Induction of Apoptosis and Fas Receptor/Fas Ligand Expression by Ischemia/Reperfusion in Cardiac Myocytes Requires Serine 727 of the STAT-1 Transcription Factor but Not Tyrosine 701*

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Previously we reported that ischemia results in apoptosis and is accompanied by phosphorylation on Tyr-701 and increased expression and transcriptional activity of the signal transducer and activator of transcription-1 (STAT-1). In the present study, we show that exposure of cardiomyocytes to ischemia induced the phosphorylation of STAT-1 at another site, Ser-727. Moreover, STAT-1 is critical for the induction of Fas receptor and Fas ligand expression by ischemia/reperfusion (I/R). Transcriptional activation of Fas and FasL was dependent on Ser-727 of STAT-1 but was independent of Tyr-701. Similarly, Ser-727 but not Tyr-701 was required for enhancement of cardiomyocyte cell death by STAT-1 during I/R. In addition, inhibition of the p38 pathway prevented the induction and transcriptional activation of Fas and FasL in cardiac cells exposed to I/R, whereas inhibition of p42/p44 MAPK had no effect. Finally, I/R also induced phosphorylation of STAT-1 on Ser-727 and expression of Fas/FasL in ventricular myocytes in the intact heart ex vivo. These results indicate that Fas/FasL genes and apoptosis are activated by STAT-1 in cardiac myocytes exposed to I/R and these effects are dependent on the Ser-727 but not the Tyr-701 phosphorylation sites of STAT-1.

Loss of cardiac myocytes by programmed cell death (apoptosis) is an important mechanism in the development of cardiac failure during injury due to ischemia/reperfusion and myocardial infarction (1, 2). Recent studies have indicated that apoptotic death occurs in cardiac cells exposed to a variety of damaging stimuli both in vitro and in the intact heart in vivo (3–6). Thus, cardiac cells exposed to a hypoxic/ischemic insult followed by reperfusion undergo apoptotic cell death in vitro (3, 6). Similarly, apoptotic cell death is also observed in the intact heart following ischemia in vivo (4, 5). Despite the convincing evidence that apoptosis occurs, the mechanisms and signaling pathways which lead to apoptosis following hypoxic/ischemic stimuli in cardiac cells are as yet unknown.

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The Fas (CD95)/Fas ligand (CD95L) system is a key regulator of apoptosis. Fas, a 48-kDa transmembrane receptor glycoprotein, is a member of the tumor necrosis factor receptor superfamily of surface molecules (7), and Fas expression is enhanced in cardiomyocytes exposed to hypoxia (8). The Fas ligand (Fasl) which binds to the Fas receptor belongs to the tumor necrosis factor superfamily of cytokines and can exist as a membrane-bound or a soluble protein. Binding of Fas by its cognate ligand Fasl mediates the recruitment of the Fas-associated death domain adapter molecule to the receptor complex. In turn, the upstream procaspase-8 is attracted to the complex and undergoes autoproteolytic activation (9). This then leads to the activation of downstream caspases including caspase-3, followed by cleavage of key regulatory proteins resulting in apoptosis (9).

Cytokines and growth factors are known to modulate growth, survival, and death in many cell types. These pro- and anti-apoptotic effects are mediated, at least in part, by signaling through a family of transcription factors called signal transducers and activators of transcription (STATs).† Six STATs have been cloned, some of which exist in different isomeric forms, and all share a high degree of conservation of their structural domains (10, 11). These include a DNA-binding domain, a site for homo- or heterodimer formation and also conserved residues for tyrosine and serine phosphorylation by Janus protein kinases (JAKs) and mitogen-activated protein kinases (p42/44 and p38 MAPKs), respectively (12–13). STATs phosphorylated by JAK/MAPK dimerize and translocate to the nucleus where they transactivate STAT responsive genes.

