Insulin-Like Growth Factor-1 Induces Mdm2 and Down-Regulates p53, Attenuating the Myocyte Renin-Angiotensin System and Stretch-Mediated Apoptosis

Annarosa Leri,* Yu Liu,* Pier Paolo Claudio,[†] Jan Kajstura,* Xiaowei Wang,* Shenglun Wang,* Parminder Kang,* Ashwani Malhotra,*[‡] and Piero Anversa*

From the Department of Medicine,* New York Medical College, Valhalla, New York, the Department of Pathology, Anatomy, and Cell Biology and Institute for Cancer Research and Molecular Medicine,[†] Jefferson Medical College, Philadelphia, Pennsylvania, and the Department of Medicine,[‡] Montefiore Medical Center and Albert Einstein College of Medicine, New York, New York

Insulin-like growth factor (IGF)-1 inhibits apoptosis, but its mechanism is unknown. Myocyte stretching activates p53 and p53-dependent genes, leading to the formation of angiotensin II (Ang II) and apoptosis. Therefore, this *in vitro* system was used to determine whether IGF-1 interfered with p53 function and the local renin-angiotensin system (RAS), decreasing stretch-induced cell death. A single dose of 200 ng/ml IGF-1 at the time of stretching decreased myocyte apoptosis 43% and 61% at 6 and 20 hours. Ang II concentration was reduced 52% at 20 hours. Additionally, p53 DNA binding to angiotensinogen (Aogen), AT₁ receptor, and Bax was markedly down-regulated by IGF-1 *via* the induction of Mdm2 and the formation of Mdm2-p53 complexes. Concurrently, the quantity of p53, Aogen, renin, AT₁ receptor, and Bax was reduced in stretched myocytes exposed to IGF-1. Conversely, Bcl-2 and the Bcl-2-to-Bax protein ratio increased. The effects of IGF-1 on cell death, Ang II synthesis, and Bax protein were the consequence of Mdm2-induced down-regulation of p53 function. In conclusion, the anti-apoptotic impact of IGF-1 on stretched myocytes was mediated by its capacity to depress p53 transcriptional activity, which limited Ang II formation and attenuated the susceptibility of myocytes to trigger their endogenous cell death pathway. (*Am J Pathol* 1999, 154:567-580)

Insulin-like growth factor (IGF)-1 interferes with the stimulation of cell death, necrotic and apoptotic in nature, in various cell types *in vitro* and *in vivo*.¹⁻⁴ This protective effect of IGF-1 has been documented in the central ner-

vous system after ischemia⁵ and in the heart after acute myocardial infarction⁴ or ischemia-reperfusion injury.⁶ Although these *in vivo* observations were restricted to the role of the ligand, similar results have been obtained by overexpressing IGF-1 receptor (IGF-1R) *in vitro*. An increase in surface IGF-1R attenuates apoptosis⁷ and a reduction in IGF-1R below wild-type levels causes massive death in tumor cell lines.^{8,9} However, the mechanisms by which IGF-1 and/or its activated receptor mediate cell survival are poorly understood. IGF-1 enhances the generation of nitric oxide in endothelial cells,¹⁰ and this adaptation may be critical for the viability of ventricular myocytes in the overloaded heart; nitric oxide decreases the formation of superoxide anion,¹¹ improving the resistance of cells to apoptosis. Additionally, this growth factor may inhibit the cleavage of interleukin-1 β -converting enzyme, hindering distal events coupled with cell death.¹²

A consistent pathological condition associated with apoptosis in the myocardium involves an increase in diastolic wall stress, resulting from the impairment in cardiac pump function, cavity dilation, and thinning of the wall.¹³⁻¹⁵ This *in vivo* state has been mimicked, at least in part, *in vitro* by stretching adult myocytes on distensible membranes,¹⁶ or exposing papillary muscles to abnormal levels of resting tension.¹¹ In both cases, the imposition of a mechanical stimulus is characterized by the initiation of programmed cell death and, in the myocyte preparation, the death signal has been identified with the synthesis and release of angiotensin II (Ang II).¹⁶ Moreover, the formation of this peptide appears to be linked to activation of the tumor suppressor gene p53 and its ability to up-regulate the cellular renin-angiotensin system (RAS) and the apoptotic gene product Bax and down-regulate the anti-apoptotic gene product Bcl-2.¹⁶⁻¹⁸ A relationship between p53 and p53-dependent genes on the one hand, and Ang II-mediated apoptosis

Supported by grants HL-38132, HL-43023, and AG-15756 from the National Institutes of Health and by a grant-in-aid (97-GIA-038) from the American Heart Association.

Accepted for publication October 14, 1998.

Address reprint requests to Dr. Piero Anversa, Department of Medicine, Vosburgh Pavilion, Room 302, New York Medical College, Valhalla, NY 10595. E-mail: piero_anversa@nymc.edu.

on the other, has been shown by employing an adenoviral vector overexpressing wild-type human p53 in myocytes.¹⁹ As IGF-1R is a tyrosine kinase receptor, its activation may transmit a signal to its major substrates that is subsequently transduced by a common effector pathway to the nucleus.²⁰ This may result in the phosphorylation of the amino-terminal region of p53, leading to the expression of the proto-oncogene *mdm2*.^{21,22} Mdm2 protein may form a complex with p53, decreasing p53 stability^{23,24} and inhibiting p53 binding activity.²⁵ On this basis, the hypothesis was advanced that IGF-1 may affect stretch-induced myocyte death by interfering with the local RAS via the suppression of p53 and p53-inducible genes through the induction of *mdm2*.

Materials and Methods

Myocyte Isolation

Hearts from 3-month-old Sprague-Dawley rats (Charles River Breeding Laboratories, North Wilmington, MA) were excised, and myocytes from the left ventricle were enzymatically dissociated. Hearts were placed on a stainless steel cannula for retrograde perfusion through the aorta. The solutions were supplements of modified commercial MEM Joklik (Sigma Chemical Co., St. Louis, MO). Hepes/MEM contained 117 mmol/L NaCl, 5.7 mmol/L KCl, 4.4 mmol/L NaHCO₃, 1.5 mmol/L KH₂PO₄, 17 mmol/L MgCl₂, 21.1 mmol/L Hepes, 11.7 mmol/L glucose, amino acids, and vitamins, 2 mmol/L L-glutamine, 10 mmol/L taurine, and 21 mU/ml insulin and adjusted to pH 7.2 with NaOH. This solution is 292 mosm, isosmolar with rat serum. Resuspension medium was Hepes/MEM supplemented with 0.5% bovine serum albumin, 0.3 mmol/L calcium chloride, and 10 mmol/L taurine adjusted to 292 mosm. The cell isolation procedure consisted of three main steps. 1) For calcium-free perfusion, blood washout and collagenase (selected type II, Worthington Biochemical Corp., Freehold, NJ) perfusion of the heart was carried out at 34°C with Hepes/MEM gassed with 85% O₂ and 15% N₂. 2) For mechanical tissue dissociation, after the heart was removed from the cannula, the left ventricle was separated from the right ventricular free wall and minced. Collagenase-perfused tissue was subsequently shaken in resuspension medium containing collagenase and 0.3 mmol/L calcium chloride. Supernatant cell suspensions were washed and resuspended in resuspension medium. 3) For separation of intact cells, intact cells were enriched by centrifugation, and the supernatant was discarded. This procedure was repeated four to five times in each preparation to remove nonmyocyte cells, cell debris, and the residual collagenase. Each centrifugation was performed at 30 × *g* for 3 minutes. Subsequently, approximately 10⁶ cells were suspended in 10 ml of isotonic Percoll and centrifuged for 10 minutes at 34 × *g*. Intact cells were recovered and washed, and smears were made to control the purity of the preparation. Rectangular, trypan-blue-excluding cells constituted nearly 80% of myocytes. The average number of myocytes obtained from the left ventricle was 6 × 10⁶. The

contribution of interstitial cells was assessed by counting 1000 cells in each left ventricle and then computing the respective fractions of myocytes and nonmyocytes encountered. Nonmyocytes accounted for less than 1% of the cell population.¹⁶

Cell Culture and Equibiaxial Stretch Apparatus

Myocytes were plated at a density of 2 × 10⁴ cells/cm² in a device that results in a homogeneous equibiaxial strain of 0% to 20% to a culture rubber substrate^{16,26} coated with 0.5 μg/cm² laminin (Sigma). Cells were incubated in serum-free medium (SFM) for 24 hours to adhere to the substrate before stretching. Stretching corresponded to a 10.3% increase in sarcomere length, measured at ×1000 by averaging groups of 10 sarcomeres each in 300 cells in each preparation. As previously described, stretching *per se* was not associated with cell injury.¹⁶ Therefore, nonstretched and stretched myocytes were examined at 20 minutes and 1, 2, 5, 6, 16, 20, and 36 hours. IGF-1 (Genzyme, Cambridge, MA) was added to myocytes at a concentration of 200 ng/ml, 15 minutes before stretch, and kept for the period of observation. For histochemistry, cells were washed with cold HBSS, fixed on ice in 1% formaldehyde, and stored in 70% ethanol at −20°C. For molecular determinations, cells were collected in cold PBS, centrifuged at 12,000 × *g*, and stored at −75°C.

