Regulation of Platelet-derived Growth Factor-A Chain by Krüppel-like factor 5\textsuperscript{a}\textsuperscript{b}

NEW PATHWAY OF COOPERATIVE ACTIVATION WITH NUCLEAR FACTOR-κB

Kenichi Aizawa\textsuperscript{a}, Toru Suzuki\textsuperscript{a}\textsuperscript{c}\textsuperscript{d}, Nanae Kada\textsuperscript{a}, Atsushi Ishihara\textsuperscript{a}, Keiko Kawai-Kowase\textsuperscript{a}, Takayoshi Matsumura\textsuperscript{a}, Kana Sasaki\textsuperscript{a}, Yoshiko Munemasa\textsuperscript{a}, Ichiro Manabe\textsuperscript{a}\textsuperscript{e}\textsuperscript{f}, Masahiko Kurabayashi\textsuperscript{a}, Tucker Collins\textsuperscript{a}, and Ryozo Nagaï\textsuperscript{a}\textsuperscript{g}

From the \textsuperscript{a}Departments of Cardiovascular Medicine and \textsuperscript{b}Clinical Bioinformatics, The University of Tokyo, 7-3-1 Honjo, Bunkyo-ku, Tokyo 113-8655, Japan, the \textsuperscript{c}Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, 3-39-15, Shawa-machi, Maebashi, Gunma, 371-8511, Japan, and the \textsuperscript{d}Department of Pathology, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

The transcription factor Krüppel-like factor 5 (KLF5) and its genetically downstream target gene platelet-derived growth factor-A (PDGF-A) chain are key factors in regulation of cardiovascular remodeling in response to stress. We show that KLF5 mediates a novel distinct delayed persistent induction of PDGF-A chain in response to the model agonist, phorbol ester, through a cis-element previously shown to mediate phorbol ester induction on to PDGF-A chain through the early growth response factor (Egr-1). Interestingly, the nuclear factor-κB (NF-κB) p50 subunit further cooperatively activates PDGF-A chain through protein-protein interaction with KLF5 but not Egr-1. RNA interference analysis confirmed that KLF5 and p50 are important for induction of PDGF-A chain. Collectively, we identify a novel regulatory pathway in which PDGF-A chain gene expression, under the control of KLF5, is cooperatively activated by the NF-κB p50 subunit and a pathophysiologic stimulus.

Adaptation to external stimuli is the hallmark feature of cardiovascular pathogenic processes ranging from atherosclerosis to heart failure (1, 2). Insult in the form of ischemia, infection, or physical stress brings about a remodeling process of cardiovascular cells and tissues to repair and compensate through activation of vascular endothelial and smooth muscle cells as well as cardiac interstitial cells.

Recent investigations by our group using genetically engineered mice have shown that the transcription factor Krüppel-like factor 5 (KLF5)\textsuperscript{1} is an important mediator of cardiovascular remodeling as manifested by a hypertrophic cardiac response to angiotensin II stimulus and a vascular inflammatory remodeling response to mechanical stress (3). Two important findings of these past studies that are relevant to the present study were that (1) the mitogenic growth factor platelet-derived growth factor-A (PDGF-A) chain gene was shown to be an endogenous target gene of KLF5 and (2) there was an attenuated inflammatory wound-healing response to vascular stimulus (cuff-injury).

PDGF, a homo- or hetero-dimer of A and B chains, is a well known mediator of cardiovascular remodeling and wound healing processes as an inducible factor controlling cell proliferation and migration (4–7). PDGF-A chain is a target gene of KLF5 based on the biochemical analysis of the knockout mouse and on the partial resemblance of the phenotypes (e.g. intestine) of the PDGF-A chain and KLF5 knockout mice (8). Despite these observations, we know little about how the PDGF-A chain is regulated by KLF5, which is important to understand a variety of pathophysiological mechanisms. We undertook the present study to dissect the mechanisms whereby KLF5 acts on PDGF-A chain, and particularly to understand their regulation in response to pathophysiological stimulus.

