 The abbreviations used are: FCS, fetal calf serum; BrdUrd, bromodeoxyuridine; RT, reverse transcription; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; PE, phenylphosphate; AH2, peptide sequence targeted to the DEF box region of the E2F heterodimerization domain; AHS2, scrambled sequence based upon AH2; AN, penetratin sequence.
Role of E2F in Cardiac Development and Hypertrophy

Fig. 2. Induction of hypertrophy in isolated cardiac myocytes. Panel a shows quantitative PCR analysis of ANF during the development of myocyte hypertrophy using two hypertrophic stimuli, i.e. 20% FCS and 100 μM PE for 24 h. Expression of ANF was normalized using the housekeeping gene GAPDH. Panel b shows the cell cycle profiles of myocytes stimulated to undergo hypertrophy with these hypertrophic stimuli compared with cells maintained in 0% FCS. Panel c contrasts the difference in cell size observed when myocytes were stimulated to undergo hypertrophy with 20% FCS and 100 μM PE for 24 h as compared with control cells maintained in 0% FCS.

Changes in E2F Activity in Cardiac Myocytes during the Development of Hypertrophy—Because our initial studies showed that the E2F and DP proteins are regulated differentially during the development of cardiac myocyte hypertrophy, we next determined whether the transcriptional activities and DNA binding activities of these proteins was similarly altered during this process by using a combination of dual luciferase assays and EMSAs. We measured global E2F activity in myocytes by transfecting a construct containing the firefly luciferase gene under transcriptional control of the mammalian cyclin E promoter into cultured neonatal myocytes. A second con-
struct, containing the Renilla luciferase gene under transcriptional control of the cytomegalovirus (CMV) promoter, was co-transfected into myocytes to provide a baseline luminescence which could be used to normalize E2F activity and provide an overall ratio of E2F-mediated luciferase activity. Fig. 4a shows that E2F activity was doubled when myocytes were stimulated to undergo hypertrophy with FCS, whereas PE increased E2F activity—6-fold compared with levels found in 0% FCS control cells 48 h post-stimulation. We confirmed that global E2F-DNA binding was increased following treatment with hypertrophic agents by performing a series of EMSAs (Fig. 4b) 24 h post-stimulation. The results of these studies confirmed those from the dual luciferase assays and showed an up-regulation in E2F-DNA binding following stimulation of myocytes with 20% FCS or 100 μM PE. To ensure probe specificity in EMSAs, appropriate competition studies were performed with an unlabeled E2F probe and probes designed to detect Sp1 and PPAR-γ (data not shown). Two E2F bands were visualized in EMSAs, namely “free E2F” that describes the E2F-DP heterodimer and “bound E2F” that describes E2F-DP in complex with pocket proteins. A nonspecific band (ns in Fig. 4), which was not competed out in appropriate competition studies, was also observed near the unincorporated nucleotide front.

Blocking E2F Activity Prevents the Induction of Hypertrophy—Because E2F activity is increased during the development of cardiac myocyte hypertrophy (Fig. 4), we next determined whether inhibiting E2F activity would affect this process. E2F-DP heterodimerization is essential for transcriptional E2F activity, and its function can be blocked completely by inhibiting this dimerization. Indeed, a specific peptide sequence named “AH2,” targeted to the DEF box region of the heterodimerization domain of E2F, has been shown previously to inhibit the growth of tumor cells consistent with its inhibition of E2F activity (15). We have used this same peptide sequence to block E2F function in myocytes stimulated to undergo hypertrophy with 20% FCS or 100 μM PE. Peptides were N-terminally linked to the penetratin sequence to aid delivery into intact cells (19). The penetratin sequence (referred to as “ANT”) and a scrambled sequence based upon AH2 (termed AH2s) were included as negative controls for this study because they do not inhibit E2F function. Using a combination of semi-quantitative (Fig. 5a) and quantitative (Fig. 5b) PCR analyses, we determined the effect of blocking E2F function on the development of hypertrophy. Fig. 5a shows that myocytes stimulated to undergo hypertrophy in the absence of any peptide or those exposed to the control peptides, AH2s (30 μM) and ANT (30 μM), showed a significant induction of ANF and BNP, confirming that hypertrophic growth had occurred. However, cells stimulated to undergo hypertrophy with 20% FCS in the presence of AH2 (30 μM) showed a reduction in ANF and BNP levels compared with cells treated with control peptides. This reduction was even more pronounced in PE-stimulated cells wherein AH2 completely abrogated the induction of both ANF and BNP. Quantitative PCR analyses (Fig. 5b) showed that, following serum stimulation, the presence of AH2 inhibited ANF induction where levels were decreased 4-fold when compared with normal serum-stimulated cells and those with se-
rum and control peptides, i.e. AHS2 and ANT. Similar to that seen with the semi-quantitative analyses, ANF induction was greatly reduced in AH2-treated cells (6-fold) when compared with PE-stimulated cells or PE-stimulated cells in the presence or absence of the E2F blocking peptide AH2 (30 μM) or the control peptides AHS2 and ANT (30 μM each). Expressions of ANF and BNP were normalized using the housekeeping gene GAPDH. PE was used at 100 μM throughout.