In Situ Terminal Deoxynucleotidyl Transferase (TdT) Assay

Cultures were incubated with 50 μl of staining solution containing 5 U of TdT, 2.5 mmol/L CoCl₂, 0.2 mol/L potassium cacodylate, 25 mmol/L Tris/HCl, 0.25% bovine serum albumin, and 0.5 nmol/L dUTP, coupled to biotin via a 16-atom spacer arm (biotin-16-dUTP) for 30 minutes. After being rinsed in PBS, samples were incubated for 30 minutes at room temperature in a solution containing 4X SSC buffer and 5% (w/v) nonfat dry milk (Sigma). Staining solution, which contained 5 μg/ml fluorescein-isothiocyanate-labeled ExtrAvidin (Sigma), 4X SSC buffer, 0.1% Triton X-100, and 5% nonfat dry milk, was applied for 30 minutes. Cells were incubated at 37°C for 30 minutes with α-sarcomeric actin antibody (clone 5C5, Sigma) diluted 1:20 in PBS containing 10% goat serum and subsequently with anti-mouse IgG tetraethylrhodamine-isothiocyanate-labeled antibody, also diluted 1:30 in PBS, containing 10% goat serum. Cells were then stained with propidium iodide, 10 μg/ml, for 15 minutes to visualize nuclei and finally embedded in Vectashield (Vector Laboratories, Burlingame, CA) mounting medium.

Confocal Microscopy

The number of myocyte nuclei labeled by TdT was determined by examining 2000 to 3000 myocytes in each condition by confocal microscopy (Bio-Rad MRC-1000). This approach allowed the simultaneous detection of

morphological alterations of nuclei and the presence of TdT staining. The distinction between myocytes and non-myocytes was obtained by α -sarcomeric actin antibody labeling of the myocyte cytoplasm.

Myosin Monoclonal Antibody Labeling

Cultures of nonstretched and stretched myocytes were exposed to 0.5 μ g/ml monoclonal antibody specific for cardiac myosin (clone CCM-52; gift from Dr. William A. Clark) for the detection of membrane damage and cell necrosis.⁴ After fixation, cells were incubated with tetraethylrhodamine-isothiocyanate-labeled anti-mouse IgG. The percentage of stained cells was determined by examining 1000 myocytes in each preparation.

DNA Gel Electrophoresis

Myocytes were fixed for 24 hours at -20°C in 70% ethanol, centrifuged at $800 \times g$ for 5 minutes, and resuspended in 40 μ l of phosphate-citrate buffer consisting of 192 parts of 0.2 mol/L Na_2HPO_4 and 8 parts of 0.1 mol/L citric acid (pH 7.8) for 1 hour. After centrifugation, the supernatant was concentrated by vacuum in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY) for 15 minutes. A 3- μ l aliquot of 0.25% Nonidet P-40 (Sigma) in distilled water was then added, followed by 3 μ l of a solution of RNase (1 mg/ml), also in water. After incubation at 37°C , 3 μ l of a solution of proteinase K (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) was added, and the extract was incubated for an additional 1 hour. Subsequently, 12 μ l of loading buffer (0.25% bromophenol blue, 30% glycerol) was added, and samples were subjected to electrophoresis on 2% agarose gel containing 5 μ g/ml ethidium bromide.

Western Blot of p53, Bax, Bcl-2, Angiotensinogen, and AT₁ Receptor

For immunoblot assay of p53, Bax, Bcl-2, angiotensinogen (Aogen), and AT₁ receptor gene products, myocytes were lysed with 150 to 200 μ l of lysis buffer (50 mmol/L Tris/HCl, pH 7.5, 5 mmol/L EDTA, 250 mmol/L NaCl, 0.1% Triton X-100) containing the protease inhibitors 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 5 mmol/L dithiothreitol, and 1 mmol/L Na_3VO_4 , incubated on ice, and spun down at 14,000 rpm. Equivalents of 50 to 120 μ g of protein were separated by 10% to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred on nitrocellulose filters, blocked with 6% powdered milk, and exposed to mouse monoclonal anti-human p53 (Pab240, Santa Cruz Biotechnology, Santa Cruz, CA), to rabbit polyclonal anti-human Bcl-2 (Δ C21, Santa Cruz), anti-human Bax (P19, Santa Cruz), mouse anti-rat Aogen (Swant, Bellinzona, Switzerland), and rabbit polyclonal anti-human AT₁ receptor (306, Santa Cruz) at a concentration of 1 μ g/ml in Tris-buffered saline/Tween 20 (TBST). Bound antibodies were detected by peroxidase-conjugated anti-mouse or anti-

rabbit IgG. p53 was detected as a 53-kd band, Bcl-2 as a 29-kd band, Bax as a 21-kd band, Aogen as a double band at 54 to 56 kd, and AT₁ as a 41-kd band.

ELISA Determination of Ang II

Ang II in conditioned medium (CM) was measured by the Peninsula ELISA procedure (Peninsula Laboratories, Belmont, CA). CM (4.5 ml) was treated with 0.2 ml of 10% trifluoroacetic acid (TFA) and centrifuged at 6,000 rpm for 15 minutes at 4°C . The supernatant was dried in a Speed Vac concentrator and the residue dissolved in 5 ml of 0.1% TFA, pH 3.0. The soluble extract was partially purified using a C18 Sep-Pak column (Waters Associates, Millford, MA). The Sep-Pak column was first equilibrated by washing sequentially with 8 ml each of methanol, tetrahydrofuran, hexane, methanol, and distilled water. The soluble extract was applied to the prewashed column and washed with distilled water and 10% acetonitrile in 0.1% TFA. The Ang II fraction was eluted from the column with 30% acetonitrile in 5 ml of 0.1% TFA, dried, and dissolved in 0.25 ml of TBST solution. Samples of 50 μ l were analyzed in a microtiter plate, coated with 2 μ g of protein A/ml of bicarbonate buffer, using Ang II antibody (1:32,000) and a tracer, biotinylated Ang II. The microtiter plate was washed five times with TBST and treated with streptavidin/horseradish peroxidase. The color reaction was developed with 100 μ l of tetramethyl-benzidine substrate and terminated by 2 N HCl. The absorbance was recorded at 450 nm within 15 to 30 minutes, and the concentration was calculated from the standard curve generated each time for Ang II, ranging from 10^{-6} mol/L to 10^{-12} mol/L.

Mobility Shift Assay

To prepare a double-stranded probe for bax, oligonucleotides 5'-AGCTTGCTCACAAGTTAGAGACAAGCCTGGCGTGGCTATATTGA-3' and 5'-AGCTTCAATATAGCCCACGCCAGGCTTGTCTCTAAGTTGTGAGCA-3',²⁷ which contain one perfect and three overlapping imperfect consensus motifs for p53 in the human bax promoter,¹⁸ were annealed and labeled with [γ -³²P]ATP and T4 polynucleotide kinase (Boehringer Mannheim). This sequence corresponds to -492 bp to -447 bp and is located 70 bp 5' of the TATAA box (GenBank U17193). To prepare a probe for AT₁, oligonucleotides 5'-ATTTAATTAACATGCCTGTGACTTT-3' and 5'-AAAGTCACAGGCATGTTAATTAAAT-3', which correspond to rat AT₁ sequence from -1862 bp to -1838 bp located 1813 bp 5' of the TATAA box (GenBank S66402) were used. To prepare a probe for Aogen, oligonucleotides 5'-CTTCATCCACAAGCCCAGAACATT-3' and 5'-AATGTTCTGGGCTTGTGGATGGAAG-3', which correspond to rat Aogen sequence from -599 bp to -575 bp located 568 bp 5' of the TATAA box (GenBank M31673) were used.^{16,19} Nuclear extracts were obtained by incubation with hypotonic buffer. Lysates were mixed with 10% Nonidet P-40 and centrifuged, and nuclear pellets were incubated in high-salt buffer. After centrifugation, the su-

pernatant was collected. Nuclear extracts (40 μ g of protein) were incubated in 10% glycerol, 20 mmol/L MgCl_2 , 10 mmol/L dithiothreitol, 200 mmol/L NaCl, 200 mmol/L HEPES, pH 7.9, 1.0 mmol/L phenylmethylsulfonyl fluoride for 10 minutes on ice, and 2 μ l of ^{32}P -labeled probe was added. The reaction mixture was incubated at room temperature. In some experiments, nuclear extracts were incubated with anti-p53 antibody, 0.5 μ g of Pab240 (Santa Cruz), or with an irrelevant antibody. Samples were subjected to electrophoresis in 4% polyacrylamide gel. Controls for specificity included the unlabeled bax, AT₁, and Aogen probes as competitors and an unlabeled mutated bax probe (5'-AAGTTAGAGATAATGCTGGGC-GAG-3' and 5'-CTCGCCGAGCATTATCTCTAACTT-3') as noncompetitor.

