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Conditional expression of anti-apoptotic protein p35 by Cre-mediated DNA recombination in cardiomyocytes from loxP-p35-transgenic mice

T Araki[1,3], M Shibata[1,4], R Takano[1], S Hisahara[1], S-i Imamura[5], Y Fukuuchi[4], T Saruta[3], H Okano[1,2] and M Miura*[,1,2]

[1] Division of Neuroanatomy, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[2] CREST of Japan Science and Technology Corporation (JST), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[3] Department of Nephrology, Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan
[4] Department of Neurology, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan
[5] Research Division, The Heart Institute of Japan, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-0054
* Corresponding author: M Miura, Division of Neuroanatomy (D12), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: +81-6-6879-3581; Fax: +81-6-6879-3589; E-mail: mmiura@nana.med.osaka-u.ac.jp

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Abstract

p35, a viral inhibitor of caspase, prevents cell death induced by various stimuli. We established an experimental system to study the involvement of caspases in cell death, using primary cultured cells from p35 transgenic mice in which the p35 open reading frame (ORF) had been disrupted by the insertion of a DNA segment flanked by loxP sites, the Cre recognition sites. In this system, p35 expression can be initiated by Cre recombinase. Cardiomyocytes, which are highly sensitive to hypoxic stress, were infected with an adenovirus carrying the *cre* gene (AxCANCre). Expression of p35 by infection with AxCANCre resulted in inhibition of caspase-3 activation and resistance to hypoxia-induced cell death. Hypoxia-induced cytochrome c release was also attenuated in p35-expressing cardiomyocytes. Our transgenic mice can be used as an experimental model for studying the involvement of caspases in various degenerative diseases as well as programmed cell death both *in vitro* and *in vivo*. *Cell Death and Differentiation* (2000) **7,** 485–492.

Keywords: p35; caspases; cardiomyocytes; hypoxia; cre/loxP

Abbreviations: PBS, phosphate-buffered saline; FBS, fetal bovine serum; RT–PCR, reverse transcription PCR; DMEM, Dulbecco's modified Eagle's medium

Introduction

Caspases are common mediators of cell death induced by diverse stimuli. Pharmaceutical caspase inhibitors such as Z-VAD-fmk and Z-DEVD-fmk, are widely used to study the involvement of caspases in cell death. However, because of their non-specific effects on proteases other than caspases, and because they have different cell permeabilities and are toxic for certain types of cells, there are substantial limitations to their applications. In contrast to pharmaceutical inhibitors, viral inhibitors of caspases are highly specific for caspases, and are considered more suitable for studying the role of caspases in physiological cell death. A viral inhibitor for cytokine response modifier A (crmA), has been shown to block the activation of caspase-1 and -8[1,2] and to prevent the cell death induced by Fas, TNF, and growth factor deprivation.[3–6] Another viral inhibitor of caspases, the baculovirus gene product p35, is a more potent suppressor of programmed cell death in *Caenorhabditis elegans* (*C. elegans*) and *Drosophila*.[7–9] Expression of *p35* in insect and mammalian cells prevents cell death induced by different signals.[10–17] *In vitro* data indicate that p35 effectively inhibits mammalian caspase-1, -2, -3, -4, -6, -7, -8, -10, and *C. elegans* Ced-3.[18–22] Inhibition of all effector phase caspases (caspase-3, -6, -7) by p35 strongly suggests that p35 can be used as a general inhibitor of cell death in mammals.

A transgenic mouse expressing *crmA* in the thymus has been produced.[1,2] While *crmA* expression inhibits *in vitro* Fas-induced apoptosis, it does not affect the negative selection driven by the minor lymphocyte stimulating antigen or the endogenous H-Y antigen. In contrast, transgenic mice expressing p35, a broad-range inhibitor of the caspase family, in thymocytes did show inhibition of MHC classII-restricted negative selection induced by staphylococcal enterotoxin B (SEB) superantigen.[23] This result raises the possibility that transgenic mice carrying *p35* can be used to study the mechanisms underlying cell death mediated by caspases.

There are some difficulties in producing p35 transgenic mice. Prevention of caspase activity in the early embryo could result in an embryonic lethal phenotype because the knockout of certain caspase family members (caspase-3, -8 and -9) causes embryonic lethality.[24–28] We have generated mice carrying a *p35* gene that can be expressed ubiquitously but that is not expressed under normal conditions because its open reading frame (ORF) has been disrupted by the insertion of a DNA segment flanked by loxP sites, the Cre recognition sites.[29] Expression of *Cre*, which mediates recombination of two loxP sites into a single site, with concomitant removal of the DNA segment they flank, restores the p35 ORF, thereby allowing production of p35 in the cell population of interest.

