Functional Interaction between the Transcription Factor Krüppel-like Factor 5 and Poly(ADP-ribose) Polymerase-1 in Cardiovascular Apoptosis*

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Krüppel-like factor 5 (KLF5) is a transcription factor important in regulation of the cardiovascular response to external stress. KLF5 regulates pathological cell growth, and its acetylation is important for this effect. Its mechanisms of action, however, are still unclear. Analysis in KLF5-deficient mice showed that KLF5 confers apoptotic resistance in vascular lesions. Mechanistic analysis further showed that it specifically interacts with poly(ADP-ribose)-polymerase-1 (PARP-1), a nuclear enzyme important in DNA repair and apoptosis. KLF5 interacted with a proteolytic fragment of PARP-1, and acetylation of KLF5 under apoptotic conditions increased their affinity. Moreover, KLF5 wild-type (but not a non-acetylatable point mutant) inhibited apoptosis as induced by the PARP-1 fragment. Collectively, we have found that KLF5 regulates apoptosis and targets PARP-1, and further, for acetylation to regulate these effects. Our findings thus implicate functional interaction between the transcription factor KLF5 and PARP-1 in cardiovascular apoptosis.

The cardiovascular adapts dynamically to metabolic and/or mechanical stresses (i.e. blood vessel remodeling in response to oxidative and hypertensive stress). Although this response initially compensates for the pathological stimulus, chronic and excessive load ultimately leads to decompensatory maladaptation, which is the underlying pathology of heart failure and atherosclerosis (1, 2). The cellular mechanisms underlying cardiovascular adaptation processes are characterized by cellular hyperplasia, hypertrophy and death. Previous studies have begun to clarify the molecular basis of the process centered on signaling pathways linking extracellular stimuli to intracellular processes characterized by the intracellular signaling cascade and downstream gene expression events, which include roles of transcription factors, such as NFAT (nuclear factor of activated T cells) through the calcineurin pathway and histone deacetylases in cardiac hypertrophy (3–6). We have recently shown that the transcription factor, Krüppel-like factor 5 (KLF5), regulates the cardiovascular response to pathological stress (e.g. angiotensin II) by modulating atherosclerosis, angiogenesis, and cardiac hypertrophy (7–10).

Although the transcriptional and signaling networks regulating the cardiac adaptation response have begun to be unraveled, further investigation is needed to better understand the pathogenic roles of the involved factors and pathways. Regulation of cell death/survival, in particular, remains poorly understood. Here, we have shown that KLF5 inhibits cell death/apoptosis and that it functionally interacts with poly(ADP-ribose)-polymerase-1 (PARP-1), a nuclear enzyme involved in the response to DNA damage (11).

MATERIALS AND METHODS

Cell Culture and Apoptotic Assays—3T3 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% serum. Human umbilical vein-derived endothelial cells were cultured in EBM-2 medium with EGM-2 supplement (Clonetics). Stable transformant cell lines derived from 3T3 and HeLa cells (12) were maintained in Dulbecco’s modified Eagle’s medium/10% serum containing 50 μg/ml G418 (Sigma). For most cell death/survival experiments, cells were treated with 50 ng/ml recombinant tumor necrosis factor-α (TNF-α) (Peprotech) and/or 4 μM actinomycin D (Sigma). Analysis in human umbilical vein-derived endothelial cells was done following transfection of expression vectors. Caspase-3 was assayed with the caspase-3 assay system (Promega). Cleaved DNA was assayed with the Cell death detection assay kit ELISA (enzyme-linked immunosorbent assay; Roche Applied Science). TUNEL

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4The abbreviations used are: KLF5, Krüppel-like factor 5; PARP-1, poly(ADP-ribose) polymerase-1; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; GST, glutathione S-transferase.
staining was done with the in situ apoptosis detection kit (Takara) after cells were fixed with 4% paraformaldehyde. For the mouse femoral artery injury sections, the in situ death detection kit (Roche Applied Science) was used. Nuclei were counterstained with propidium iodide (Sigma). Sections were mounted with the ProLong antifade kit (Molecular Probes, Eugene, OR) and observed under a confocal microscope (Fluo-view FV300; Olympus, Tokyo, Japan).

Mouse Femoral Artery Injury Model—Eight-week-old male KLF5 heterozygous knock-out mice (9) and wild-type littermates were subjected to femoral artery injury and analyzed as described previously (13).

RNA Interference Analysis—RNA interference analysis of KLF5 was done as described previously (14).