Immunoprecipitation and Immunoblot of Mdm2 and p53

Aliquots of myocyte lysates prepared from nonstretched and stretched myocytes, in the presence or absence of IGF-1, were obtained at 5, 16, and 36 hours after the imposition of the mechanical stimulus (see above). Two separate immunoprecipitation assays were performed: 1) 200 to 300 μ g of soluble protein extracts were incubated with 3 μ g of mouse monoclonal anti-human mdm2 antibody (Smp14, Santa Cruz) and 250 μ l of buffer (20 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol) containing the protease inhibitors phenylmethylsulfonyl fluoride (0.2 mmol/L), aprotinin (2 μ g/ μ l), and Na_3VO_4 (0.2 mmol/L) overnight at 4°C. Subsequently, 50 μ l of protein A-agarose (Pierce, Rockford, IL) was added to each sample. After several washes with a buffer containing 20 mmol/L Tris/HCl, pH 7.4, 300 mmol/L NaCl, 2 mmol/L EDTA, and 2 mmol/L EGTA, samples were spun at 14,000 rpm for 2 minutes. Loading buffer (40 μ l) was added to each pellet, and immunoprecipitated proteins were separated by 10% SDS-PAGE. Proteins were transferred on nitrocellulose filters and exposed to rabbit polyclonal anti-human mdm2 antibodies (C-18 and K-20, Santa Cruz) and rabbit polyclonal anti-human p53 antibody (FL393, Santa Cruz) at a concentration of 1 μ g/ml TBST. Samples were then treated as described above for Western blot. 2) A procedure identical to that detailed above was followed here with one exception. This consisted of the use of mouse monoclonal anti-human p53 antibody (Pab240, Santa Cruz) to immunoprecipitate the myocyte lysates. p53 was detected as a 53-kd band, and the four spliced forms of Mdm2 as a 57- to 58-kd, 76-kd, 85-kd, and 90-kd band, respectively.

Data Collection and Analysis

Results are presented as mean $\pm$ SD. Autoradiograms and gels were analyzed densitometrically by an image analyzer (Gel Doc 1000, Bio-Rad, Hercules, CA). Statistical significance for comparisons between two measurements was determined by the unpaired, two-tailed Student's *t*-test. Statistical significance for comparison

among distinct culture conditions was determined using the analysis of variance and the Bonferroni method.²⁸ Values of $P < 0.05$ were considered significant; *n* values for each determination are listed in the text or in the legend to each figure.

Results

IGF-1 and Stretch-Induced Myocyte Apoptosis

Myocytes stretched in an equibiaxial stretch device were characterized by a 10.3% ($P < 0.001$) increase in sarcomere length from 1.84 ± 0.03 μ m ($n = 70$) to 2.03 ± 0.04 μ m ($n = 90$). This change in sarcomere length persisted for the duration of the experiment and was uniformly distributed to cells in the center and at the periphery of the distensible membrane.²⁶ Lengthening of sarcomeres was assessed at 1, 10, and 20 hours after stretching, and identical values were obtained in the same preparations at these intervals. The addition of IGF-1 to the medium at concentrations varying from 50 to 400 ng/ml had no influence on sarcomere length. However, to achieve a 10.3% sarcomere elongation, a 20% degree of strain was applied. Similar results have previously been reported with this system.¹⁶ Myocyte cell death at 6 and 20 hours was determined by TdT labeling and confocal microscopy. This approach permits the simultaneous assessment of morphological alterations in chromatin structure and TdT staining of nuclei.^{15,16,29} The possibility of myocyte necrosis under this setting was also measured by the addition to the cultures of myosin monoclonal antibody. Necrotic myocytes allow anti-myosin to enter the cell and bind to myofibrillar myosin.^{4,30} Conversely, uninjured cells remain unlabeled.

Preliminary studies established the effects of different concentrations of IGF-1 on stretch-induced myocyte apoptosis at 20 hours after the imposition of the mechanical stimulus (Figure 1). The percentage of TdT-positive myocytes with stretching alone varied in these experiments from a minimum of 11% to a maximum of 20%. IGF-1 at 50 ng/ml decreased apoptosis modestly, whereas doses of growth factor of 100, 200, and 400 ng/ml reduced programmed cell death by 40% ($P < 0.05$), 57% ($P < 0.001$), and 54% ($P < 0.001$), respectively. In view of these observations, a concentration of IGF-1 of 200 ng/ml was used.

The methodology used to measure apoptosis is illustrated in Figure 2. By confocal microscopy, nuclei are recognized by the red fluorescence of propidium iodide staining (Figure 2A), and TdT labeling is depicted by the green fluorescence (Figure 2B). Myocytes are identified by the red fluorescence of α -sarcomeric actin antibody staining (Figure 2C). In this example, margination and initial fragmentation of chromatin are apparent, and this structural damage is associated with the detection of DNA strand breaks in the same nucleus. Horseshoe appearance of nuclei and nuclear fragmentation were observed (Figure 2, D–F). Sarcolemmal blebbing (Figure 2, G and H) and early phases of nuclear damage (Figure 2, I and J) were also noted. On the basis of these criteria,

Figure 1. Effects of different doses of IGF-1 on stretch-mediated apoptosis at 20 hours. Results are presented as means $\pm$ SD. * $P < 0.05$, difference from stretched myocytes not exposed to IGF-1; $n = 5$ in each condition.

values of apoptosis were obtained at baseline and after sarcomere stretching in the presence and absence of IGF-1 in the medium.

The effects of IGF-1 on the magnitude of stretch-induced apoptosis are shown in Figure 3. The collection of the data illustrated in this figure involved the counting of 12,000 myocyte nuclei by confocal microscopy at both 6 and 20 hours (nonstretched = 3000, $n = 6$; nonstretched plus IGF-1 = 3000, $n = 6$; stretched = 3000, $n = 6$ at 6 hours, $n = 8$ at 20 hours; stretched plus IGF-1 = 3000, $n = 6$ at 6 hours, $n = 8$ at 20 hours). Low levels of apoptosis were detected in nonstretched myocytes, and IGF-1 did not alter this baseline degree of cell death. Conversely, sarcomere elongation increased markedly the extent of cell death at 6 and 20 hours, but the addition of IGF-1 attenuated apoptosis by 43% ($P < 0.001$) and 61% ($P < 0.001$) at the earlier and later time points, respectively. The histochemical and morphological evaluations of apoptosis were complemented with the detection of low molecular weight DNA by agarose gel electrophoresis.^{4,11,16} This analysis included three separate experiments. DNA fragments consistent with internucleosomal DNA cleavage were apparent in stretched myocytes, and this pattern of DNA damage was more evident at 20 than at 6 hours. DNA laddering was noticeably reduced in IGF-1-treated cells (Figure 4). Finally, myocyte necrosis was modest in nonstretched myocytes at 6 and 20 hours in the absence (6 hours = $0.66 \pm 0.16\%$, $n = 5$; 20 hours = $0.78 \pm 0.21\%$, $n = 5$) and presence (6 hours = $0.54 \pm 0.14\%$, $n = 5$; 20 hours = $0.76 \pm 0.34\%$, $n = 5$) of IGF-1. Similarly, myocyte necrosis was low in stretched myocytes without IGF-1 (6 hours = $0.70 \pm 0.20\%$, $n = 5$; 20 hours = $0.81 \pm 0.25\%$, $n = 5$) and after the addition of the growth factor (6 hours = $0.77 \pm 0.29\%$, $n = 5$; 20 hours = $0.79 \pm 0.21\%$, $n = 5$). In summary, IGF-1 markedly attenuated stretch-induced myocyte apoptosis.