Here, we evaluated the p35 expression system in cardiomyocytes undergoing cell death under hypoxic conditions. Several studies have reported the involvement of caspases in hypoxia-induced cardiomyocyte injury using the pan-caspase inhibitor Z-VAD-fmk.[30] However, Z-VAD-fmk has been reported to also inhibit calpain,[31] it is difficult to

conclude that caspases were specifically involved. To address this issue more directly, we examined the anti-cell death function of p35 in cardiomyocytes derived from p35 transgenic mice. Cardiomyocytes were infected with an adenovirus carrying the *cre* gene (AxCANCre),[32] and their sensitivity to hypoxia-induced cell death was tested. Expression of *p35* after infection with AxCANCre resulted in a significant resistance to hypoxia-induced cell death. Consistent with this observation, activation of caspase-3 and release of cytochrome c were attenuated in p35-expressing cardiomyocytes under hypoxic conditions. Our results indicate that caspase activation plays a crucial role in hypoxia-induced cardiomyocyte injury. Our transgenic mice can be used as an experimental model for studying the involvement of caspases in various degenerative diseases as well as programmed cell death both *in vitro* and *in vivo*.

Results

Experimental design of Cre-loxP ON/OFF system-mediated p35 expression

In our p35 transgenic mice, a *p35* transgene that can not produce p35 protein until it has been 'activated' by Cre-mediated DNA recombination was constructed by inserting the *neo* gene as a stuffer flanked by loxP sites between the CAG promoter, and the p35 coding sequence (Figure 1A). The CAG promoter consists of the cytomegalovirus IE enhancer and the chicken β-actin promoter, and enables efficient production of the gene of interest in most mouse tissues.[33] Cre-mediated recombination of the two loxP sites yields a single loxP site with the concomitant deletion of the *neo* gene, placing the *p35* gene under the control of the CAG promoter (Figure 1A).

We examined the expression of the *neo* gene by RT–PCR to determine whether or not the CAG promoter was working ubiquitously in the transgenic mice. *neo* expression could be detected by RT–PCR as an 800-bp fragment using the neo-2 and neo-3 primers. Expression of the *neo* gene was detected in most of the adult tissues tested, except for the liver and spleen (Figure 1B). It is unclear why *neo* was not expressed in the liver and spleen. Transgenic mice carrying a CAG-loxP-βGeo-loxP-human placental alkaline phosphatase gene has been made and examined the expression of transgene.[34] Although the transgene is widely expressed, to date, transgenic lines with proven ubiquitous expression have not been made.[34] Since the activity of most promoters is