EXPERIMENTAL PROCEDURES

Plasmid Construct Preparation—Luciferase reporter deletion constructs were made by cleaving the PDGF-A chain luciferase promoter construct (PDGF-900) (3) into the following restriction fragments; SacI-HindIII (630 to +10), XhoI-HindIII (+260 to +10), Sac-HindIII (+1 to +10), and Sac-HindIII (+55 to +10) fragments. The resultant plasmids were named PDGF-630, PDGF-260, PDGF-71, and PDGF-55, respectively. The KLF5 expression vector pCAG-KLF5 has been described (3), and p50 and p65 expression vectors were a kind gift of Dr. Kunisch (9).

Cell Culture and Transfection Assays—HeLa cells (1 × 10\textsuperscript{5} cells) were transfected with 100 μg reporter and 1 μg expression plasmids by Tfx-20 reagent (Promega). Cells were incubated for 48 h and then subjected to luciferase assay (Promega) by luminometry (Lumat LB9507, Berthold). Luciferase activity was normalized to protein concentration of cell lysates. Shown are the results of a representative assay as done in duplicate and reproduced in at least two other occasions. Error bars denote standard deviation.

Protein Expression and Purification—The recombinant His\textsuperscript{b}-tagged KLF5 DNA-binding domain protein (KLF5DBD) was generated by PCR on pBK-ILKLF (4) (a kind gift of Dr. Teng) using primers (5’-CCGGAT-CCGGGAAAGACGCAGCCTACATC-3’ and 5’-CCGGATCCCTCGTTGCGCTCTC-3’) containing BamHI restriction endonuclease sites, inserted into the vector 6HIS-pET11d, and then expressed and purified essentially according to the described methods (10).

Gel-shift DNA-binding Assay—Gel-shift DNA-binding assays were done essentially as described previously (10). A DNA oligomer con-
RESULTS

Transcriptional Activation of the PDGF-A Chain Promoter by KLF5—We first investigated whether KLF5 transcriptionally regulates PDGF-A chain. Using reporter constructs harboring the PDGF-A chain promoter upstream of a luciferase reporter, cell co-transfection studies showed that KLF5 activates the PDGF-A chain promoter (containing 900 bp upstream of the transcription initiation site) (Fig. 1A, A and B). To next determine the cis-element responsible for mediating the KLF5 response, serial deletion PDGF-A chain promoter-reporter constructs were made and subjected to co-transfection analysis. Results showed that a region between −71 bp and −55 bp of the transcription initiation site mediated the KLF5 response as shown by a dramatic decrease in promoter activation for constructs not containing this region. This region between −71 bp and −55 bp is well characterized as an element that mediates various stimuli including phorbol ester on to the PDGF-A chain promoter and is known to bind the Sp1-binding sites as well as the similar Egr-1-binding sites. Using gel-shift analysis, we show that KLF5 binds this region between −71 bp and −55 bp of the PDGF-A chain promoter in a sequence-specific manner (Fig. 1D) thus showing that KLF5 has the ability to directly bind this cis-element and mediate promoter activation.