Recent studies have implicated STATs in both pro- and anti-apoptotic signaling. For example, a STAT-1 deficient cell line is resistant to tumor necrosis factor a-induced apoptosis (14), and STAT-1 has been implicated in transcriptional activation of some caspases (15). In contrast, a dominant negative form of STAT-3 has been shown to prevent interleukin-6-dependent cell growth and induce apoptosis in a myeloid cell line (16). In addition, the ability of IFN a/b to rescue T cell clones from apoptosis induced by interleukin-2 withdrawal is dependent on STAT-2 (17), and the anti-apoptotic effects of interleukin-9 appear to involve homo/hetero dimers of STAT-1, -3, and -5 (18).

Recently we have started to assess the role of STATs in...
mediating pro- and anti-apoptotic signals in the heart. For example, we have recently demonstrated that STAT-1 activation plays a critical role in inducing apoptosis in cardiac cells exposed to ischemia/reperfusion (I/R) in a caspase-1-dependent manner (19). In addition, we have shown that the phosphorylated active form of STAT-1 reduces the basal activity of the promoters of the anti-apoptotic genes, Bcl-2 and Bcl-x, whereas STAT-3 increases promoter activity. Hence, STAT-1 and STAT-3 have opposing actions on the Bcl-2 and Bcl-x promoters (20). To extend these studies we have investigated the role of STAT-1 in regulating expression of Fas and Fas ligand during I/R of cardiac myocytes and in the intact perfused heart ex vivo.

MATERIALS AND METHODS

Plasmid Constructs and DNA Transfection—The STAT-1, STAT1-Ser-727 mutant (serine to alanine) and STAT1-Tyr-701 mutant (tyrosine to phenylalanine) expression vectors were kindly provided by Curt Horvath (Mount Sinai Medical Center, New York). The STAT-1 anti-sense vector was constructed by inserting the STAT-1 cDNA in the antisense orientation into the pcDNA3 vector. The dominant negative MKK6 construct was kindly provided by Ei skuo Nishida (Kyoto University, Kyoto, Japan). Genomic DNA fragments including the Fas (21) and FasL promoters regions were obtained from a human cDNA library, and plasmid upstream of the firefly luciferase gene. The Fas and FasL promoter regions extended to nuclear localization sites –1436 and –2373, respectively, with respect to the transcription start site as +1. The structure of the FasL promoter will be described elsewhere. The STAT-1 reporter gene, was constructed by ligating a STAT-1-binding site from SIEm67 DNA (5'-GATCTGATTACGGGAAATG-3') into pGL2-luciferase (Promega). Transfection of reporter construct and expression vectors into cardiac cells, as performed by the calcium phosphate method. The STAT-1-deficient cells (U3A) was kindly provided by Dr. Ian Kerr (ICRF, London).

Neonatal Rat Cardiac Cultures and Isolated Intact Heart Using the Langendorf Perfusion Apparatus ex Vivo—Ventricular myocytes isolated from the hearts of neonatal rats (Harlan Sprague-Dawley) that were less than 2 days old were cultured as described previously (22) with the following modifications. After collagenase digestion the cells were pre-plated in medium consisting of Dulbecco’s modified Eagle’s medium (1000 μg of glucose/liter, 1 mmol/liter l-glutamine, 100 units/ml penicillin/streptomycin (all Life Technologies, Inc.) supplemented with 15% (v/v) fetal calf serum on 10-cm tissue culture dishes. Pre-plating of the cell suspension for 30 min allows contaminating fibroblasts to attach and the myocytes remain free within the culture media. Subsequent to this incubation, the cardiac myocyte cell suspension was transferred onto six-well (3 cm) gelatin-coated plates (Falcon) at a density of 10^5 cells per well. This plating system yields cell cultures that are more than 95% myocytes as determined by indirect staining with a monoclonal mouse antibody to desmin. Transient transfection experiments were performed in neonatal cardiac myocytes following 24 h in culture and the DNA precipitates were left for 16 h. The cells were then washed in 1 time in phosphate-buffered saline and the media was replaced by the above media containing reduced fetal calf serum at 1% (v/v) (hereafter referred to as growth medium) for an additional 24 h before experimentation. Within 3 days a confluent monolayer of spontaneously beating myocytes was formed.