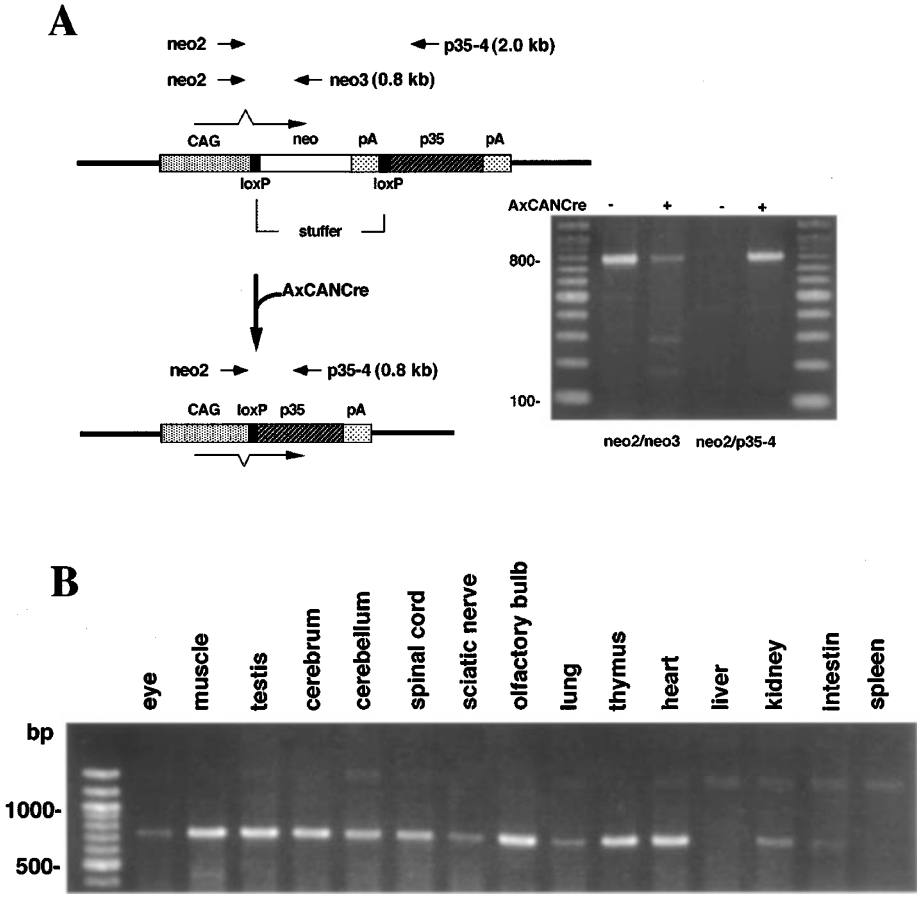

Figure 1 (**A**) Schematic representation of the structure of the *p35* transgene, and Cre-mediated removal of the stuffer sequence. Right panel shows the expression of *p35* revealed by RT–PCR using neo2 and p35-4 primers. Cardiomyocytes were infected with adenovirus (2×10^5 pfu/ml) for 24 h, then total RNA was isolated. Expression of *p35* was initiated by infection of cardiomyocytes with AxCANCre. (**B**) Expression of the *neo* gene was detected by RT–PCR using the neo2 and neo3 primers in various tissues

integration-site dependent, the integration site of our p35 transgenic mice could affect the activity of the CAG promoter in liver and spleen.

Cre-mediated expression of p35 prevents cardiomyocyte cell death induced by hypoxia

To evaluate the anti-cell death function of p35 in response to hypoxia, cardiomyocytes were infected with a replication-

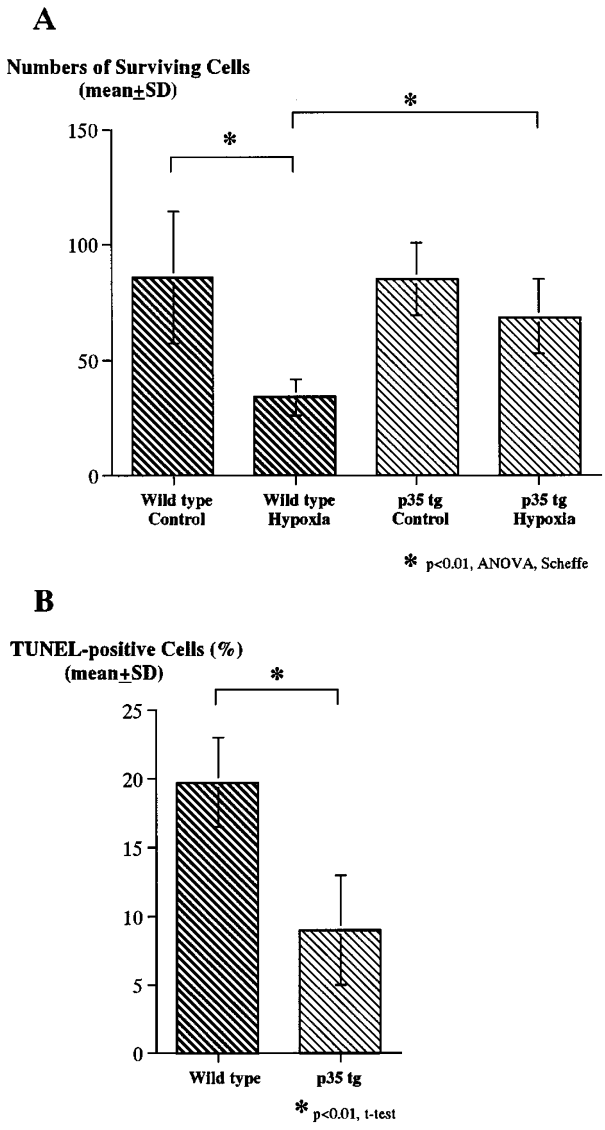

Figure 2 (**A**) Changes in numbers of cardiomyocytes in response to hypoxia. Cardiomyocytes from wild-type and p35-transgenic (p35 tg) mice were infected with adenovirus (2×10^5 pfu/ml) for 24 h, then placed under hypoxic conditions for 48 h. The cells were counted and the data were expressed as means $\pm$ S.D. The results were obtained from 20 independent visual fields. The asterisks in the figure represent *P*-values below 0.01. (**B**) Hypoxia-induced DNA fragmentation. Cardiomyocytes from wild-type and p35-transgenic (p35 tg) mice were infected with adenovirus (2×10^5 pfu/ml) for 24 h, then placed under hypoxic conditions for 48 h. DNA fragmentation was detected by TUNEL staining. The data were expressed as means $\pm$ S.D. The results were obtained from six independent visual fields. The asterisks in the figure represent *P*-values below 0.01