KLF5 Mediates Activation of PDGF-A Chain by Phorbol Ester—Phorbol ester, a model agonist to investigate inducible pathophysiological stimulation, is known to stimulate PDGF-A chain promoter activation by the Egr-1 transcription factor, which is transiently and rapidly expressed at one hour after stimulation through this cis-element (12, 13). KLF5 is also transcriptionally activated by phorbol ester stimulation. We therefore investigated if KLF5 may mediate phorbol ester stimulation on to the PDGF-A chain promoter (18, 19). Under conditions in which the region between −71 bp and −55 bp of the transcription initiation site mediated phorbol ester activation of PDGF-A chain consistent with previous studies (Fig. 2A), co-transfection analysis showed that KLF5 strongly activates PDGF-A chain promoter in cooperation with phorbol ester (Fig. 2A). Expression analysis of KLF5 after phorbol ester stimulation showed that KLF5 expression showed a gradual increase that was still increasing at six hours after stimulation, in contrast to Egr-1 that was transiently increased only in the first hour after stimulation (Fig. 2B). Importantly, PDGF-A chain expression paralleled the expression of KLF5. To test the direct causal relationship between KLF5 and PDGF-A chain expression, we tested whether forced expression of KLF5 would induce endogenous PDGF-A gene expression. By use of adenoviral-mediated transfer of KLF5, although forced expression of KLF5 alone was insufficient, co-treatment by phorbol ester with forced expression of KLF5 induced PDGF-A chain gene expression as shown by RT-PCR analysis (Fig. 2C). Collectively, KLF5 mediates phorbol ester stimulation of PDGF-A chain in a manner that is delayed, yet persistent, as compared with the acute transient induction mediated by Egr-1. This finding implicates a new pathway for the regulation of PDGF-A chain by KLF5 in response to a pathophysiological stimulus.

Cooperative Activation of PDGF-A Chain by KLF5 and NF-κB—KLF5 mediates a persistent induction of PDGF-A chain in response to phorbol ester stimulation suggesting an important role in mediating pathologic stress. Among the other signaling pathways that are activated by phorbol ester, the NF-κB pathway is well known for mediating external stimuli, particularly in inflammatory responses (20). However, a pathway linking NF-κB and PDGF-A chain has not been described. As the KLF5 knockout mice showed a defect in inflammatory vascular response, we envisioned that there may be cooperative interaction between these downstream factors to regulate transcription in the nucleus. We first examined whether NF-κB cooperates with KLF5 in regulating PDGF-A chain expression. Using co-transfection reporter analysis, we show that although NF-κB p50 and p65 either alone or together do not activate the PDGF-A chain promoter-reporter construct, cooperative activation of the PDGF-A chain promoter is seen specifically when the p50 subunit, but not the p65/c-rel subunit of NF-κB, is co-expressed with KLF5 (Fig. 3A). This is not due to the effects of p50 on KLF5 expression levels as shown by absence of effects on immunoblot analysis. Administration of an NF-κB inhibitor, parthenolide (21), blocked this cooperative activation with p50 (Fig. 3B).