To subject the cells to simulated ischemia, the normal growth media of the cardiomyocyte cultures was replaced with 1 ml of ischemic buffer; 137 mM NaCl, 12 mM KCl, 0.49 mM MgCl2, 0.9 mM CaCl2·H2O, 4 mM HEPES, 20 mM Na lactate, 10 mM deoxyglucose (Sigma, Dorset, United Kingdom) (pH 6.2) and the cells were incubated at 37 °C in an ischemic chamber for 4 h in an atmosphere of 0% oxygen, 5% CO2, balance gas (BOC gases). In some experiments cells exposed to simulated ischemia for 4 h were returned to a normoxic environment for a further 16 h or re-oxygenation to simulate reperfusion. In some cases cardiomyocytes were also treated for 16 h with IFN-γ (50 ng/ml) (Sigma) following transient transfection with the indicated reporter constructs. The MEK1 chemical inhibitor PD98095 and the p38 MAPK chemical inhibitor SB203580 were purchased from New England BioLabs (Beverly, MA).

For the ex vivo studies, hearts were quickly removed and mounted in a Langendorf perfusion apparatus and perfused with an oxygenated Krebs-Henseleit buffer as previously described (23). The isolated hearts were subsequently exposed to 35 min of zero-flow global ischemia followed by 2 h of reperfusion. Thereafter treated hearts cross-sectioned from the base to the apex into slices which were either frozen directly for Western blot analysis or placed in 4% formaldehyde and embedded in molten paraffin until immunofluorescence analysis.

Western Blotting—Cells were exposed to a 4 h ischemic insult as described above and harvested immediately or following 16 h of reoxygenation. Approximately 1 x 10^6 cells were harvested in 100 μl of 2% trichloroacetic acid (TCA) prechilled on dry ice. The precipitated SDS–polyacrylamide gel electrophoresis sample buffer. Cardiac ventricular tissue (~0.2 g) was homogenized in 0.2 ml of ice-cold lysis buffer, then after centrifugation the supernatant was removed and the cells transferred into 1.5-ml microcentrifuge tubes. Cells were lysed by three 20-s bursts of sonication in 1 ml of ice-cold lysis buffer (50 mM HCl, 0.1% (v/v) 2-mercaptoethanol, 0.1% Triton X-100, 10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride) cooled on ice between each sonication. Tissue debris was removed by centrifugation (12,000 rpm, 10 min 4 °C) and an equal volume of SDS buffer was added to the supernatant which was boiled for 3 min. Samples were then electrohoresed on a 8% SDS-polyacrylamide gel, transferred to nitrocellulose filters, and subjected to Western blotting with specific antibodies against anti-phospho-STAT1Ser-727 (Zymed Laboratories Inc.), anti-STAT-1, anti-Fas, anti-FasL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-p38 and anti-p38 (New England BioLabs).

Immunofluorescence—Immunofluorescence staining was performed as described previously (24). Briefly, ventricular sections were dewaxed, deparaffin in xylene, and subsequently rehydrated with 100% ethanol, 90% ethanol, 70% ethanol, and distilled water. Sections were then pre-treated in TBS containing 1 μg/ml trypsin, washed with TBS and incubated with anti-phospho-STAT1Ser-727 antibody (1:50) for 1 h followed by a further 1 h with either anti-Fas or anti-FasL antibody and subsequently with rhodamine or fluorescein-conjugated secondary antibody for a further 1 h. Sections were thoroughly washed in TBS and slides were mounted under glass coverslips using antifade mounting medium and examined by confocal fluorescent microscopy.

Assessing Apoptosis—For assessing the effects of STAT-1, STAT1-701, or STAT1–727 I/R-induced cell death in primary neonatal cardiac myocytes, 5 μg of expression vectors were co-transfected with the calcium phosphate method with 2 μg of pCMV-β-galactosidase vector to mark the transfected cells by the calcium phosphate method. As assayed by this method 10–15% of cells were successfully transfected. 24 h following transfection, cells were fixed in 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-galactopyranoside to test for β-galactosidase activity. TUNEL assays were also performed on the fixed cells. Percentage of apoptotic cells were determined by calculating the fraction of cells positive for both β-galactosidase and TUNEL as a fraction of total β-galactosidase positive cells. In all cases a total of 200 cells were counted per well.