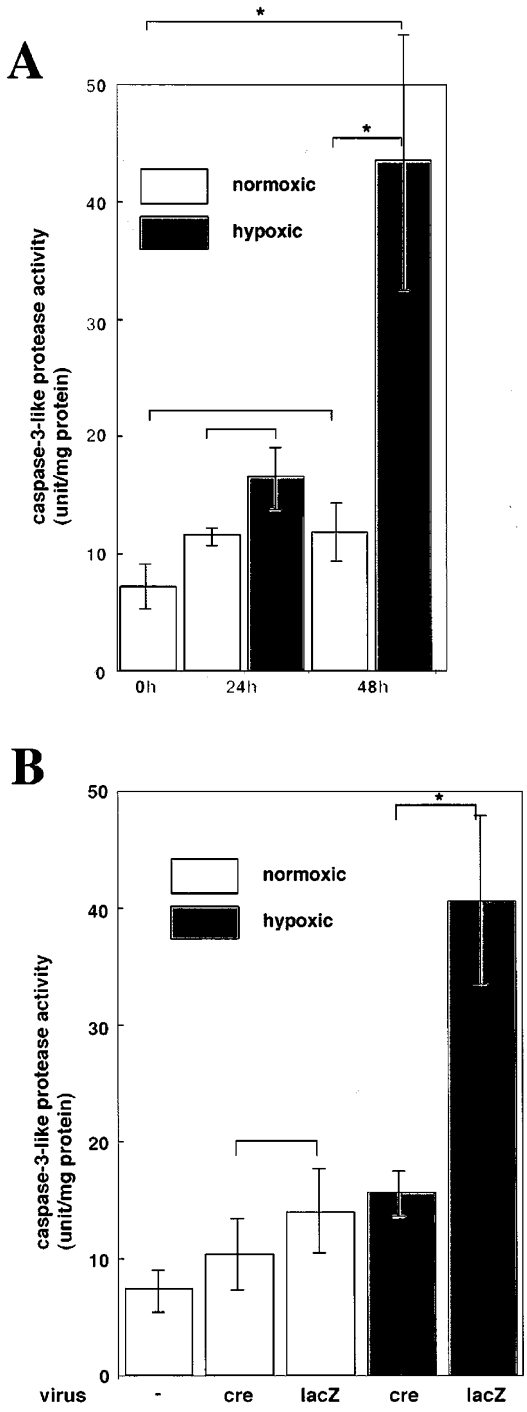

Figure 3 (**A**) Caspase-3-like protease activity of cardiomyocytes in hypoxia. At 48 h in culture, caspase-3-like activity was 43.5 ± 21.8 (n=3) in hypoxic conditions and 11.8 ± 5.0 (n=3) in normoxic conditions. Statistical analysis was performed using Student's *t*-test. The asterisks in the figure represent *P*-values below 0.05. (**B**) Inhibition of caspase-3-like protease activity in cardiomyocytes expressing p35. Cardiomyocytes from p35 transgenic mice were infected with adenovirus (2×10^5 pfu/ml) for 24 h, then placed under hypoxic conditions for 48 h. Cell lysates were extracted and caspase activity was measured. Caspase-3-like activity was 40.5 ± 6.6 (n=3) in AxCALacZ-infected cardiomyocytes and 15.7 ± 3.6 (n=3) in AxCANCre-infected cardiomyocytes. Statistical analysis was performed using Student's *t*-test. The asterisks in the figure represent *P*-values below 0.05