Immunoprecipitation and Western Blot Analysis—Whole cell lysate or nuclear extract was immunoprecipitated with anti-FLAG M2 affinity gel (Sigma), prepared anti-KLF5 antibody, or anti-PARP antibody (R & D Systems) with protein G-Sepharose (GE Healthcare), subjected to SDS-PAGE analysis, and then immunoblotted as described previously (12, 14). For Western blot analysis, antibodies from the apoptosis sampler kit (BD Biosciences) were used in addition to FLAG M2 monoclonal antibody (Sigma), anti-PARP-1 monoclonal antibody (BD Biosciences and R & D systems), anti-acetylated lysine antibody (Santa Cruz Biotechnology), and anti-KLF5 antibody (KM1785) (9). Plasmid transfections were done using Lipofectamine 2000 (Invitrogen). Adenoviral transfections were done as described previously (14).

Preparation of Anti-KLF5 Antibody—Anti-KLF5 antibody was prepared by immunizing rabbits with 100 μg of full-length purified recombinant His6-KLF5 for 8 times at 1-week intervals, after which serum was extracted. Antibody specificity was confirmed by Western blot (data not shown).

Preparation of Recombinant Epitope-tagged Protein—Human KLF6 was PCR-amplified and subcloned into the pGEX vector (Amersham Biosciences). Expression and purification of bacterial recombinant proteins for GST-KLF6, KLF5, KLF5-K369R, zinc fingers, and His6 24-kDa PARP-1 were done essentially as described previously (12, 15, 16).

Protein-Protein Interaction Assay and Acetylation Assay—Acetylation reactions and the GST pull down assay were done as previously described (12, 16).

Statistical Analysis—All data were analyzed by the non-paired t test. p < 0.05 was considered significant.

RESULTS

Apoptotic Resistance Is a Pathophysiological Function of KLF5—The present study began with the initial observation of an attenuated response to vascular injury in KLF5 knock-out mice subjected to a mouse femoral artery injury model (9) (Fig. 1, A and B). To understand the mechanisms underlying this effect on neointimal hyperplasia, we questioned whether cell

FIGURE 1. Apoptotic resistance as a pathophysiological function of KLF5. A, mouse femoral artery injury model in KLF5 wild type (a, KLF5-wt) and heterozygous knock-out (b, KLF5-KO+/−) mice. Hematoxylin and eosin staining. B, intima to media (I/M) ratio (n = 6). Error bars represent S.E. Scale bar, 50 μm. **, p < 0.01. C, TUNEL staining of femoral artery injury samples. TUNEL is shown in green and propidium iodide (PI) in red. Arrowheads indicate merged TUNEL-positive nuclei (yellow). Arrows indicate the internal elastic lamina. NI, neointima; PC, phase contrast. D, graphical representation of TUNEL-positive apoptotic cells. *, p < 0.05. E, TUNEL staining in apoptotic cells subjected to RNA interference. HeLa cells were used on the basis that they contained the highest known endogenous levels of KLF5. F, graphical representation of TUNEL-positive apoptotic cells.* p < 0.05.
Increased apoptosis at the cellular level, we next used an RNA interference approach to knockdown KLF5 under apoptotic stimulation (TNF-α). TUNEL staining showed an increase in positive staining cells when subjected to KLF5 small interfering RNA as compared with control small interfering RNA (secreted alkaline phosphatase) (Fig. 1, E and F). Thus, KLF5 insufficiency is associated with increased apoptosis.

**Cellular Apoptotic Resistance of KLF5**—To characterize the apoptotic resistance mechanisms of KLF5, stable transformants (cloned) expressing KLF5 in human HeLa and murine 3T3 cells were used for further investigations. Both KLF5-expressing cells showed resistance to induced cell death by TNF-α as compared with mock cells (Fig. 2A). We then measured apoptotic caspase-3 activity, which was reduced in both KLF5-expressing cells (29% for TNF-α stimulation and 44% for anisomycin treatment in HeLa cells and 34% in 3T3 cells for TNF-α stimulation) (Fig. 2B). Further, TUNEL staining showed that KLF5-expressing cells were resistant to DNA cleavage by apoptotic stimulation as shown by quantification of TUNEL-positive cells (15% by TNF-α and 40% by anisomycin) (Fig. 2C). Thus, cells expressing KLF5 were consistently resistant to apoptosis by criteria including morphology, caspase-3 activity, and DNA cleavage (TUNEL). Apoptotic resistance was confirmed in at least two independent clones for both HeLa and NIH3T3 cells as well as in a resistance-selected heterogenous noncloned colony (data not shown).

**Mechanisms of Apoptosis-resistant Activity of KLF5**—Next, to investigate the molecular mechanisms of the apoptosis-resistant effects of KLF5, the expression levels of a panel of proteins related to the apoptotic signaling cascade were examined in the HeLa stable transfomant with apoptotic stimulation by TNF-α. Although there were no apparent effects on the expression of most of these apoptosis-related proteins, the protein level of poly(ADP-ribose) polymerase-1 (PARP-1) was markedly affected in KLF5-expressing cells as compared with the control (Fig. 3A). PARP-1 is a 113-kDa nuclear enzyme involved in DNA repair that catalyzes the initiation, elongation, and branching of poly(ADP-ribose) onto its target protein (11).
PARP-1 is cleaved by caspase into a 24-kDa amino-terminal DNA-binding domain and an 89-kDa carboxyl-terminal catalytic domain under apoptotic conditions. This result was not unexpected, given that caspase cleaves PARP-1, and as caspase-3 activity was reduced in KLF5-expressing cells (Fig. 2B). RNA interference experiments confirmed involvement of PARP-1 in apoptosis of the tested HeLa cells (data not shown).