Band Shift Assays—U3A cells transfected with wild-type STAT-1, STAT1–701, or STAT1–727 mutant were exposed to ischemia for 4 h, and cells were harvested in cold phosphate-buffered saline. Cell extracts were prepared for band shift assay with a STAT-1 SIEm67 DNA probe that serves as a binding site for STAT-1. Samples were also incubated with either a STAT-1 or STAT-3 antibody for 30 min prior to incubation with the DNA probe to determine the specificity of the complex.

Statistical Analysis—All results are expressed as mean ± S.E. of at least three independent experiments unless stated otherwise. Paired data were evaluated by Student’s t test. A one-way ANOVA was used for multiple comparisons. A value of p < 0.05 was considered significant.

RESULTS

Ischemia Induces Phosphorylation of Both p38 MAPK and STAT-1 Ser-727 and Increases Expression of Fas and FasL—Our previous studies have demonstrated the enhanced expression and activation by phosphorylation on tyrosine 701 (via the JAK pathway) of the transcription factor STAT-1 in cardiomyocytes exposed to I/R (19). We also showed that the pro-apoptotic factor caspase-1 was one STAT-1 target gene involved in promoting the apoptosis effect of STAT-1 (19). In the present study we assessed whether apoptosis induced by simulated I/R in cultured neonatal cardiac myocytes also induced the phosphorylation of STAT-1 on serine 727 mediated via the p42/44 or p38 MAPK pathway. As shown in Fig. 1, cardiac myocytes exposed to simulated ischemia alone showed enhanced STAT-1 Ser-727 phosphorylation as well as STAT-1 enhanced expression and the intensity of STAT-1 Ser-727 phosphorylation was
increased further following subsequent re-oxygenation without any change in total STAT-1 level. Recently phosphorylation of STAT-1 at Ser-727 was shown to be dependent on the activation of p38 MAPK (25–27). Therefore we also examined the levels of active phosphorylated p38 MAPK in cardiomyocytes exposed to simulated ischemia or I/R. As shown in Fig. 1, exposure of cardiomyocytes to simulated ischemia showed enhanced phosphorylation of p38 MAPK. However, the level of phospho-p38 MAPK reverted to undetectable control levels following I/R. The above data suggests that the phosphorylation of p38 MAPK occurs as a rapid and acute response, while induction of phospho-STAT-1 Ser-727 is a progressive and sustained response to ischemia and I/R.

We next determined whether the observed changes in phosphorylation of p38 MAPK and STAT-1 Ser-727 in cardiomyocytes exposed to I/R correlated with changes in the levels of both Fas and FasL. Exposure of cardiomyocytes to simulated I/R induced the expression of Fas and FasL (Fig. 1). The induction of Fas and FasL was not observed in cardiomyocytes exposed to ischemia alone, suggesting a time-dependent response with the re-oxygenation/reperfusion phase required for maximal induction of Fas and FasL. Interestingly, the p38 MAPK inhibitor SB203580 attenuated the enhancement of both Fas and FasL protein levels exposed to I/R. Furthermore, the p38 inhibitor also abrogated the enhancement of STAT-1 Ser-727 phosphorylation (Fig. 2).

**Fas and FasL Are Target Genes for STAT-1 Signaling**—The above data demonstrate that both STAT-1 phosphorylation on Ser-727 and Fas/FasL are induced in cardiomyocytes exposed to I/R correlated with changes in the levels of both Fas and FasL. Exposure of cardiomyocytes to simulated I/R induced the expression of Fas and FasL (Fig. 1). The induction of Fas and FasL was not observed in cardiomyocytes exposed to ischemia alone, suggesting a time-dependent response with the re-oxygenation/reperfusion phase required for maximal induction of Fas and FasL. Interestingly, the p38 MAPK inhibitor SB203580 attenuated the enhancement of both Fas and FasL protein levels exposed to I/R. Furthermore, the p38 inhibitor also abrogated the enhancement of STAT-1 Ser-727 phosphorylation (Fig. 2).