IGF-1, Stretch, p53, and p53-Dependent Genes

The effects of sarcomere stretching alone, or in combination with IGF-1, on the expression of p53, Bax, Bcl-2, Aogen, and AT₁ receptor subtype in myocytes were examined by Western blot at different time points after the imposition of the mechanical stimulus. These determinations were performed because one perfect and three imperfect consensus sites for p53 binding are present in the bax promoter,¹⁷ a p53-dependent negative response element has been identified in the bcl-2 gene,¹⁸ and the promoters of angiotensinogen and AT₁ receptor each contain one imperfect motif with homology to the consensus sequence of p53.^{16,19} Additionally, the changes in renin were measured to characterize further the local RAS in this *in vitro* model.

The protein levels of p53, Bax, Bcl-2, Aogen, renin, and AT₁ receptor did not vary in nonstretched myocytes at 5, 10, and 20 hours in culture. Similarly, IGF-1 did not influence the amount of these proteins in the absence of stretching. For each gene product, a Western blot and densitometric analysis are shown. The detection and quantification of p53 is depicted in Figure 5. In comparison with control myocytes, sarcomere elongation increased 126% ($P < 0.01$), 205% ($P < 0.001$), and 406% ($P < 0.001$) the quantity of p53 protein at 5, 10, and 20 hours, respectively. The addition of IGF-1 decreased the amount of p53 in stretched myocytes: 67% ($P < 0.001$) at 5, 82% ($P < 0.001$) at 10, and 84% ($P < 0.001$) at 20 hours after the mechanical stimulus.

Figure 6, A–D, illustrates the effects of IGF-1 on the expression of Bax and Bcl-2 in myocytes after stretch. With respect to nonstretched myocytes, the amount of Bax increased 219% ($P < 0.001$), 200% ($P < 0.001$), 368% ($P < 0.001$), and 300% ($P < 0.001$) at 1, 5, 10, and 20 hours after stretch (Figure 6, A and B). IGF-1 attenuated these increases of Bax by 57% ($P < 0.001$), 59% ($P < 0.001$), 74% ($P < 0.001$) and 57% ($P < 0.001$) at 1, 5, 10, and 20 hours, respectively. Sarcomere elongation did not alter Bcl-2 at 5 hours but decreased the quantity of this protein 38% ($P < 0.05$) and 49% ($P < 0.01$) at 10 and 20 hours (Figure 6, C and D). IGF-1 reversed the impact of stretch on myocytes, increasing the amount of Bcl-2 in these cells 77% ($P < 0.001$), 296% ($P < 0.001$), and 305% ($P < 0.001$) at 5, 10, and 20 hours, respectively.

Aogen increased progressively with stretch. A 43% (nonsignificant), 95% ($P < 0.001$), and 98% ($P < 0.001$) increase was measured at 5, 10, and 20 hours after the imposition of the mechanical stimulus (Figure 7, A and B). The addition of IGF-1 reduced in a time-dependent fashion Aogen quantity in stretched myocytes. A 44% ($P < 0.001$), 64% ($P < 0.001$), and 68% ($P < 0.001$) reduction in the expression of this protein was noted at 5, 10, and 20 hours. Renin was detectable in minimal amounts by Western blot (Figure 7C). Stretching increased the expression of renin in myocytes, and IGF-1 did not affect this enzyme at 5 hours but reduced its amount by 31% ($P < 0.05$) at 10 hours and 76% ($P < 0.001$) at 20 hours (Figure 7D).

Figure 2. Four examples of apoptosis in mononucleated and binucleated myocytes after stretch at 6 (A to F) and 20 (G to J) hours. **A to C:** Mononucleated myocyte with chromatin margination and initial fragmentation, depicted by the red fluorescence of propidium iodide staining (**A**, arrow) and TdT labeling (**B**, arrow). The combination of these two stainings of the nucleus (arrow) with the red fluorescence of α -sarcomeric actin antibody labeling is shown in **C**. A horseshoe image of one nucleus (arrow) and fragmentation of the other (arrowhead) are illustrated by the same procedure in a binucleated myocyte (**D** to **F**). **G and H:** TdT labeling of a nucleus shown by green fluorescence (**G**, arrow) and the combination of TdT and propidium iodide staining of the same nucleus by yellow fluorescence (**H**, arrow). This latter image is associated with α -sarcomeric actin antibody labeling of the peripheral region of the myocyte, which exhibits loss of myofibrils in a large portion of the cytoplasm and blebbing of the sarcolemma (**H**, arrowhead). **I and J:** TdT labeling of two nuclei shown by green fluorescence (**I**, arrows) and the combination of TdT and propidium iodide staining of the same nuclei by yellow fluorescence (**J**, arrows). This latter image is associated with α -sarcomeric actin antibody labeling of portion of the cell mostly in the area adjacent to the sarcolemma. Note that TdT labeling of one of the two nuclei involves most of the chromatin but not the entire structure (arrowhead). Magnification, $\times 1500$ (A to F) and $\times 1000$ (G to J).

Figure 3. Effects of 200 ng/ml IGF-1 on stretch-mediated apoptosis at 6 and 20 hours after the imposition of the mechanical stimulus. * $P < 0.05$, difference from nonstretched myocytes (NS) and nonstretched myocytes exposed to IGF-1 (NS+IGF-1); † $P < 0.05$, difference from stretched myocytes (S). S+IGF-1, stretched myocytes in the presence of IGF-1.

The influence of IGF-1 on AT_1 receptor subtype in stretched myocytes is shown in Figure 7, E and F. Sarcomere elongation resulted in a 1.5-fold ($P < 0.05$), 1.8-fold ($P < 0.001$), and 2.0-fold ($P < 0.001$) increase in AT_1 receptor at 5, 10, and 20 hours. The addition of IGF-1 was characterized by a 33% ($P < 0.01$), 27% ($P < 0.05$), and 25% ($P < 0.05$) decrease in AT_1 receptor at 5, 10, and 20 hours, respectively. In summary, IGF-1 attenuated the stretch-mediated up-regulation of p53, Bax, Aogen, and AT_1 receptor and the down-regulation of Bcl-2 in myocytes.

Figure 4. DNA gel electrophoresis of myocytes exposed to stretch for 20 hours in the presence of 200 ng/ml IGF-1 (S+IGF-1) or in the absence of the growth factor (S). DNA laddering is noted in stretched cells but is more apparent in the absence of IGF-1. MW, molecular weight markers; arrowheads indicate multiples of 200 bp.

Figure 5. A: Effects of IGF-1 on the quantity of p53 protein measured by Western blot (top) in nonstretched myocytes at 20 hours (NS) and stretched myocytes (S) at 5, 10, and 20 hours (h). IGF-1 markedly attenuated the amount of p53 in stretched cells at all time points. SV-T2 was used as positive control. Loading of proteins is illustrated by Coomassie blue staining (bottom). B: Densitometric analysis of p53 protein in myocytes. Data are presented as means $\pm$ SD. * $P < 0.05$, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; † $P < 0.05$, difference from stretched myocytes in the absence of the growth factor; $n = 5$ in each determination.

IGF-1, Stretch, and Ang II Formation

To determine whether IGF-1 attenuated Ang II secretion after sarcomere stretching, Ang II was measured in CM obtained from cultures of nonstretched and stretched myocytes in the presence and absence of IGF-1. The intervals examined included 20 minutes and 2, 6, and 20 hours. Figure 8 illustrates that sarcomere elongation progressively increased Ang II concentration in CM from 20 minutes to 20 hours. IGF-1 did not interfere with the release of Ang II from myocytes at the earliest time point, but it decreased the formation of this peptide at the subsequent intervals. At 2 and 6 hours, IGF-1 diminished the level of Ang II to values comparable to those detected in nonstretched myocytes. At 20 hours, the concentration of Ang II in CM of IGF-1-treated cells was 52% ($P < 0.001$) lower than in stretched myocytes but 55% ($P < 0.05$) higher than in nonstretched cells. In summary, IGF-1 depressed the ability of myocytes to generate Ang II as a result of sarcomere stretching.

Figure 6. A: Effects of IGF-1 on the quantity of Bax protein measured by Western blot (top) in nonstretched (NS) and stretched (S) myocytes at 1, 5, 10, and 20 hours (h). IGF-1 markedly attenuated the amount of Bax in stretched cells at all time points. Loading of proteins is illustrated by Coomassie blue staining (bottom). B: Densitometric analysis of Bax protein in myocytes. Data are presented as means $\pm$ SD. * P < 0.05, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; † P < 0.05, difference from stretched myocytes in the absence of the growth factor; n = 5 in each determination. C: Effects of IGF-1 on the quantity of Bcl-2 protein measured by Western blot (top) in nonstretched myocytes at 20 hours (NS) and stretched myocytes (S) at 5, 10, and 20 hours (h). IGF-1 significantly increased the amount of Bcl-2 in stretched cells at the time points examined. Loading of proteins is illustrated by Coomassie blue staining (bottom). D: Densitometric analysis of Bcl-2 protein in myocytes. Data are presented as means $\pm$ SD. * P < 0.05, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; † P < 0.05, difference from stretched myocytes in the absence of the growth factor. n = 5 in each determination.