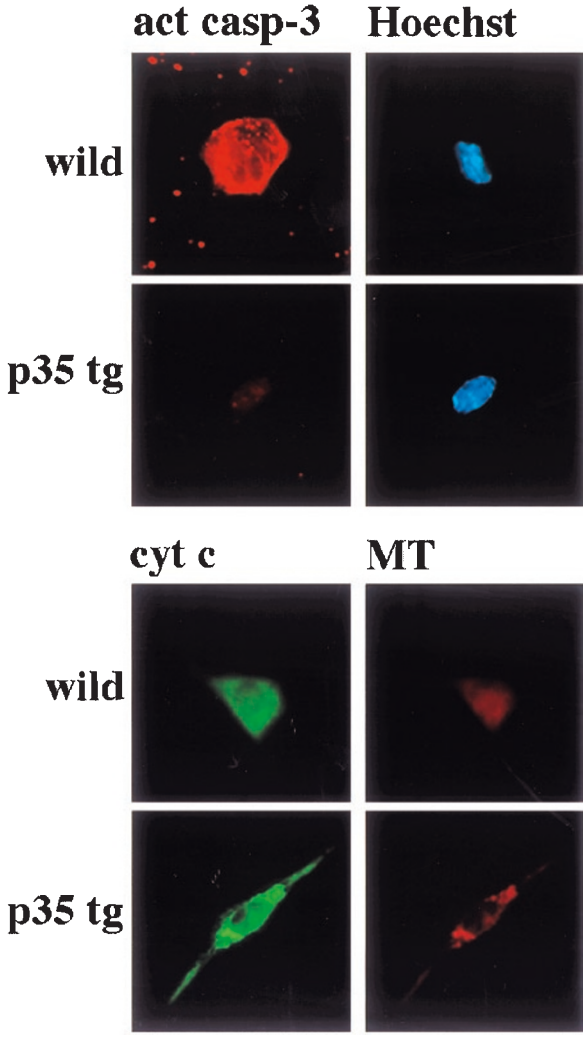

Figure 4 Immunostaining of the activated form of caspase-3 and cytochrome c. A wild-type cardiomyocyte subjected to 48-h hypoxia exhibited immunoreactivity for the activated form of caspase-3, which was located diffusely in the cytoplasm. A p35-expressing cardiomyocyte exhibited no significant immunoreactivity for the activated form of caspase-3 after 48-h hypoxia. Cytochrome c immunoreactivity showed a punctate pattern, consistent with a mitochondrial localization as indicated by MitoTracker Red CMXRos, in a p35-expressing cell subjected to 48-h hypoxia. In a wild-type cardiomyocyte subjected to 48-h hypoxia, a diffuse pattern of cytochrome c immunoreactivity was observed. act casp-3: anti-activated caspase-3 staining. Hoechst: Hoechst 33342 staining. cyt c: anti-cytochrome c staining. MT: Mitotracker Red CMXRos staining. wild: cardiomyocytes from wild-type mice. p35 tg: cardiomyocytes from p35-transgenic mice

defective adenovirus encoding Cre recombinase under the control of the CAG promoter (AxCANCre).[32] Infection efficiency was determined by β-galactosidase staining after infection with AxCALacZ at 2×10^5 pfu/ml, and was observed to be $>90\%$ at 48 h (data not shown). The number of cardiomyocytes was similar in wild-type and p35-transgenic mice before hypoxia (85.9 ± 28.4 and 85.2 ± 15.8, wild-type and p35-transgenic mice, respectively). Under hypoxia, the number of remaining cardiomyocytes was significantly ($P<0.01$) reduced to 34 ± 7.9 in wild-type mice. However, there was only a slight decrease in p35-transgenic mice (68.8 ± 16.0) (Figure 2A). To determine whether the infected

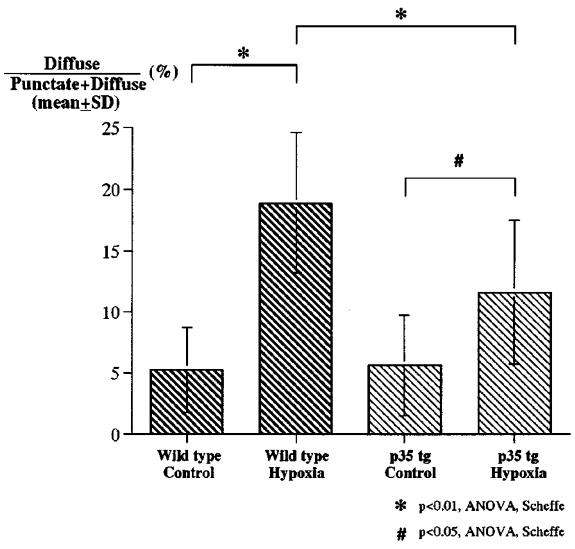

Figure 5 Cytochrome c translocation in response to hypoxia. Cardiomyocytes from wild-type and p35-transgenic (p35 tg) mice were infected with AxCANCre (2×10^5 pfu/ml) for 24 h, then placed under hypoxic conditions for 48 h. The data were expressed as means $\pm$ S.D. The results were obtained from 20 independent visual fields

Cre effectively mediated the switching on of *p35* expression in the cardiomyocytes derived from transgenic mice, we performed RT–PCR, using the p35-4 and neo-2 primers (see Materials and Methods). Expression of *p35* transgene could not be detected in cardiomyocytes under normal culture conditions. After infection with AxCANCre, a single 800-bp PCR product was detected (Figure 1A). These results show that the Cre-mediated induction of *p35* expression was achieved in the cardiomyocytes. We then used TUNEL staining to examine the DNA fragmentation of cardiomyocytes attached to the dish 48 h after hypoxic stress. The proportions of cardiomyocytes showing DNA fragmentation after hypoxia were 19.7 ± 3.2 and $9.0\pm4.0\%$ in wild-type and p35-transgenic mice, respectively (Figure 2B). These results indicate that p35 prevents the hypoxia-induced cell death of cardiomyocytes.