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FIG. 4. **a**, Western blots of protein extracts from STAT-1-deficient cells (U3A) transfected with the STAT1-S727A (727) mutant or the STAT1-Y701F (701) mutant and treated with IFN-γ for 30 min and blotted with phosphospecific antibodies recognizing the Phospho-STAT1-Ser-727 (ST1-p727) or phospho-STAT1-Tyr-701 (ST1-p701) or unphosphorylated STAT-1 (ST1). **b**, band shift assay using nuclear extracts from U3A cells transfected with wild-type STAT-1 (ST1), STAT1–701 (ST1701), or STAT1–727 (ST1727) mutant and exposed to simulated ischemia for 4 h. Nuclear extracts were also incubated with an anti-STAT-1 (Ab-ST1) or anti-STAT-3 (Ab-ST3) antibody. Effects of IFN-γ or simulated I/R with either wild-type STAT-1 (ST1), mutant STAT-1–701 (701), or mutant STAT-1–727 (727) on transactivating the Fas (**c**) and FasL (**d**) promoter activity in neonatal cardiomyocytes. In all cases luciferase activity was assessed in a total of three experiments. **e**, luciferase promoter activity with the STAT-1 reporter construct transfected into the U3A cells and co-transfected with wild-type STAT-1 (ST1) alone or together with the STAT1–701 mutant (ST1701). Cells were exposed to INF-γ for 24 h and luciferase activity was assessed from a total of three experiments.
IFN-γ/STAT-1 and simulated I/R-induced activation of the Fas and FasL promoters (Fig. 3). These results indicate that the Fas and FasL genes are target genes for STAT-1. In agreement with the analysis, both the Fas and FasL promoters reveal several potential STAT-1-binding sites.

Ser-727 but Not Tyr-701 Is Essential for STAT-1 Activation of the Fas and FasL Promoters—Recently it has been demonstrated that phosphorylation of STAT-1 at Ser-727 by p38 MAPK is able to modulate STAT-1 signaling pathways (25–27). STAT-1 Ser-727 has been shown to be a target for both ERK and p38 MAPKs, while STAT-1 Tyr-701 is a target for JAKs. Our previous study also demonstrated that STAT-1 enhanced the response to I/R-induced cell death (19). Therefore, in the present study we assessed the functional activity of two STAT-1 mutants in which Ser-727 had been mutated to alanine or Tyr-701 had been mutated to phenylalanine for their ability to modulate the activity of the Fas and FasL promoters. Each of these mutants was first transfected into STAT-1-deficient cells and treated with IFN-γ and cell extracts blotted with antibodies to total STAT-1 or STAT-1 phosphorylated on Ser-727 or Tyr-701. As shown in Fig. 4a, the Phe-701 mutant was not phosphorylated on position 701 but was phosphorylated at position 727, whereas the Ala-727 mutant had the opposite pattern of phosphorylation. Moreover, in accordance with previous results (29), both the wild-type and the two mutant forms of STAT-1 were able to bind to DNA in a DNA mobility shift assay using nuclear extracts from the transfected STAT-1-deficient cells (Fig. 4b). In addition, this retarded band was abolished by an anti-STAT-1 antibody, but not anti-STAT-3 antibody, in nuclear extracts from STAT-1-deficient U3A cells exposed to ischemia, thus confirming it as STAT-1.

Most interestingly, transfection of STAT1–727 mutant greatly reduced the activation of both Fas and FasL promoters compared with the wild-type STAT-1 in cardiac cells following exposure to either IFN-γ or simulated I/R. In contrast, STAT1–

**TABLE I**

Proportion of cardiac myocytes positive for phopho-STAT1–727, Fas, and FasL labeling in ischemic reperfused rat heart

|           | STAT-1 | FasL | Fas |
|-----------|--------|------|-----|
| Control   | 0.1%   | 0.1% | 0.1%|
| Ischemia (35') | 26% ± 3 | 3.3% ± 1 | 4.9% ± 1 |
| I/R (35 min + 60' min) | 43% ± 5 | 27% ± 2 | 36% ± 4 |

FIG. 5. The p38 MAPK chemical inhibitor SB203580 (SB), 10 μM, or the vector expressing a dominant-negative upstream MAPK activator of p38, MKK6 (dn-MKK6), or a dominant negative JNK (dn-JNK) but not a dn-MEK1 vector or the MEK1 chemical inhibitor PD98059 (25 μM) abrogated simulated I/R-induced Fas (a) and FasL (b) promoter activity. In all cases luciferase activity was assessed in a total of three experiments.