IGF-1, Stretch, and p53 DNA Binding

The results described above have documented that IGF-1 affected the expression of p53, p53-dependent genes, such as bax and bcl-2, and various components of the local RAS, including Aogen, renin, and AT₁ receptors. However, these observations did not prove that IGF-1-mediated down-regulation of p53 constituted the primary event responsible for the inhibitory impact of the growth factor on the myocyte RAS and other gene products. Changes in the quantity of p53 do not necessarily imply corresponding changes in the activity of this transcription factor.³¹ Therefore, the consequences of IGF-1 on p53 binding to the promoter of bax, Aogen, and AT₁ receptor were determined by gel retardation assays. Figure 9A illustrates that, in comparison with nonstretched myocytes, stretch was associated with an increase in p53 binding to the bax promoter at 6 and 16 hours. IGF-1

markedly decreased the optical density of the p53 shifted complex at both intervals after sarcomere elongation. The addition of IGF-1 to nonstretched myocytes did not change p53 DNA binding (not shown). The specificity of the assay was established by subjecting the p53 band to competition with an excess of unlabeled self oligonucleotide and by preincubating nuclear extract with a monoclonal p53 antibody. Under these conditions, the p53 shifted complex decreased significantly. Conversely, the addition of an unlabeled mutated form of bax or irrelevant antibody did not interfere with DNA binding. These controls were obtained using nuclear extracts prepared from myocytes stretched for 16 hours in the absence of IGF-1.

The gel shift analysis shown in Figure 9B documents that the Aogen probe resulted in the formation of two p53 shifted bands. With respect to nonstretched myocytes, the optical density of the shifted complexes increased in

Figure 7. A: Effects of IGF-1 on the quantity of Aogen protein measured by Western blot (top) in nonstretched myocytes at 20 hours (NS) and stretched myocytes (S) at 5, 10, and 20 hours (h). IGF-1 markedly attenuated the amount of Aogen in stretched cells at all time points. Serum was used as positive control. Loading of proteins is illustrated by Coomassie blue staining (bottom). B: Densitometric analysis of Aogen protein in myocytes. Data are presented as means $\pm$ SD. * P < 0.05, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; $^{\dagger}P$ < 0.05, difference from stretched myocytes in the absence of the growth factor; n = 5 in each determination. C: Effects of IGF-1 on the quantity of renin protein measured by Western blot (top) in nonstretched myocytes at 20 hours (NS) and stretched myocytes (S) at 5, 10, and 20 hours (h). IGF-1 significantly decreased the amount of renin in stretched cells at 10 and 20 hours. Loading of proteins is illustrated by Coomassie blue staining (bottom). D: Densitometric analysis of renin protein in myocytes. Data are presented as means $\pm$ SD. * P < 0.05, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; $^{\dagger}P$ < 0.05, difference from stretched myocytes in the absence of the growth factor; n = 5 in each determination. E: Effects of IGF-1 on the quantity of AT₁ protein measured by Western blot (top) in nonstretched myocytes at 20 hours (NS) and stretched myocytes (S) at 5, 10, and 20 hours (h). IGF-1 attenuated the amount of AT₁ in stretched cells at the three time points examined. Loading of proteins is illustrated by Coomassie blue staining (bottom). F: Densitometric analysis of AT₁ protein in myocytes. Data are presented as means $\pm$ SD. * P < 0.05, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; $^{\dagger}P$ < 0.05, from stretched myocytes in the absence of the growth factor; n = 5 in each determination.

Figure 8. Effects of IGF-1 on the quantity of Ang II in the medium of stretched myocytes. Data are presented as means $\pm$ SD. * $P < 0.05$, difference from nonstretched myocytes and nonstretched myocytes exposed to IGF-1; † $P < 0.05$, difference from stretched myocytes in the absence of the growth factor; $n = 5$ in each determination.

stretched cells at 6 and 16 hours, but IGF-1 significantly decreased the intensity of the bands at the two intervals examined. IGF-1 did not modify p53 binding activity of nonstretched myocytes (not shown). When nuclear extracts from myocytes stretched for 16 hours were exposed to an excess of unlabeled self oligonucleotide, or to a p53 antibody, the mobility-shifted complexes were barely visible. In contrast, the addition of an irrelevant antibody had no effect on the p53 bands. Similarly, the AT₁ probe resulted in the generation of two p53 shifted bands that were much more apparent in stretched than in nonstretched myocytes (Figure 9C). IGF-1 decreased markedly the optical density of stretch-induced p53 DNA binding. IGF-1 had no effect on p53 DNA binding of nonstretched myocytes (not shown). The specificity of the assay was determined as described above for Aogen. In summary, IGF-1 decreased not only the quantity but also the activity of p53 in stretched myocytes.

Stretch, IGF-1, and Proportion of p53 and Mdm2

The experiments in the preceding sections have established that IGF-1 can decrease p53 quantity and activity. However, the mechanism by which IGF-1 depresses p53 function, the myocyte RAS, and apoptosis remained to be demonstrated. In an attempt to address this issue, the changes in the expression of Mdm2 after stretch and IGF-1 treatment were evaluated. This approach was followed because Mdm2 can affect the stability and function of p53.^{23–25} To identify the formation of Mdm2-p53 complexes, cell lysates were obtained in the absence of SDS. This preparation allowed the preservation of protein complexes during the procedure and the subsequent identification of the individual components when co-immunoprecipitated proteins were exposed to SDS and run on SDS-PAGE.³² Immunoprecipitation with anti-mdm2

antibody resulted in the formation of five bands with different molecular weights (Figure 10). The protein detected at 53 kd corresponded to the p53 protein that co-precipitated with Mdm2, ie, the fraction of p53 bound to Mdm2. The higher molecular weight proteins, 57 to 58 kd and 90 kd, most likely reflected the amount of Mdm2 linked to p53, as both Mdm2 proteins possess an amino-terminal hydrophobic cleft, which is required for the interaction with the hydrophobic face of the p53 molecule.³³ The 90-kd protein represents the full-length Mdm2 and the 57- to 58-kd protein constitutes a spliced form of Mdm2 that lacks the carboxyl-terminal region.³⁴ However, it cannot be excluded that portions of the 57- to 58-kd and 90-kd proteins detected here were not associated with p53. Moreover, the 76-kd and 85-kd proteins have lost the amino terminus and cannot bind to p53.³⁴

Figure 10 illustrates that IGF-1 increased significantly the expression of Mdm2 p76 ($P < 0.001$) in nonstretched myocytes; Mdm2 p57–58, p76, p85, and p90 in myocytes stretched for 5 hours ($P < 0.001$); and Mdm2 p57–p58, p85, and p90 in myocytes stretched for 16 hours ($P < 0.05$ to $P < 0.001$). In view of the low levels of Mdm2 proteins in myocytes, a longer exposure of the same blot shown in the upper panel is depicted in the lower panel of Figure 10. This micrograph documents in a more apparent manner the difference in optical density of Mdm2 bands in the presence and absence of IGF-1 treatment in nonstretched and stretched myocytes. Moreover, p53 was not detected in nonstretched and stretched myocytes in the absence of IGF-1 and in nonstretched myocytes exposed to IGF-1 but was apparent in IGF-1-treated stretched myocytes at 5 and 16 hours. At 36 hours after a single addition of IGF-1 to stretched myocytes, p53 was barely seen, and the most prominent Mdm2 band, 90 kd, was similarly markedly attenuated. This may reflect a decrease of the growth factor in the medium with time as a single dose of IGF-1 was used at the beginning of the experiment.

Figure 11 illustrates that myocyte lysates immunoprecipitated with anti-p53 antibody were characterized by three bands, two of which corresponded to Mdm2 90 kd and Mdm2 57 to 58 kd. The third band, at 53 kd, reflected total p53. IGF-1 treatment increased Mdm2 p90 in nonstretched myocytes ($P < 0.001$) and Mdm2 p57–58 and p90 in myocytes stretched for 5 hours ($P < 0.001$) and 16 hours ($P < 0.001$). Total p53 decreased 50% ($P < 0.001$) after IGF-1 administration in nonstretched myocytes, 40% ($P < 0.001$) in stretched myocytes at 5 hours, and 80% ($P < 0.001$) at 16 hours after the imposition of the mechanical stimulus. The lower panel of Figure 11 shows the same blot after longer exposure to emphasize the low levels of expression of Mdm2. Although the Mdm2 forms detected in Figure 10 could have been only in part bound to p53, the Mdm2 proteins identified in Figure 11 were unequivocally linked to p53 as they were co-immunoprecipitated with an anti-p53 antibody. In summary, IGF-1 was coupled with the induction of Mdm2, which formed complexes with p53 in stretched myocytes.