To determine the cis-element which mediates this response, PDGF-A chain promoter deletion constructs were tested, which showed that the cooperative activation was mediated by the −71 bp to −55 bp region (Fig. 3C). This region mediates KLF5 property to bind similar GC-rich binding sites (14–17), we thought that KLF5 would also bind the Sp1-binding sites as well as the similar Egr-1-binding sites. Using gel-shift analysis, we show that KLF5 binds this region between −71 bp and −55 bp of the PDGF-A chain promoter in a sequence-specific manner (Fig. 1D) thus showing that KLF5 has the ability to directly bind this cis-element and mediate promoter activation.
Co-immunoprecipitation analysis further showed that KLF5 bound p50 (Fig. 3E). Importantly, Egr-1 did not bind to p50 under similar conditions attesting to the specificity of the reaction (Fig. 3E). To further confirm the specificity of this functional interaction, reporter assays using various promoters and factors were tested. Under conditions in which KLF5 and p50 cooperatively activated the PDGF-A chain promoter, neither Sp1 nor Egr-1 showed cooperative action with p50. Other KLF5-responsive promoters such as vascular cell adhesion molecule-1, inducible nitric oxide synthase, and non-muscle myosin heavy chain-B did not show cooperative activation, but tissue factor and plasminogen activator inhibitor-1 also showed
this phenomenon which may be suggestive of the generality of this mechanism (Fig. 3F and supplemental figure). Collectively, we show that KLF5 and the p50 subunit of NF-κB show cooperative activation of PDGF-A chain by forming a complex on the KLF5-binding element of the PDGF-A chain promoter. To our knowledge, this is the first report of NF-κB-mediated
FIG. 3. KLF5 and NF-κB p50 subunit cooperatively activate the PDGF-A chain promoter. A, co-transfection reporter analysis showing cooperative activation of PDGF-A chain promoter by KLF5 and NF-κB. Note that NF-κB p65/c-rel subunit does not show cooperative activation (lane 6) as seen for p50 with KLF5 (lane 7). B, the NF-κB inhibitor, parthenolide, inhibits cooperative activation by KLF5 and p50 (lane 6 versus 7). C, mapping the KLF5-p50-activated region of the PDGF-A chain promoter. Note that there is a significant decrease in cooperative activation between PDGF-71 and PDGF-55 (lane 12 versus 15). D, gel-shift assay showing KLF5 and p50 form a DNA-protein complex on PDGF-A chain promoter between −71 bp and −55 bp. Note that under conditions that KLF5 binds this region (lane 2) and p50 does not (lane 3), reaction with both proteins results in a slower mobility DNA-protein complex (lane 4), which is supershifted by anti-p50 antibody (lane 5). E, co-immunoprecipitation assay showing binding of KLF5 and p50. Under conditions in which cell lysate was immunoprecipitated with control IgG (lane 2) and p50 (lane 3) antibodies, immunoblot with KLF5 showed interaction with p50. Similar analysis was done with Egr-1 showing lack of association. F, specificity of cooperative activation between KLF5 and p50. Co-transfection analysis to test the specificity of KLF5, Sp1, and Egr-1 in combination with p50 on the PDGF-A chain promoter as well as various other promoters as shown (see supplemental figure for raw data). Combinations showing cooperative activation are shown in bold.
Fig. 4. Requirement of KLF5 and p50 for PDGF-A chain expression and induction in vitro. A, RNA interference analysis of KLF5. Note lack of effect on the 18S internal control and SEAP negative control attesting to the specificity of the knockdown reaction as shown by RT-PCR analysis. Constructs #1–#3 denote three independent constructs against KLF5. B, specificity of anti-KLF5 siRNA constructs is shown by lack of effect on KLF2, KLF4, KLF6, and Egr-1. C, RNA interference analysis of p50. Constructs #1–#3 denote three independent constructs against p50. D, KLF5 RNA interference attenuates PDGF-A chain expression as shown by RT-PCR analysis. E, p50 RNA interference attenuates PDGF-A chain expression as shown by RT-PCR analysis. F, selected siRNA constructs (numbers correspond to constructs shown for KLF5 in A and p50 in C) reduce PDGF-A chain promoter activation as shown by luciferase reporter assays. Note that the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA interference had no effect on PDGF-A chain promoter activation as compared with KLF5 (#1) and p50 (#1). G, summary of results. A new pathway of PDGF-A chain activation by KLF5 is seen in the late stages of phorbol ester stimulated expression, in contrast to the rapid induction of Egr-1 seen at early stages. Also, a new pathway of cooperative activation of KLF5 and the NF-κB p50 subunit showing regulation of PDGF-A chain by KLF5 at multiple stages.
regulation of PDGF-A chain. Multiple pathways, one involving direct stimulation of KLF5 activation by phorbol ester and another mediated by the p50 subunit of NF-xB on KLF5, therefore converge on KLF5 to activate the PDGF-A chain promoter.