**Fig. 5.** Blocking E2F-DP heterodimerization prevents the induction of hypertrophy. Panel a shows semi-quantitative and panels b and c show quantitative RT-PCR analyses of ANF and/or BNP expression in myocytes stimulated to undergo hypertrophy in the absence or presence of the E2F blocking peptide AH2 (30 μM) or the control peptides AHS2 and ANT (30 μM each). Expressions of ANF and BNP were normalized using the housekeeping gene GAPDH. PE was used at 100 μM throughout.

**Fig. 6.** Blocking E2F-DP heterodimerization prevents the increase in protein content associated with hypertrophic growth. Myocytes were stimulated to undergo hypertrophy with 20% FCS (panel a) or 100 μM PE (panel b) in the presence or absence of AH2, AHS2, or ANT peptides (30 μM each) compared with control cells (0% FCS-stimulated myocytes). Total protein content was determined by Bradford assay 24 h following treatment. Results show mean ± S.D. obtained from three separate experiments.

that blocking E2F function inhibits the protein synthesis that is known to occur during cardiac myocyte hypertrophy. In addition, AH2-treated myocytes did not show any increase in size 24 h post stimulation with 20% FCS, whereas cells treated with 20% FCS alone or 20% FCS plus AHS2 or ANT peptide approximately doubled in size (data not shown).

**DISCUSSION**

In this study, we have shown for the first time that E2F and DP family members are regulated differentially during normal rat cardiac development and during the development of hypertrophy in cultured, neonatal rat myocytes. It has been shown previously that neonatal rat myocytes exit the cell cycle between days 3 and 4 after birth and accumulate in the G0/G1 phases of the cell cycle (1, 20). Previous work from our laboratory also has shown that certain cell cycle molecules that act upstream of E2F are down-regulated during normal development but are re-activated transiently during adaptive hypertrophic growth (2, 4, 16). Our current data demonstrate that E2F (with the exception of E2F-5) and DP family members are down-regulated in the cardiac myocyte at the mRNA and protein levels from the fetal to adult stages of ventricular development. In contrast, E2F-5 protein expression was increased throughout development. This unique expression pattern for E2F-5 is consistent with a potential role for this cell cycle molecule in myocyte differentiation. Indeed, in neuronal tissue, which also has a terminally differentiated phenotype, E2F-5 similarly maintains significant levels of expression through to adulthood (21). Because E2F-5 mRNA levels were down-regulated progressively during development, E2F-5 expression is likely to be controlled at the translational level in adult cardiac myocytes.

Using an in vitro model of myocyte hypertrophy, we determined the expression patterns of E2F and DP family mem-
bers during this process. Interestingly, a differential expression pattern of E2F and DP members was observed in myocytes undergoing hypertrophic growth such that there was a reversal toward the fetal pattern of protein expressions consistent with the transient up-regulation in specific cyclins and cyclin D-cyclin-dependent kinases during the development of left ventricular hypertrophy (4). E2F-1 expression levels were not changed significantly when myocytes were treated with PE, although a significant up-regulation was seen with FCS stimulation. Previously, E2F-1 has been implicated in cardiac myocyte cell cycle progression because adenoviruses expressing the E2F-1 gene caused a G1/S phase transition (10). However, cells did not progress further than the G2 phase of the cell cycle. The reason for the differential regulation of E2F-1 with serum compared with PE might reflect differences in signaling pathways (a growth factor-mediated pathway for FCS versus the a1-adrenergic receptor for PE) that mediate the hypertrophic response.