Discussion

The results of the present study indicate that IGF-1 improved myocyte survival in stretch-induced apoptosis. This protective action of IGF-1 was exerted by down-regulating the myocyte RAS, which was up-regulated by sarcomere elongation. The growth factor was capable of decreasing the expression of Aogen and renin, reducing the generation of Ang II in stretched myocytes. Additionally, the synthesis of AT₁ receptors was depressed by IGF-1, interfering with the receptor system implicated in the transmission of the Ang II death signal. The proximal event responsible for increased cell viability with IGF-1 consisted in the induction of Mdm2 and the formation of Mdm2-p53 complexes. This protein-protein interaction decreased p53 function, which was activated by stretch and had the ability to increase transcription of Aogen and AT₁ receptors in myocytes. Moreover, p53-mediated up-regulation of Bax and down-regulation of Bcl-2 after stretch were inhibited by IGF-1 that also enhanced the expression of Bcl-2, resulting in an increased Bcl-2-to-Bax protein ratio in the cells. Thus, IGF-1 opposed myocyte apoptosis after stretch; this was accomplished by limiting the consequences of physical forces on p53-

Figure 9. A: Gel mobility assay illustrating p53 binding to its consensus sequence in the bax promoter. Nuclear extracts were obtained from nonstretched myocytes (NS) at 16 hours and stretched myocytes (S) at 6 and 16 hours (h) in the absence and presence of IGF-1. IGF-1 decreased p53 DNA binding activity at both time points. The **arrow** indicates the position of p53 shifted band. The p53-specific band, corresponding to nuclear extract obtained at 16 hours after stretch, was subject to competition with an excess of unlabeled self oligonucleotide (C) and with a monoclonal p53 antibody (Ab). The addition of an irrelevant antibody (Irr) or preincubation with an unlabeled mutated form of bax (Bax mut) did not interfere with p53 binding. Bax, bax probe in the absence of nuclear extracts. Optical density data were as follows: NS = 0.46 ± 0.22 ($n = 5$), S-6 hours = 1.83 ± 0.37 ($n = 5$), $P < 0.002$; S-16 hours = 2.94 ± 0.74 ($n = 5$), *versus* NS $P < 0.001$, *versus* S-6 hours $P < 0.02$; NS+IGF-1 = 0.41 ± 0.18 ($n = 5$), S-6 hours+IGF-1 = 0.80 ± 0.27 ($n = 5$), *versus* NS+IGF-1 not significant, *versus* S-6 hours $P < 0.02$; S-16 hours+IGF-1 = 0.79 ± 0.37 ($n = 5$), *versus* NS+IGF-1 not significant, *versus* S-16 hours $P < 0.001$. **B:** Gel mobility assay illustrating p53 binding to its consensus sequence in the Aogen promoter. Nuclear extracts were obtained from nonstretched myocytes (NS) at 16 hours and stretched myocytes (S) at 6 and 16 hours (h) in the absence and presence of IGF-1. IGF-1 decreased p53 DNA binding activity at both time points. **Arrows** indicate the position of p53 shifted bands. p53-specific bands, corresponding to nuclear extract obtained at 16 hours after stretch, were subject to competition with an excess of unlabeled self oligonucleotide (C) and with a monoclonal p53 antibody (Ab). The addition of an irrelevant antibody (Irr) did not interfere with p53 binding. Ao, Aogen probe in the absence of nuclear extracts. Optical density data of the two bands combined were as follows: NS = 0.76 ± 0.25 ($n = 5$), S-6 hours = 1.59 ± 0.27 ($n = 5$), $P < 0.001$; S-16 hours = 2.38 ± 0.25 ($n = 5$), *versus* NS $P < 0.001$, *versus* S-6 hours $P < 0.001$; NS+IGF-1 = 0.65 ± 0.15 ($n = 5$), S-6 hours+IGF-1 = 0.68 ± 0.18 ($n = 5$), *versus* NS+IGF-1 not significant, *versus* S-6 hours $P < 0.001$; S-16 hours+IGF-1 = 1.15 ± 0.16 ($n = 5$), *versus* NS+IGF-1 $P < 0.05$, *versus* S-16 hours $P < 0.001$. **C:** Gel mobility assay illustrating p53 binding to its consensus sequence in the AT₁ promoter. Nuclear extracts were obtained from nonstretched myocytes (NS) at 16 hours and stretched myocytes (S) at 6 and 16 hours (h) in the absence and presence of IGF-1. IGF-1 decreased p53 DNA binding activity at both time points. **Arrows** indicate the position of p53 shifted bands. p53-specific bands, corresponding to nuclear extract obtained at 16 hours after stretch, were subject to competition with an excess of unlabeled self oligonucleotide (C) and with a monoclonal p53 antibody (Ab). The addition of an irrelevant antibody (Irr) did not interfere with p53 binding. AT₁, AT₁ probe in the absence of nuclear extracts. Optical density data of the two bands combined were as follows: NS = 0.41 ± 0.12 ($n = 5$), S-6 hours = 1.16 ± 0.26 ($n = 5$), $P < 0.001$; S-16 hours = 2.18 ± 0.29 ($n = 5$), *versus* NS $P < 0.001$, *versus* S-6 hours $P < 0.001$; NS+IGF-1 = 0.43 ± 0.19 ($n = 5$), S-6 hours+IGF-1 = 0.35 ± 0.06 ($n = 5$), *versus* NS+IGF-1 not significant, *versus* S-6 hours $P < 0.001$; S-16 hours+IGF-1 = 0.56 ± 0.11 ($n = 5$), *versus* NS+IGF-1 not significant, *versus* S-16 hours $P < 0.001$.

Figure 10. Effects of stretch and IGF-1 on the quantity of the various forms of Mdm2 and p53 measured by Western blot analysis of myocyte lysates immunoprecipitated with Mdm2 antibody. Nonstretched: 90 kd; no IGF-1: not detectable, $n = 5$; IGF-1: not detectable, $n = 5$; 85 kd; no IGF-1: not detectable, $n = 5$; IGF-1: not detectable, $n = 5$; 76 kd; no IGF-1: OD = 0.04 ± 0.05 , $n = 5$; IGF-1: OD = 2.9 ± 0.9 , $n = 5$, $P < 0.001$; 57 to 58 kd; no IGF-1: OD = 2.1 ± 0.4 , $n = 5$; IGF-1: OD = 2.4 ± 0.5 , $n = 5$, NS; stretched myocytes for 5 hours: 90 kd; no IGF-1: not detectable, $n = 5$; IGF-1: OD = 6.5 ± 1.5 , $n = 5$, $P < 0.001$; 85 kd; no IGF-1: not detectable, $n = 5$; IGF-1: OD = 1.5 ± 0.5 , $n = 5$, $P < 0.001$; 76 kd; no IGF-1: OD = 2.7 ± 0.4 , $n = 5$; IGF-1: OD = 6.6 ± 0.9 , $n = 5$, $P < 0.001$; 57 to 58 kd; no IGF-1: OD = 0.9 ± 0.3 , $n = 5$; IGF-1: OD = 13 ± 2.4 , $n = 5$, $P < 0.001$; stretched myocytes for 16 hours: 90 kd; no IGF-1: not detectable, $n = 5$; IGF-1: OD = 5.2 ± 0.6 , $n = 5$, $P < 0.001$; 85 kd; no IGF-1: not detectable, $n = 5$; IGF-1: OD = 1.3 ± 0.5 , $n = 5$, $P < 0.001$; 76 kd; no IGF-1: OD = 8.6 ± 0.8 , $n = 5$; IGF-1: OD = 9.4 ± 1.6 , $n = 5$, NS; 57–58 kd; no IGF-1: OD = 9.2 ± 2.0 , $n = 5$; IGF-1: OD = 14 ± 3 , $n = 5$, $P < 0.05$. p53 in nonstretched myocytes: no IGF-1: not detectable, $n = 5$; IGF-1: not detectable, $n = 5$; stretched myocytes for 5 hours: no IGF-1: not detectable, $n = 5$; IGF-1: 10 ± 2 , $n = 5$, $P < 0.001$; stretched myocytes for 16 hours: no IGF-1: OD = 0.02 ± 0.04 , $n = 5$; IGF-1: OD = 9.8 ± 1.6 , $n = 5$, $P < 0.001$.

dependent Ang II formation and by attenuating the impact of this peptide on the initiation of the suicide program in stressed myocytes.