Nevertheless, we further pursued actions of KLF5 on PARP-1 under apoptotic conditions given the specific effects on PARP-1. We hypothesized that PARP-1 and KLF5 might functionally interact, given that they are zinc finger proteins that often physically and functionally interact (17). Immunoprecipitation experiments showed that KLF5 interacts with PARP-1, and strikingly, for this interaction to be specific with the 24-kDa fragment under apoptotic conditions (Fig. 3B). Note that loading amounts of protein were normalized to show the difference in binding affinities. KLF5 did not interact with the 89-kDa carboxyl-terminal catalytic domain under these conditions (data not shown).

As these experiments were done using the stable transformant, further immunoprecipitation experiments were done to confirm interaction by endogenous proteins (Fig. 3C). Immunoprecipitation experiments using anti-KLF5 and PARP-1 antibodies confirmed that KLF5 and the PARP-1 24-kDa fragment interact in the cell. We thus sought to understand the functional implications and regulation of this interaction.

Interaction between KLF5 and PARP-1—To characterize the interaction between KLF5 and PARP-1, we next examined the specificity and site of interaction. For specificity, we compared the binding of PARP-1 between KLF5 and KLF6, the latter being a similar Krüppel-like factor (18, 19) (Fig. 4, A and B). GST pulldown assay under conditions in which KLF5 bound the 24-kDa pro-apoptotic fragment of PARP-1 (Fig. 4B, lane 4) showed a lack of interaction with KLF6 (Fig. 4B, lane 5). Thus, interaction of KLF5 with PARP-1 was direct and specific.

We further determined the site of interaction between KLF5 and PARP-1. To confirm our initial expectations that the zinc
finger motif is the protein-protein interaction interface (12, 17), we tested whether the zinc finger 24-kDa fragment of PARP-1 directly interacts with the zinc finger DNA-binding domain of KLF5 (Fig. 4, C and D). The GST pulldown assay showed that the zinc finger region of KLF5 directly and specifically bound the 24-kDa fragment of PARP-1 (Fig. 4D, lane 4). Given that the zinc finger regions of KLF5 and PARP-1 (which mediate their interaction) comprise their DNA-binding domains, we tested the requirement of DNA by competitively adding DNA to protein interaction assays, which showed that DNA is not necessary for this interaction and for this interaction to thus be mediated by protein-protein interaction (data not shown).

As the zinc finger DNA-binding domain of KLF5 contains three zinc finger motifs, we next examined whether there is specific binding of individual zinc fingers to PARP-1 (Fig. 4E). The GST pulldown assay showed the first zinc finger but not the second nor third zinc finger peptides to interact with PARP-1 (Fig. 4F, lanes 4–6).

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**Acetylation Is Important for the Apoptosis-resistant Effects of KLF5—**

As the interaction with PARP-1 was mediated through the first zinc finger of KLF5 (Fig. 4F) (which contains a lysine residue whose acetylation we have previously shown to be important for the cell growth stimulatory effects of KLF5 (12)), we next asked whether acetylation is important for apoptosis-resistant actions and interaction with PARP-1.

First, we examined whether a non-acetylatable point mutant of KLF5 (K369R, lysine → arginine substitution at residue 369) would lack resistance to apoptosis. Stable transformants in 3T3 cells of wild type and that of the point mutant K369R of KLF5 were subjected to apoptotic stimulus (TNF-α), and morphology was examined in addition to quantification of caspase-3 activity and DNA cleavage. As compared with wild-type KLF5-expressing cells, cells expressing the point mutant KLF5-K369R were less viable in response to apoptotic stimulus (TNF-α) (Fig. 5, A and B). Caspase-3 and DNA cleavage assay both showed that the point mutant KLF5-K369R did not inhibit apoptosis under conditions in which KLF5 wild type showed significant inhibition. Adenoviral transfer of the wild type and point mutant K369R into balloon-injured rat carotid arteries confirmed that the wild type but not the point mutant K369R can inhibit pathophysiological vascular apoptosis. These findings suggest acetylation of KLF5 is important for its apoptosis-resistant cellular effects.