Our studies show that E2F-3a and, to a lesser extent E2F-4, are the predominant E2F species up-regulated both 24 and 48 h post stimulation with both hypertrophic agonists, suggesting an important role for these transcription factors in the hypertrophic growth of myocytes. It is possible that, during the development of hypertrophy, cardiac myocytes exhibit a temporal expression pattern of E2F as described by Takahashi et al. for human T98G glioblastoma cells (22). In this study, T98G cells were synchronized into the G0/G1 phase of the cell cycle by serum starvation and then stimulated to traverse the cell cycle by subsequent serum stimulation. The results showed that E2F-1 promoter activity was at its greatest 16 h post-stimulation and then declined, with E2F-3 taking a leading role late in the G1 phase when it bound to promoters for cyclin A, cdc2, cdc6, p107, and E2F-1, all of which are required for S phase progression. E2F-5 activity was not detected in these cells, possibly as a consequence of the fact that they were a proliferating population. It is possible that a similar scenario exists in cardiac myocytes such that E2F-1 could be dominant early on in hypertrophy and eventually become down-regulated to control levels, leaving E2F-3a to continue cell cycle progression and modulate growth of stimulated myocytes. Currently, E2F-4 is considered to be important for differentiation, alongside E2F-5 (7). However, we observed a significant presence of the E2F-4 protein in myocytes undergoing hypertrophy with both agonists. We therefore hypothesize that E2F-4 may be acting in conjunction with E2F-1 and E2F-3 to promote hypertrophy or that it could be removed from the nucleus following hypertrophic stimulation. Indeed, unlike the proliferative E2Fs (E2F-1, -2, and -3), both E2F-4 and E2F-5 contain nuclear export signals (NES) (7). Therefore, it is possible that E2F-4 is removed from the nucleus upon hypertrophic stimulation of the cardiac myocyte, thereby repressing its differentiation function.

In addition to determining the expression levels of the E2F proteins, we also investigated the global activity status of these transcription factors. Using a cyclin E promoter-luciferase assay, myocytes stimulated with 20% FCS showed a 2-fold increase in overall E2F activity, whereas cells stimulated with PE showed a 6-fold increase in overall E2F activity when compared with control cells (p < 0.05). These results strongly suggest that E2F’s play an important role in the development of hypertrophy following growth factor- or a1-adrenergic receptor-mediated stimulation. EMSAs confirmed these findings and showed an increase in E2F-DNA binding during the development of hypertrophy with serum and PE.

Inhibiting functional E2F activity with a specific peptide sequence that inhibits E2F-DP heterodimerization led to a significant inhibition in the development of myocyte hypertrophy as determined by a combination of ANF and BNP mRNA expressions and protein synthesis measurements. Both semi-quantitative (Fig. 5a) and quantitative PCR (Fig. 5b) analyses showed that blocking E2F function led to significant inhibition of hypertrophic growth, as levels of ANF and BNP were reduced to those seen in non-stimulated myocytes. Interestingly, semi-quantitative analysis showed that blocking E2F activity in PE-stimulated cells with AHS2 has a greater effect, because ANF and BNP induction were reduced to undetectable levels (Fig. 5a). This finding is consistent with our luciferase assay results showing that PE-mediated hypertrophic growth is strongly E2F-dependant (Fig. 4a).

Furthermore, inhibiting E2F function in 20% FCS-stimulated cells prevented protein synthesis, which doubled in cells exposed to FCS alone and in cells treated with FCS and the control peptides AHS2 or ANT. Other investigators have shown previously that targeting E2F can affect the growth of cardiovascular cells. Thus, Morishita et al. (24) showed that transfecting an E2F decoy oligonucleotide into rat vascular smooth muscle cells decreased proliferation of these cells and decreased the expression of E2F-dependent genes, namely, c-myc, cdc2, and the proliferating cell nuclear antigen. More recently, Mann et al. (10) used a similar approach to prevent neointimal hyperplasia in autologous vein grafts, a common problem that contributes to vein graft failure. Using an ex vivo pressure-mediated delivery of an E2F decoy oligonucleotide into veins, a transfection efficiency of 89% was achieved, and overall graft failure event rates fell to 29% compared with 69% for the control group. Clearly, blocking E2F function in the vascular system can provide significant clinical benefits for patients. In the present study, we have shown that blocking E2F function in cardiac myocytes may provide similar benefits for patients with hypertrophic cardiac disease.