Hypoxia-induced caspase activation was prevented by p35

Cultured cardiomyocytes from neonatal mice exposed to hypoxic conditions showed higher caspase-3 like protease activity than normoxic time-matched controls (Figure 3A). This rise in caspase activity was more robust at 48 h hypoxia (3.8 times control) than 24 h (1.4 times control). The high caspase activity of cardiomyocytes under hypoxia was in proportion to cardiomyocyte viability. In contrast, caspase-1 like protease activity did not change as much as the caspase-3 like protease activity did (data not shown). To test the effect of p35 on changes in caspase activity, cardiomyocytes were infected with AxCANCre or AxCALacZ for 24 h before hypoxic stress, then cultured under hypoxic conditions for 48 h. At 48 h of hypoxic stress, 40.5 units of caspase-3 like protease activity were detected in the lysate from cells infected with AxCALacZ, while only 15.7 units of DEVDase activity were

detected in the lysate from AxCANCre-infected cells (Figure 3B). Cardiomyocytes from wild-type mice, but not from p35-transgenic mice, were positive for activated caspase-3 (Figure 4). These results suggest that p35 prevents the hypoxia-induced activation of caspase-3 in cardiomyocytes.

p35 prevents cytochrome c release induced by hypoxic stress

A number of reports have shown that the release of cytochrome c from mitochondria to the cytosol initiates caspase-3 activation through the aggregation of Apaf-1, procaspase-9, and dATP, and the subsequent activation of caspase-9, which in turn activates caspase-3.[35,36] In healthy cardiomyocytes, immunostaining for cytochrome c showed a punctate pattern that corresponded with the localization of mitochondria, as revealed by the mitochondrial marker MitoTracker Red (data not shown). Under hypoxic conditions, a diffuse pattern of cytochrome c staining was seen, indicating cytochrome c translocation into the cytoplasm (Figure 4). In *p35*-expressing cells, a punctate staining pattern for cytochrome c remained under hypoxic conditions (Figure 4). Under normal conditions, the proportion of cardiomyocytes displaying a diffuse staining pattern for cytochrome c was similar in both wild-type and p35-transgenic mice (5.3 ± 3.5 and 5.6 ± 4.1% for wild-type and p35-transgenic mice, respectively). Under hypoxia, 18.9 ± 5.7% of the remaining cardiomyocytes exhibited a diffuse pattern in wild-type mice, indicating an increased occurrence of cytochrome c translocation. In p35-transgenic mice, the proportion of cardiomyocytes showing a diffuse pattern was 11.6 ± 5.9% (Figure 5). These results suggest that p35 can reduce hypoxic stress-induced cardiomyocyte injury upstream of cytochrome c release.

Discussion

We could successfully express *p35* in cardiomyocytes from p35 transgenic mice by infecting them with a recombinant adenovirus bearing the Cre recombinase, AxCANCre. Recombinant adenovirus vectors are widely used for the delivery of genes of interest into postmitotic cells such as neurons and cardiomyocytes.[37–39] However, some foreign genes can not be produced using recombinant adenoviruses if the gene products result in growth discharge, toxicity, or have other adverse effects on the host cells. Since overexpression of *p35* results in growth suppression in mouse 3T3 fibroblasts,[40] we speculated that the production of a recombinant adenovirus carrying *p35* could be difficult. In such a case, a dual infection strategy using the Cre-loxP system could be applied to produce p35 using a recombinant adenovirus. The Cre-loxP system was used to deliver recombinant adenovirus-mediated exogenous Bcl-2 into postmitotic neurons.[41] The authors simultaneously infected neurons with two recombinant retroviruses; one was AxCANCre and the other was a loxP-*bcl-2* adenovirus. Only neurons infected with both recombinant adenoviruses could express *bcl-2*.[41] However, there is a limitation to achieving high infection efficiency: the dual virus infection sometimes exhibits cytotoxicity.[41] In contrast, the cells from our p35 transgenic mice require only a single infection with AxCANCre to activate the expression of *p35*. Therefore, we can use a relatively low titer of recombinant adenovirus to achieve a high efficiency of infection and *p35* expression. Expression of foreign genes are transient in case of adenovirus-mediated gene transfer. Since adenovirus can not be integrated into chromosome, replication defective recombinant adenovirus can be diluted through cell proliferation. Even in post-mitotic cells, difficulty of long term expression has been reported.[42] Once stuffer sequence is removed from the genome of cells derived from loxP-p35 transgenic mice, expression of *p35* is expected to continue as long as cell survives. This is another advantage of our strategy compared to dual infection method.

A number of Cre-transgenic mice or Cre-knock-in mice have been reported to express Cre under the regulation of a variety of tissue specific promoters e.g., the insulin promoter for pancreatic β-cells; the myosine light chain 2v locus for cardiac ventricular muscle; and the Ca^{2+}/calmodulin-dependent protein kinase IIa-promoter for the pyramidal cells of the hippocampal CA1 region.[43–45] By crossing these mice with loxP-p35 mice, we can express p35 in many tissues of interest. Our p35 transgenic mice can provide a useful model for examining the involvement of caspases in development and/or in various models of degenerative diseases *in vitro* as well as *in vivo*.