IGF-1, Ang II, and Apoptosis

Observations in this study document that the addition of a single dose of IGF-1 15 minutes before myocyte stretching reduced markedly the magnitude of apoptosis generated by the induction of this mechanical stimulus. This attenuation in cell death was coupled with a significant decrease in the concentration of Ang II in the medium. However, at 6 hours after stretching in the presence of IGF-1, the level of this peptide was essentially identical to that measured in control cultures of nonstretched myocytes, whereas apoptosis was decreased by only 43%. This apparent inconsistency most likely reflected the extent of apoptosis, triggered by the release of Ang II stored in the cells at the onset of myocyte stretching. Despite the administration of IGF-1, Ang II was significantly increased at 20 minutes after stretch. Moreover, an almost identical value of cell death has been detected previously, earlier than 6 hours after sarcomere elongation in the absence of IGF-1.¹⁶ At 20 hours of stretching, the inclusion of IGF-1 was associated with a 61% decrease in myocyte apoptosis and an amount of Ang II that was 52% lower than in stretched cells without IGF-1 but 55% higher than in control cultures. Thus, IGF-1 interfered only in part with the myocyte RAS; the initial block in Ang II formation noted at 6 hours was less apparent at 20 hours, when newly generated Ang II was again detectable in the medium. Importantly, losartan

Figure 11. Effects of stretch and IGF-1 on the quantity of the various forms of Mdm2 and p53 measured by Western blot analysis of myocyte lysates immunoprecipitated with p53 antibody. Nonstretched: 90 kd; no IGF-1: OD = 1.0 ± 0.4 , $n = 5$; IGF-1: OD = 2.8 ± 0.4 , $n = 5$, $P < 0.001$; 57 to 58 kd; no IGF-1: OD = 10 ± 3 , $n = 5$; IGF-1: OD = 13 ± 4 , $n = 5$, NS; stretched myocytes for 5 hours: 90 kd; no IGF-1: OD = 0.4 ± 0.2 , $n = 5$; IGF-1: OD = 12 ± 3 , $n = 5$, $P < 0.001$; 57 to 58 kd; no IGF-1: OD = 6.6 ± 1.4 , $n = 5$; IGF-1: OD = 14 ± 4 , $n = 5$, $P < 0.005$; stretched myocytes for 16 hours: 90 kd; no IGF-1: OD = 2.5 ± 0.5 , $n = 5$; IGF-1: OD = 5.9 ± 1.6 , $n = 5$, $P < 0.001$; 57 to 58 kd; no IGF-1: OD = 14 ± 3 , $n = 5$; IGF-1: OD = 42 ± 6 , $n = 5$, $P < 0.001$. Total p53 in nonstretched myocytes: no IGF-1: OD = 24 ± 5 , $n = 5$; IGF-1: OD = 15 ± 3 , $n = 5$, $P < 0.001$; stretched myocytes for 5 hours: no IGF-1: OD = 31 ± 5 , $n = 5$; IGF-1: OD = 20 ± 4 , $n = 5$, $P < 0.001$; stretched myocytes for 16 hours: no IGF-1: OD = 33 ± 6 , $n = 5$; IGF-1: OD = 10 ± 4 , $n = 5$, $P < 0.001$.

blocks completely stretch-mediated apoptosis¹⁶ by preventing ligand binding to surface AT₁ receptor on myocytes.

Findings here in this *in vitro* model, which mimics diastolic overload *in vivo*,^{35,36} provide some explanation for the beneficial effects of IGF-1 administration in the failing heart. Experimentally, therapeutic interventions with IGF-1 and growth hormone enhance cardiac hypertrophy and limit ventricular remodeling, improving myocardial function after infarction.³⁷ Similar adaptations, consisting of an increased muscle mass and reduced cavity volume, have been found in patients with idiopathic dilated cardiomyopathy.³⁸ Abnormal increases in resting tension of the myocardium *in vitro*,¹¹ comparable to those occurring acutely after myocardial infarction,³⁹ are characterized by myocyte apoptosis and side-to-side slippage of cells, the major cause of cardiac dilation in the overloaded heart. Elevations in diastolic wall stress may be coupled with the local release of Ang II and apoptosis.^{16,36} The ability of IGF-1 to prevent myocyte death is consistent with a more efficient preservation of cardiac mass and less restructuring of the wall. Additionally, IGF-1 operates through the down-regulation of the myocyte RAS, which affects negatively the evolution of the cardiomyopathic heart of ischemic and nonischemic origin.^{40,41} Constitutive overexpression of IGF-1 in myocytes inhibits the activation of cell death in the surviving myocardium after infarction, attenuating ventricular dilation, myocardial loading, and reactive hypertrophy.⁴ The current results may provide the basis for these *in vivo*

findings and for future use of IGF-1 in the decompensated heart.

IGF-1, p53, and Mdm2-p53 Complexes

Observations in the current study indicate that the anti-apoptotic action of IGF-1 on stretched myocytes was dependent on its capacity to depress p53 quantity and activity in the cells. This was documented by attenuation of p53 DNA binding to the promoter of bax, Aogen, and AT₁ receptor, which was paralleled by a decrease in the quantity of the respective gene products in the stressed myocytes. The mechanism by which IGF-1 affects p53 function was in part investigated here. Activation of the IGF-1 receptor by its ligand transmits a signal to its major substrates, which is subsequently transduced *via* a common effector pathway to the nucleus.²⁰ This may result in the phosphorylation of the amino terminus of p53,²¹ leading to the expression of mdm2^{22,27} that may form a complex with p53, inhibiting its DNA binding activity.²⁵ This contention is consistent with the present results documenting that the forms of Mdm2 capable of interacting with p53 were increased by IGF-1. Serine phosphorylation of specific sites on the amino terminus may occur *via* Raf-1 kinase, Jun-kinase, or MAP-kinase.²¹ Additionally, the association of p53 with Mdm2 decreases the stability of the tumor suppressor, accelerating its degradation.^{23,24} Both of these events may have been implicated in the inhibitory action of IGF-1 on p53 in stretched myocytes.

The expression of mdm2 is controlled by p53 that binds to two consensus motifs present in the first intron of the mdm2 promoter.⁴² Endogenous low levels of Mdm2 are sufficient to regulate p53 stability, providing a rapid turnover and a short half-life of the transcription factor.⁴² Enhanced expression of Mdm2 is characterized by a reduction in the cellular amount of p53, which occurs *via* an up-regulated proteasome-dependent degradation.^{23,24} The repression of p53-mediated transcription by Mdm2 involves a protein-protein interaction that masks the activation domain of p53, or disrupts functional association between the p53 domain and multiple components of the basal transcription machinery.⁴³ Moreover, Mdm2 promotes cell survival and proliferation.⁴⁴ In addition to its action on p53, Mdm2 stimulates the transcription of cell-cycle-related genes through the activation of E2F-1⁴⁴ and interacts with the retinoblastoma protein, inhibiting its growth suppressor effect.⁴⁵

Although the signaling cascade by which IGF-1 down-regulates p53 remains to be identified, the impact of p53 and Mdm2-p53 complexes on the modulation of the local RAS has been characterized here. By opposing p53 function in stretched myocytes, Mdm2 attenuated the transcriptional activation of Aogen and AT₁ receptors and the generation of Ang II in myocytes. In a manner opposite to IGF-1, Ang II may phosphorylate through protein kinase C the carboxy terminus of p53, enhancing its transcriptional activity.⁴⁶ This may result in expression of pro-apoptotic genes and repression of anti-apoptotic genes in myocytes, increasing the susceptibility of cells

to die. Such hypothesis is supported by results obtained with forced expression of wild-type p53 in adult ventricular myocytes.¹⁹

In conclusion, IGF-1 induces Mdm2 and, by this mechanism, may attenuate the function of p53, down-regulating the expression of several p53-inducible genes implicated in the modulation of apoptosis and local RAS. The inhibitory effect of Mdm2 on p53 decreases the expression of Bax and increases the expression of Bcl-2, enhancing the resistance of myocytes against apoptotic stimuli. The down-regulation of Aogen, renin, and AT₁ receptors on myocytes and the reduced synthesis and secretion of Ang II in the presence of IGF-1 appear to be critical in the prevention of cell death by this growth factor with sarcomere elongation. Although the extrapolation of *in vitro* results to the *in vivo* state requires considerable caution, the possibility may be advanced that therapeutic interventions with IGF-1 may protect, at least in part, from the stimulation of apoptosis after sudden increases of diastolic load in the diseased heart.

Acknowledgment

The expert technical assistance of Maria Feliciano is greatly appreciated.