In summary, we have shown that E2F and DP family members are regulated differentially during normal rat cardiac myocyte development such that all transcription factors, with the exception of E2F-5, are down-regulated as the heart matures. This pattern of expression is reversed during the development of myocyte hypertrophy, because E2F-1, -3a, and -4 and DP-1 are up-regulated, whereas E2F-5 is down-regulated following mitogenic stimulation. E2F activity is increased during the development of hypertrophy, and blocking E2F function inhibits the development of hypertrophy, suggesting that inhibiting E2F activity in certain cardiac diseases might provide a novel therapeutic approach for inhibiting the development of myocyte hypertrophy that might delay, and possibly prevent, the onset of heart failure.

REFERENCES

1. Poolman, R. A., and Brooks, G. (1998) J. Mol. Cell. Cardiol. 30, 2121–2135
2. Li, J.-M., and Brooks, G. (1999) Eur. Heart J. 20, 406–420
3. Ponn, M. R., Bondmass, M., and Schwartz, D. W. (1996) Heart Lung 25, 3–19
4. Li, J.-M., Poolman, R. A., and Brooks, G. (1998) Am. J. Physiol. 275, H814–H822
5. Dyson, N. (1996) Genes Dev. 12, 2245–2262
6. Nevins, J. R., Leone, G., DeGregori, J., Jakoi, L., and Nevins, J. R. (1997) J. Cell. Physiol. 172, 233–236
7. Trimarchi, J. M., and Lees, A. J. (2002) Nat. Rev. Mol. Cell Biol. 3, 11–20
8. Rogers, K. T., Higgins, P. D., Mills, M. M., Phillips, B. S., Horowitz, J. M., Rogers, R. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7594–7599
9. Deleted in proof
10. Mann, M. J., Whittemore, A. D., Donaldson, M. C., Belkin, M., Conte, M. S., Polak, J. F., Orav, E. J., Ehsan, A., Dell’Acqua, G., Dzau, V. J., and Mann, M. J. (1999) Lancet 354, 1493–1498
11. Flintk, I., Oana, S., Maitra, N., Bahl, J. J., and Morkin, E. (1998) J. Mol. Cell. Cardiol. 30, 563–578
12. Agah, R., Kirshenbaum, L. A., Abdellatif, M., Truong, L. D., Chakraborty, S., Michael, L. H., and Schneider, M. D. (1997) J. Clin. Invest. 100, 2722–2728
13. von Harsdorf, R., Hauck, L., Mehrhof, F., Wegenka, U., Cardoso, M. C., and Dietz R. (1999) Circ. Res. 85, 128–136
14. Cloud, J. E., Rogers, C., Reza, T. L., Ziebold, U., Stone, J. R., Picard, M. H.
Caron, A. M., Brunson, R. T., and Lees, J. A. (2002) Mol. Cell. Biol. 22, 2663–2672
15. Bandara, L. R., Girling, R., and La Thangue, N. B. (1997) Nat. Biotechnol. 15, 896–901
16. Brooks, G., Poolman, R. A., McGill, C. J., and Li, J. M. (1997) J. Mol. Cell. Cardiol. 28, 2261–2271
17. La Thangue, N. B, Thimmappaya, B., and Rigby, P. W. (1990) Nucleic Acids Res. 18, 2929–2938
18. Kusek, J. C., Greene, R. M., Nugent, P., and Pisano, M. M. (2000) Int. J. Dev. Biol. 44, 267–277
19. Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) J. Biol. Chem. 269, 10444–10450
20. Li, F., Wang, X., Capasse, J. M., and Gerdes, A. M. (1996) J. Mol. Cell. Cardiol. 28, 1737–1746
21. Kusek, J. C., Greene, R. M., and Pisano, M. M. (2001) Brain Res. Bull. 54, 187–198
22. Takahashi, Y., Rayman, J. B., and Dynlacht, B. D. (2000) Genes Dev. 14, 804–816
23. Deleted in proof
24. Morishita, R., Gibbons, G. H., Horiuchi, M., Ellison, K. E., Nakama, M., Zhang, L., Kaneda, Y., Oghara, T., and Dzau, V. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5855–5859
Inhibition of E2F Abrogates the Development of Cardiac Myocyte Hypertrophy
Dharmesh Vara, Katrina A. Bicknell, Carmen H. Coxon and Gavin Brooks

J. Biol. Chem. 2003, 278:21388-21394.
doi: 10.1074/jbc.M212612200 originally published online April 6, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212612200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 20 references, 7 of which can be accessed free at
http://www.jbc.org/content/278/24/21388.full.html#ref-list-1