The former experiments suggested that KLF5 is likely acetylated under apoptotic conditions. To test this, Western blot analysis using antibody against acetylated lysine was done that showed KLF5 is markedly acetylated under apoptotic conditions (Fig. 5C, lane 3), although we did see some acetylation under basal conditions (lane 2). We next asked whether acetylation might regulate interaction between KLF5 and the PARP-1 fragment. A protein-protein interaction assay using in vitro acetylated KLF5 was done that showed acetylation of KLF5 increased its binding affinity with the PARP-1 fragment (Fig. 5D, lane 4 versus lane 3), although we did note that the addition of the p300 acetyltransferase region alone resulted in a

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marginal increase (data not shown). We further examined whether acetylation augments interaction between KLF5 and the PARP-1 fragment in the cell. Immunoprecipitation experiments in endothelial cells transfected by adenovirus expressing similar amounts of wild-type or the point mutant KLF5-K369R followed by immunoprecipitation of similar amounts of the 24-kDa PARP-1 fragment by anti-PARP-1 antibody showed that wild-type KLF5 but not the point mutant KLF5-K369R to selectively interact with the PARP-1 24-kDa fragment (Fig. 5E, lane 2 versus lane 3). Acetylation of KLF5 is thus induced under apoptotic conditions and is important for its apoptosis-resistant activity as well as its interaction with PARP-1.

We further characterized the functional effects of this selective interaction. We reasoned that wild-type but not the point mutant KLF5-K369R may inhibit apoptosis induced by the 24-kDa pro-apoptotic fragment of PARP-1 (16, 20). The 24-kDa pro-apoptotic fragment of PARP-1 and wild-type KLF5 or the point mutant KLF5-K369R were transfected into endothelial cells, and effects on apoptosis were determined by examining caspase-3 activity. Under conditions in which the 24-kDa pro-apoptotic fragment of PARP-1 stimulated apoptosis, although marginally in our hands (Fig. 5F, lane 1 versus lane 2), wild-type KLF5 inhibited caspase-3 activity in contrast to the point mutant KLF5-K369R in which suppression of caspase-3 activity was not seen (Fig. 5F, lane 3 versus lane 4). These experiments showed that wild-type KLF5 but not the point mutant KLF5-K369R can inhibit apoptotic activity as stimulated by overexpression of the 24-kDa pro-apoptotic fragment of PARP-1.

DISCUSSION

Functional Interaction between KLF5 and PARP-1—The cardiovascular transcription factor, Krüppel-like factor 5 (KLF5), inhibits cell death/apoptosis and functionally interacts with PARP-1, a highly abundant nuclear enzyme that functions as a sensor of DNA damage. To our knowledge, KLF5 is the first protein to interact specifically and functionally with the 24-kDa PARP-1 fragment. This PARP-1 fragment also, at least partially, inhibits physical interaction with the Werner syndrome protein and also likely with DNA ligase III (21, 22), but neither the specificity nor the functional effect of the interaction had been addressed.

The PARP-1 fragment has been reported to harbor pro-apoptotic activity (16). We have found that KLF5 is able to inhibit the marginal pro-apoptotic effects of the PARP-1 fragment, that KLF5 interacts with this peptide, and that acetylation of KLF5 stimulates this interaction. It is tempting to speculate that sequestration of the PARP-1 proteolytic fragment by KLF5 may be a novel target for regulation of PARP-1 actions. However, we do note that the effects of PARP-1 on apoptosis remain controversial. Gene ablation studies in mice have shown that PARP-1 is not essential for apoptosis (23, 24). Additionally, cells exhibiting cleaved PARP-1 can divide normally (25), making its instructive role in apoptosis unclear. The fragment being produced after the apoptotic commitment step of caspase activation makes it further unlikely to be a critical determinant of apoptotic progression. Further investigation of the functional effect of the interaction with KLF5 will require a better understanding on the precise role of the PARP-1 fragment.

Regulatory Effects of Acetylation—Another important finding of the present study is that the signaling modification (acetylation) was shown to play an important role in the effects of KLF5 on cell death and interaction with PARP-1. Acetylation is a nucleus specific signaling modification that affects protein-protein as well as protein-DNA interactions by various nuclear factors (e.g. Armadillo and T-cell factor, Importin α and β) (26, 27). Although the biological role of this modification is not well understood, we show that it affects multiple activities of KLF5.

A recent study showed that acetylation of Sp1, a close relative of KLF5 that we previously showed to be acetylated (28–30), can be similarly induced by an apoptosis-inducing anti-cancer agent (31), which together with our findings may suggest that acetylation plays a key role in regulation of cell death/survival pathways in this family of factors. Further, as deacetylase (6) as well as acetylase and its activity (32) have been implicated in the cardiovascular remodeling response in the heart, this signaling pathway may have general implications for regulating the cardiovascular cell phenotype in response to pathological stress. KLF5, therefore, through acting on apoptotic pathways, may tip the balance between survival/repair and death/apoptosis under cardiovascular pathophysiological settings.

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