References

- Rodriguez-Tarduchy G, Collins MKL, Garcia I, Lopez-Rivas A: Insulin-like growth factor 1 inhibits apoptosis in IL-3 dependent hemopoietic cells. *J Immunol* 1992, 149:535-540
- D'Mello SR, Galli C, Ciotti T, Calissano P: Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc Natl Acad Sci USA* 1993, 90:10989-10993
- Chun SY, Billig H, Tilly JL, Furuta I, Tsafiri A, Hsueh AJ: Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor 1. *Endocrinology* 1994, 135:1845-1853
- Li Q, Li B, Wang X, Leri A, Jana KP, Liu Y, Kajstura J, Baserga R, Anversa P: Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. *J Clin Invest* 1997, 100:1991-1999
- Gluckman P, Klempt N, Guan J, Mallard C, Sirimanee E, Dragunow M, Klempt M, Singh K, Williams C, Nikolics K: A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun* 1992, 182:593-599
- Buerke M, Murohara T, Skurk C, Nuss C, Tomaselli K, Lefer AM: Cardioprotective effect of insulin-like growth factor 1 in myocardial ischemia followed by reperfusion. *Proc Natl Acad Sci USA* 1995, 92:8031-8035
- Sell C, Baserga R, Rubin R: Insulin-like growth factor (IGF-1) and the IGF-1 receptor prevent etoposide-induced apoptosis. *Cancer Res* 1995, 55:303-306
- Resnicoff M, Abraham D, Yutanawiboonchai W, Rotman H, Kajstura J, Rubin R, Zoltick P, Baserga R: The insulin-like growth factor 1 receptor protects tumor cells from apoptosis *in vivo*. *Cancer Res* 1995, 55:2463-2469
- Resnicoff M, Burgaud J-L, Rotman HL, Abraham D, Baserga R: Correlation between apoptosis, tumorigenesis and levels of insulin-like growth factor-1 receptor. *Cancer Res* 1995, 55:3739-3741
- Zeng G, Quon MJ: Insulin-stimulated production of nitric oxide is inhibited by wortmannin: direct measurement in vascular endothelial cells. *J Clin Invest* 1996, 98:894-898
- Cheng W, Li B, Kajstura J, Li P, Wolin MS, Sonnenblick EH, Hintze TH,

- Olivetti G, Anversa P: Stretch-induced programmed myocyte cell death. *J Clin Invest* 1995, 96:2247–2259
12. Jung Y-K, Miura M, Yuan J: Suppression of interleukin-1 β -converting enzyme-mediated cell death by insulin-like growth factor. *J Biol Chem* 1996, 271:5112–5117
13. Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, Kjaw B-A: Apoptosis in myocytes in end-stage heart failure. *N Engl J Med* 1996, 335:1182–1189
14. Colucci WS: Apoptosis in the heart. *N Engl J Med* 1996, 335:1224–1226
15. Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, DiLoreto C, Beltrami CA, Krajewski S, Reed JC, Anversa P: Apoptosis in the failing human heart. *N Engl J Med* 1997, 336:1131–1141
16. Leri A, Claudio PP, Li Q, Wang X, Reiss K, Wang S, Malhotra A, Kajstura J, Anversa P: Stretch-mediated release of angiotensin II induces myocyte apoptosis by activating p53 that enhances the local renin-angiotensin system and decreases the Bcl-2-to-Bax protein ratio in the cell. *J Clin Invest* 1998, 101:1326–1342
17. Miyashita T, Reed J: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995, 80:293–299
18. Miyashita T, Harigai M, Hanada M, Reed JC: Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* 1994, 54:3131–3135
19. Pierzchalski P, Reiss K, Cheng W, Cirielli C, Kajstura J, Nitahara JA, Rizk M, Capogrossi MC, Anversa P: p53 induces myocyte apoptosis via the activation of the renin-angiotensin system. *Exp Cell Res* 1997, 234:57–65
20. Baserga R, Resnicoff M, D'Ambrosio C, Valentini B: The role of IGF-1 receptor in apoptosis. *Vitam Horm* 1997, 53:65–98
21. Milczarek GJ, Martinez J, Bowden GT: p53 phosphorylation: biochemical and functional consequences. *Life Sci* 1997, 60:1–11
22. Lohrum M, Scheidtmann KH: Differential effects of phosphorylation of rat p53 on transactivation of promoters derived from different p53 responsive genes. *Oncogene* 1996, 12:2527–2539
23. Haupt Y, Maya R, Kazaz A, Oren M: Mdm2 promotes the rapid degradation of p53. *Nature* 1997, 387:296–299
24. Kubbutat MHG, Jones SN, Vousden KH: Regulation of p53 stability by Mdm2. *Nature* 1997, 387:299–303
25. Momand J, Zambetti GP: Mdm-2: "Big Brother" of p53. *J Cell Biochem* 1997, 64:343–352
26. Lee AA, Delhaas T, Waldman LK, MacKenna DA, Villarreal FJ, McCulloch AD: An equibiaxial strain system for cultured cells. *Am J Physiol* 1996, 271:1400–1408
27. Hecker D, Page G, Lohrum M, Weiland S, Scheidtmann KH: Complex regulation of the DNA-binding activity of p53 by phosphorylation: differential effects of individual phosphorylation sites on the interaction with different binding motifs. *Oncogene* 1996, 12:953–961
28. Wallenstein S, Zucker CL, Fleiss JL: Some statistical methods useful in circulation research. *Circ Res* 1980, 47:1–9
29. Leri A, Liu Y, Malhotra A, Li Q, Stiegler P, Claudio PP, Giordano A, Kajstura J, Hintze TH, Anversa P: Pacing-induced heart failure in dogs enhances the expression of p53 and p53-dependent genes in ventricular myocytes. *Circulation* 1998, 97:194–203
30. Benjamin LJ, Jalil JE, Tan LB, Cho K, Weber KT, Clark WA: Isoproterenol-induced myocardial fibrosis in relation to myocyte necrosis. *Circ Res* 1989, 67:657–670
31. Ko LJ, Prives C: p53: puzzle and paradigm. *Genes Dev* 1996, 10:1054–1072
32. Momand J, Zambetti GP: Analysis of the proportion of p53 bound to mdm-2 in cells with defined growth characteristics. *Oncogene* 1996, 12:2273–2289
33. Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavletich NP: Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Nature* 1996, 274:948–953
34. Olson DC, Marechal V, Momand J, Cheng J, Romocki C, Levine AJ: Identification and characterization of multiple mdm-2 proteins and mdm-2-p53 protein complexes. *Oncogene* 1993, 8:2353–2360
35. Grossman W, Jones D, McLaurin LP: Wall stress and patterns of hypertrophy in the human left ventricle. *J Clin Invest* 1975, 56:56–64
36. Sadoshima J, Xu Y, Slayter HS, Izumo S: Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* 1993, 75:977–984
37. Duerr RL, Huang S, Miralikiabar HR, Clark R, Chien KR, Ross J Jr: Insulin-like growth factor-1 enhances ventricular hypertrophy and function during the onset of experimental cardiac failure. *J Clin Invest* 1995, 95:619–627
38. Fazio S, Sabatini D, Capaldo B, Vigorito C, Giordano A, Guida R, Pardo F, Biondi B, Saccà L: A preliminary study of growth hormone in the treatment of dilated cardiomyopathy. *N Engl J Med* 1996, 334:809–814
39. Pfeffer MA, Braunwald E: Ventricular remodeling after myocardial infarction. *Circulation* 1990, 81:1161–1172
40. Pfeffer MA, Braunwald E, Moye LA, Basta L, Brown EJ, Cuddy TE, Davis BR, Geltman EM, Goldman S, Flaker GC, Klein M, Lamas GA, Packer M, Rouleau J, Rouleau JL, Rutherford J, Wertheimer JH, Hawkins CM: Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med* 1992, 327:669–677
41. Iwai N, Shimoike H, Kinoshita M: Cardiac renin-angiotensin system in the hypertrophied heart. *Circulation* 1995, 92:2690–2696
42. Wu X, Bayle JH, Olson D, Levine AJ: The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* 1993, 7:1126–1132
43. Thut CJ, Goodrich JA, Tjian R: Repression of p53-mediated transcription by MDM2: a dual mechanism. *Genes Dev* 1997, 11:1974
44. Piette J, Neel H, Maréchal V: Mdm2: keeping p53 under control. *Oncogene* 1997, 15:1001–1010
45. Xiao Z-X, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR, Livingston DM: Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* 1995, 375:694–698
46. Hupp TR, Lane DP: Regulation of the cryptic sequence-specific DNA-binding function of p53 by protein kinase. *Cold Spring Harbor Symp Quant Biol* 1994, 59:195–206