FIG. 6. Modulation of apoptotic death (TUNEL assay) in cardiac myocytes overexpressing wild-type STAT-1, mutant STAT1–701, or mutant STAT1–727 following exposure to I/R. Results represent a total of three independent experiments.

FIG. 7. Phosphorylation of STAT-1 on Ser-727, p38 MAPK, and the induction of Fas and FasL in the intact perfused heart exposed to I/R. Western blots were performed with specific antibodies to the indicated protein. Similar results were seen in three independent experiments.
and JNK but not the ERK1/ERK2 MAPKs are potential promoters following I/R (Fig. 5). These studies indicate that no significant effect on the activation of either the Fas or FasL promoter containing a STAT-1-binding site (Fig. 4c). Hence, this clearly demonstrates that the effect of different STAT-1 mutants is different on different STAT-1 responsive genes.

To investigate the possible role of the p38 MAPK in this effect pathway we repeated our transfection experiments in the presence or absence of co-transfected dominant negative dn-MEKK6, an upstream activator of p38 MAPK. Inhibition of p38 MAPK via dn-MEKK6 reduced the level of activation of Fas and FasL promoter activity in cardiac cells exposed to simulated I/R (Fig. 5, a and b). Furthermore, addition of the chemical inhibitor of p38 MAPK, SB203580, also reduced the activation of Fas and FasL promoter activity following I/R (Fig. 5).

Recently the other two major MAPK, JNK, and p42/p44 MAPK have also been shown to modulate the expression of Fas and FasL in non-cardiac cells (31, 32). Accordingly we tested the effects of JNK and ERK MAPKs in modulating STAT-1-mediated transactivation of the Fas and FasL promoters in primary cardiomyocytes. As shown in Fig. 5, transfection of a dominant-negative JNK together with the STAT-1 expression vector reduced the level of activation of both the Fas and FasL promoters in cardiac cells exposed to simulated I/R. However, co-transfection of STAT-1 together with a dominant-negative MEK1 expression vector (the upstream activator of p42/p44), or pretreatment of cardiac cells with the specific MEK1 chemical inhibitor PD98059 in cardiac cells exposed to simulated I/R had no significant effect on the activation of either the Fas or FasL promoters following I/R (Fig. 5). These studies indicate that p38 and JNK but not the ERK1/ERK2 MAPKs are potential candidates for modulating STAT-1 activation of the Fas and FasL genes acting via the phosphorylation at Ser-727 of STAT-1.

Serine Phosphorylation of STAT-1 at 727 Is Required for Mediating Apoptosis in Cardiac Myocytes—We have previously demonstrated that overexpression of STAT-1 induced apoptotic death in cardiac myocytes and overexpression of an antisense STAT-1 (to reduce endogenous levels of STAT-1) reduced cell death (19). Therefore we investigated whether the two STAT-1 mutants STAT1–701 or STAT1–727 can also modulate the levels of apoptotic death in cardiac myocytes following exposure to I/R. Overexpression of wild-type or STAT1–701 mutant enhanced the levels of apoptotic death in cardiac myocytes (Fig. 6). However, overexpression STAT1–727 mutant in cardiac myocytes resulted in much lower levels of apoptotic cell death than those observed with either wild-type or the STAT1–701 mutant (Fig. 6). Thus, these results demonstrate that the STAT1–727 and not STAT1–701 phosphorylation site is critical not only for mediating the enhancement of Fas and FasL gene expression but also in promoting apoptosis following exposure to cardiac cells to I/R.