The involvement of caspases in heart development has been examined.[46] In cardiomyocytes, NO-mediated apoptosis is cGMP dependent and involves caspases.[47] Caspase-3-like protease is involved in staurosporine-induced apoptosis in cardiomyocytes.[48] Z-VAD-fmk was effective in reducing myocardial reperfusion injury, which could be at least partially attributed to the attenuation of cardiomyocyte apoptosis.[30] However, all the results mentioned above were obtained using pharmaceutical caspase inhibitors. Since Z-VAD-fmk has been reported to inhibit calpain as well,[31] we decided to use the pan-caspase inhibitor p35 to evaluate the involvement of caspases in hypoxia-induced cardiomyocyte injury. To our knowledge, our present data is the first demonstration that caspase-3-like protease activity is involved in hypoxia-induced cardiomyocyte cell death, and that inhibition of caspase-3-like protease activity by p35 can prevent cardiomyocyte death. We further showed that p35 prevents hypoxic stress-induced cardiomyocyte death upstream of cytochrome c release. It has recently been reported that ZVAD-fmk fails to prevent cytochrome c release in hypoxia- and ischemia-induced cardiomyocyte apoptosis.[49,50] but ZDEVD-fmk prevents cytochrome c release.[50] These results further support our p35-expressing system as a useful experimental model to investigate the physiological roles of caspases in cell death.

Materials and Methods

Transgenic mice

A transgene consisting of the CAG promoter, loxP-*neo*-loxP, *p35* coding sequence and a polyadenylation signal was microinjected into fertilized eggs of C57BL/6 (B6) mice to generate transgenic mice as previously described.[29] The presence of the *p35* transgene was also detected by PCR. The primers used in the

PCR were as follows: forward: 5′-TGGATGGATTCCACGATAGC-3′ (p35-3); reverse: 5′-TGCACACTCTCCACGTAAGC-3′ (p35-4). The DNA was denatured for 5 min at 94°C prior to 35 PCR cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were fractionated by electrophoresis in 1.2% agarose gels and a band of approximately 500 bp was identified in the presence of p35 the transgene. Expression of the transgene was detected by Reverse Transcription PCR (RT–PCR). Total RNA was prepared from the tissues of a transgenic mouse by using Trizol (Gibco BRL). One μg of total RNA was incubated at 65°C for 10 min, then cooled on ice for 5 min. First-strand cDNAs were synthesized by mixing the RNA with 9 pM random deoxynucleotide hexamers, 1 × reverse transcription buffer (Gibco BRL), 6.7 mM dithiothreitol (Gibco BRL), and 0.625 mM deoxynucleotide triphosphates (Gibco BRL) and incubating for 120 min at 37°C in the presence of 0.8 unit of RNase inhibitor (Promega) and 4 units of MoMLV reverse transcriptase (Promega). The primer sequences used for RT–PCR were as follows: forward 5′-TCTGACTGACCGCGTTACTCC-3′ (neo2); reverse 5′-TATTCGGCAAGCAGGCATCG-3′ (neo3). The following conditions were used for PCR reactions: 1 × Ex Taq PCR buffer (TaKaRa), 0.2 mM deoxynucleotide triphosphates (TaKaRa), 0.5 mM each primer, and 1 unit of Ex Taq DNA polymerase (TaKaRa) in a total volume of 20 μl. The DNA was denatured for 5 min at 94°C prior to 35 PCR cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

p35 expression by Cre-recombinase

Cardiomyocytes from the p35 transgenic mice do not express p35 under normal conditions. Cre-recombinase excised the neomycin stuffer, allowing the *p35* positioned behind the 2nd loxP site to express. To confirm *p35* mRNA expression under Cre-recombinase expressing conditions, Reverse Transcription PCR (RT–PCR) was performed. Total RNA was prepared from p35 transgenic cardiomyocytes infected with the Cre-recombinase-expressing adenovirus (AxCANCre) using Trizol. Synthesis of the first-strand cDNAs was as above. The primer sequences used for RT–PCR were as follows: forward 5′-TCTGACTGACCGCGTTACTCC-3′ (neo2); reverse 5′-TGCACACTCTCCACGTAAGC-3′ (p35-4). The conditions for the PCR reactions were as described above.

Cell culture

The heart was removed from 1 to 3-day-old C57/BL6 mice, rinsed with phosphate-buffered saline (PBS), minced with scissors, dispersed with 0.08% trypsin in PBS for 10 min at 37°C, and pipetted approximately 30 times. The suspension was spun at 1300 r.p.m. for 3 min and the supernatant fraction was removed. Three additional incubations with the trypsin solution were performed, and the supernatant fractions from each digestion were combined with Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml ampicillin. The combined solution was spun at 1300 r.p.m. for 3 min. The pellet was resuspended in DMEM/F12/FBS, and the cells were plated onto a petri dish (CORNING) and incubated at 37°C for 60 min (to remove non-cardiomyocytes by differential adhesion). The non-attached cells were then seeded into 6 cm tissue culture dishes (CORNING), then incubated at 37°C for 48–72 h under a humidified atmosphere of 95% air, 5% CO_2. When the cells exhibited synchronous contractions, they were utilized for hypoxic stress.