Requirement of KLF5 and p50 for PDGF-A Chain Expression in Vivo—To confirm that KLF5 and p50 mediate PDGF-A chain expression in vivo, we used an RNA interference (RNAi) knockdown approach to examine the requirement of these two factors in mediating this response. We first established conditions in which KLF5 could be specifically knocked down by siRNA transfer (Fig. 4A). Specific effects of the constructs were established by absence of effect on 18 S RNA and the negative control, SEAP. The specificity of the siRNA constructs against KLF5 were confirmed by lack of effect on related factors KLF2, KLF4, KLF6, and Egr-1 (Fig. 4B). We next established conditions in which p50 could be specifically knocked down by siRNA transfer (Fig. 4C) and then examined whether KLF5 and p50 siRNA constructs could inhibit PDGF-A chain expression by RT-PCR analysis. Both KLF5 and p50 siRNA constructs could inhibit PDGF-A chain expression (Figs. 4, D and E). Effective constructs were selected and further used to show that they could inhibit PDGF-A promoter activation by reporter assay, supporting and confirming that these factors act at the level of transcription (Fig. 4F). These findings confirm that KLF5 and p50 siRNA constructs inhibit PDGF-A chain expression and establish the importance of these two factors in regulating expression of PDGF-A chain. It is tempting to envision further animal experiments using RNAi approaches, but at present, technical limitations hamper use of this technology in animals with limitation to cellular experiments.

DISCUSSION

New Pathway of Regulation of PDGF-A Chain Induction by KLF5—The present study shows that KLF5 transcriptionally regulates the PDGF-A chain with cooperative activation by phorbol ester. Interestingly, phorbol ester induces expression of KLF5 in a delayed yet persistent manner that follows a rapid yet transient response as mediated by Egr-1 (Fig. 4G). In fact, forced expression of KLF5 in combination with phorbol ester administration was sufficient to induce PDGF-A chain expression in the cell, which strongly suggests that this combination of factors is relevant in the pathophysiological situation. We envision that this two-stage regulation of PDGF-A chain expression as manifested by initial stimulation by Egr-1 followed by that of KLF5 allows for assemblage and convergence of multiple levels of cascading regulation on PDGF-A chain. A 16 bp short region between −71 bp and −55 bp of the PDGF-A chain promoter integrates these signals. Importantly, this region contains only GC-rich binding sites that have been shown to bind Egr-1 and Sp1. Like KLF5, both of these zinc finger transcription factors bind GC-rich binding sites. Although we cannot exclude the possibility that both Egr-1 and KLF5 may act in concert on the PDGF-A chain promoter simultaneously (e.g. hetero-dimerization), their distinct non-overlapping expression patterns in response to phorbol ester stimulation suggests that they act independently. We therefore envision that although Egr-1 acts as the DNA-transcription factor to mediate signals during the early acute phase, KLF5 mediates signals at later stages for sustained activation of the PDGF-A chain promoter.

What signals are distinctly mediated by Egr-1 and KLF5? Indeed, it is important to understand the different regulation and pathways that converge on Egr-1 and KLF5 to understand their different functions. Our novel finding in the present study that the NF-xB p50 subunit mediates cooperative activation on PDGF-A chain by KLF5 but not by Egr-1 may be one of the answers to this important question. Given the well known role of NF-xB to mediate inflammatory responses secondary to pathological stimuli, it is an ideal nuclear factor to regulate the pathologically inducible PDGF-A chain. Our finding that p50 cooperatively activates the PDGF-A chain promoter through protein-protein interaction with KLF5 was therefore a welcome surprise. This implicates not only a direct pathway involving activation of PDGF-A chain through a delayed increase in expression amounts of KLF5, but also a cooperative effect as manifested by convergence of the NF-xB signal on to KLF5 to further provide regulation. Again, this cooperative response is not seen with Egr-1, which strongly suggests that this is a specific response that is finely tuned for robust activation of PDGF-A chain in response to pathological stimuli in a complex pathophysiological situation.

In closing, we have identified a novel pathway regulating transcriptional regulation of PDGF-A chain by KLF5 (Fig. 4G). Convergence of multiple signaling pathways on to KLF5 (e.g. phorbol ester and NF-xB) amplifies the qualitative as well as quantitative nature of the temporal and spatial regulation of PDGF-A chain. This new pathway through KLF5 is a tempting target for therapeutic intervention aimed at modulating PDGF-A chain and its associated pathologies in the cardiovascular system. Further, cooperative activation between NF-xB and KLF5 may also be a promising target for modulation of inducible pathophysiological responses.

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