I/R Induces Phosphorylation of p38 MAPK/STAT-1 Ser-727 and Fas/FasL Expression in the Intact Heart—Finally we examined whether our in vitro data with cardiac cells on simulated I/R-induced Fas and FasL expression together with the induction of phospho-STAT-1 Ser-727 and phospho-p38 MAPK were also reproducible in the intact heart. Experiments were performed using 35-min coronary occlusion to produce ischemia in the intact heart ex vivo in a Langendorff perfusion system. After exposure to 35-min ischemia the hearts were reperfused for a further 2 h. The left ventricles were then removed and Western blotting performed to assess the levels of phospho-STAT1 Ser-727, phospho-p38 MAPK, Fas, and FasL. As shown in Fig. 7, the levels of phospho-STAT Ser-727, phospho-p38 MAPK, Fas, and FasL all increased following I/R in the intact heart, whereas the total level of STAT-1 did not change. Furthermore, immunofluorescent staining also demonstrated an increase in phospho-STAT-1 Ser-727 positive staining in ventricular tissue sections in response to ischemia/reperfusion which was accompanied by an increase in the number of Fas- and FasL-positive myocytes (Table I). In addition, immunofluorescent confocal microscopy also demonstrated cardiomyocyte staining positive for STAT1–727 in the nucleus and cytoplasm (Fig. 8). A similar co-localized staining pattern was also seen with FasL and STAT-1 Ser-727 (data not shown). Thus, the increased levels of Fas and FasL during I/R may be the result of the transcriptional activation of STAT-1 translocated to the nucleus switching on both the Fas and FasL genes. Hence, these results further support a key role for STAT-1 in ischemia/reperfusion-induced cell death in the intact heart acting by inducing the expression of Fas and FasL.

**Discussion**

The Fas/FasL system plays an important role in the regulation of physiological homeostasis in the immune system. In addition, the Fas/FasL system also participates in various types of stress-induced apoptosis. For example, exposure of cardiomyocytes to hypoxia enhances both Fas expression and cell death (8). Furthermore, isolated hearts from mice lacking functional Fas display marked reduction in cell death following exposure to I/R compared with wild-type control hearts (33). Thus, the Fas/FasL system is important in modulating apoptosis in the heart following I/R. The molecular mechanisms regulating apoptosis via the Fas/FasL system have not yet been identified. The present study demonstrates that exposure of cardiac myocytes to I/R in vitro and in the intact heart ex vivo results in the induction of Fas and FasL expression via a STAT-1-dependent pathway. Overexpression of antisense

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**Fig. 8.** Section of ischemia-reperfused rat hearts showing nuclear staining (red) for the specific STAT1-Ser-727 antibody co-localized with cytoplasmic anti-Fas staining (green) (x63).
Apoptosis and Fas Receptor/Fas Ligand Expression

STAT-1 vector in cardiac cells abrogated the enhancement of I/R-induced Fas and FasL promoter activity, while functional STAT-1 enhanced it. In turn, the STAT1–727 mutant, which is not phosphorylated on Ser-727, abrogated the I/R-induced promoter activation and enhanced protein levels of Fas and FasL, whereas the STAT1–701 mutant behaved in the same way as wild-type STAT-1. Similarly, overexpression of wild-type STAT-1 or the mutant STAT1–701 and not the STAT1–727 mutant enhanced apoptotic cell death in cardiac cells. We also demonstrate enhanced phosphorylation of both p38 MAPK and STAT-1 on Ser-727 and expression of Fas/FasL in the intact heart ex vivo exposed to I/R. This is paralleled by detection of activated phospho-Ser-727 STAT-1 co-localized with increased Fas and FasL positivity in cardiomyocytes in the intact heart exposed to I/R. Hence, these results demonstrate that the Ser-727 phosphorylation site of STAT-1 is important in mediating cardiomyocyte-induced cell death by a mechanism involving the induction of Fas and its cognate ligand, whereas tyrosine 701 is not required. Tyrosine 701 has generally been regarded as critical for gene activation by STAT-1 and we showed that this is indeed the case on a test promoter containing a STAT-1-binding site. However, a recent study using gene arrays showed that mutation of this site has no effect on the STAT-1 inducibility of genes such as LMP2, while preventing the induction of other genes such as IRF1 (29). Our results support these findings indicating that the effect of this mutation is promoter specific and can still activate the Fas and FasL gene promoters.