Hypoxic stress

An anaerobic jar was used to expose the cells to hypoxic stress (Gas Pak system, BECTON DICKINSON). The medium for cardiomyocytes was replaced with DMEM/F12/5%FBS before the cells were exposed to hypoxic stress. The cells were then placed in the anaerobic jar for 24 or 48 h. Cardiomyocytes were infected with an aliquot of adenovirus carrying either the *Cre*-recombinase gene (AxCANCre)[32] or the *lacZ* gene (AxCALacZ)[32] at a concentration of 2×10^5 pfu/ml. After 24 h, cells were subjected to oxygen deprivation in the anaerobic chamber.

Immunocytochemistry

Immediately after oxygen deprivation, the cells were washed three times with PBS (pH 7.4), and fixed in PBS containing 4% paraformaldehyde for 10 min. Following three washes with PBS, the cells were permeabilized with 0.1% Triton-X/PBS for 10 min. After three washes with PBS, the cells were incubated in 4% normal goat serum (NGS)/PBS at room temperature for 1 h, then rabbit polyclonal anti-caspase-3 antibody (Pharmingen) at a dilution of 1 : 200 at 4°C overnight. After washing with PBS three times, the cells were incubated with rhodamine-conjugated anti-rabbit IgG at a dilution of 1 : 200 at room temperature for 2 h. Nuclear morphology was visualized by Hoechst 33342 (10 μM) staining.

Cardiomyocytes harvested as described above were subjected to hypoxia for 48 h. Cardiomyocytes grown under identical conditions except for hypoxia were used as controls. Prior to fixation, the cells were incubated in DMEM containing 200 nM Mitotracker Red CMXRos (Molecular Probes) for 30 min. Subsequently, the cells were fixed in 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS, and blocked with 4% normal goat serum/PBS. Mouse monoclonal anti-cytochrome c antibody (Pharmingen) at a dilution of 1 : 200 was applied at 4°C overnight. After washing with PBS three times, the cells were incubated with FITC-conjugated anti-mouse IgG at a dilution of 1 : 200 at room temperature for 2 h. The cardiomyocytes were observed using a Carl-Zeiss Axiophoto 2 fluorescence microscope. To assess the translocation of cytochrome c from mitochondria, the number of cardiomyocytes showing a diffuse staining pattern was determined from 20 independent random samplings of approximately 100 cells each and expressed as a percentage of the total cell number. The statistical analysis was performed using ANOVA followed by Scheffé's *post hoc* test.

TUNEL method

The TUNEL method was performed according to the manufacturer's instructions with these modifications (Apoptag, INTERGEN). After fixation, cells were incubated in Equilibration Buffer at room temperature for 3 min. A working-strength terminal deoxynucleotidyl transferase mixture containing digoxigenin-labeled dUTP was applied in a humidified chamber at 37°C for 1 h. The reaction was terminated using STOP/WASH Buffer. After three washes with PBS, the cells were incubated with FITC-conjugated anti-digoxigenin IgG at a dilution of 1 : 200 at 4°C overnight. Counterstaining with Hoechst 33342 was carried out as described above. To assess the proportion of cardiomyocytes exhibiting DNA fragmentation, TUNEL-positive cells were counted from six independent random samplings of approximately 150 cells each and expressed as a percentage of the total cell number. The statistical analysis was performed using a non-paired *t*-test.

Caspase activity

Cultured cardiomyocytes were washed with PBS, then detached from the dish with trypsin-EDTA (Gibco BRL) at 37°C for 5 min. An equal volume of DMEM/F12/5% FBS was added to stop the trypsinization. Dissociated cells were collected by centrifugation at 1500 r.p.m. for 5 min. The cell pellet was resuspended in 30 μl of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM EGTA. Cells were disrupted by freezing and thawing (three cycles). Digitonin was added (15 μM final concentration) to the cell suspension followed by incubation at 37°C for 30 min. Cell lysates were collected after centrifugation at 15 000 r.p.m. for 3 min. Lysates containing 3 μg protein were preincubated with 10 μl of cleavage buffer (20 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 0.05% NP40, 5 mM $MgCl_2$) at 37°C for 60 min, then 180 μl of cleavage buffer containing 10 μM Ac-DEVD-7-amino-4-methylcoumarin (Peptide Institute, Osaka, Japan) was added and the mixtures were incubated at 37°C for 60 min. The amount of 7-amino-4-methylcoumarin (AMC) released was measured using a spectrofluorometer (Biolumin 960, Molecular Dynamics) with excitation at 380 nm and emission at 460 nm. One unit was defined as the amount of enzyme required to release 0.22 nmol AMC per minute at 37°C.

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