The transcriptional activity of STAT-1 dually phosphorylated on Ser-727 and Tyr-701 is greater than that of STAT-1 phosphorylated on Tyr-701 alone. Interestingly, several studies have shown various stresses including IFN-γ to induce phosphorylation of STAT-1 on Ser-727 via a p38 MAPK-dependent pathway in noncardiac cells (25–27, 34). In the present study p38 activity appeared to be required for serine 727 phosphorylation of STAT-1 in cardiomyocytes in response to I/R. Furthermore, we show that the STAT1–727 mutant is transcriptionally inactive in enhancing Fas and FasL promoters in cardiomyocytes exposed to I/R, whereas the STAT1–701 mutant retains full transcriptional activity. Hence, this suggests that in the heart, Ser-727 phosphorylation of STAT-1 is also crucial for gene transactivation by STAT-1. In non-cardiac cells the MAPK pathways mediated by p38, JNK, and p42/p44 have been implicated in the regulation of Fas/FasL expression. For example, withdrawal of survival factors leads to activation of JNK pathway in neuronal cells resulting in FasL induction and cell death (32). Similarly, stimulation of T cells leading to activation-induced cell death results in both p38 and JNK activation and is followed by the induction of FasL (31). I/R injury to cardiac myocytes is associated with p38 MAPK phosphorylation. Inhibition of the p38 pathway reduced the level of cardiomyocyte apoptosis in vitro and also in the intact heart ex vivo (35, 36).

In the present study, chemical and molecular inhibition of the p38 pathway abolished Ser-727 phosphorylation of STAT-1 I/R-induced activation of the Fas and FasL promoters, suggesting that p38 MAPK activation is involved in STAT-1 Ser-727 phosphorylation and gene transactivation. JNK activation is also enhanced following ischemia and I/R (36, 37). Inhibition of the JNK pathway also reduced the level of cardiomyocyte apoptosis in vitro (38). A dominant-negative JNK expression vector also reduced I/R-induced Fas and FasL promoter activity. Thus, in addition to p38 MAPK, JNK also contributes to the modulation of Fas and FasL via the activation of STAT-1. In contrast, to the pro-apoptotic effects in the heart of p38 and JNK, the other stress-activated MAPK family p42/p44 (ERK1/ERK2) appears to be involved in cardiac cell survival. Factors which activate p42/p44, such as cardiotoxin-1 and urocortin, are cardioprotective both in vitro and ex vivo, and these protective effects are abolished by pretreatment with the p42/p44 pathway inhibitor, PD98059 (39, 40). Moreover, infarct size in the isolated heart exposed to I/R was increased by pretreatment with PD98059 (39, 40). Inhibition of p42/p44 in cardiac myocytes cultured under normoxic conditions did not increase the level of cell death, suggesting that p42/p44 is not required for cell survival in non-stressed conditions (39, 40). In the present study blocking p38/JNK but not the p42/p44 pathway inhibited I/R-induced Fas and FasL promoter activity. Hence, these studies together with our present study suggests that the p38/JNK but not the p42/p44 pathway may promote apoptosis in cardiac myocytes via the modulation of Fas and its cognate ligand, FasL, which in turn is dependent on the phosphorylation of STAT-1 on Ser-727.

Cardiac myocyte injury and death, at least in part by apoptosis, following I/R injury, is an important cause of morbidity and mortality. The present study reinforces our earlier work on the importance of STAT-1 in the induction of cardiac myocyte apoptosis and identifies the signaling pathways by which STAT-1 is activated during I/R in the heart as well as identifying the Fas and FasL genes as targets for STAT-1 in the heart. Understanding the molecular mechanisms by which the apoptotic program is initiated in the heart may lead to new therapeutic approaches for this common clinical problem.

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Induction of Apoptosis and Fas Receptor/Fas Ligand Expression by Ischemia/Reperfusion in Cardiac Myocytes Requires Serine 727 of the STAT-1 Transcription Factor but Not Tyrosine 701

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