Y-Box Protein 1 Mediates PDGF-B Effects in Mesangioproliferative Glomerular Disease

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The pivotal role of PDGF-B for mesangioproliferative glomerular disease is well established. Here, Y-box protein-1 (YB-1) was identified as a downstream signaling target of PDGF-B. In healthy kidney cells, YB-1 was located predominantly within the nuclear compartment. Subsequent to PDGF-B infusion and in the course of anti–Thy1.1-induced mesangioproliferative glomerulonephritis, relocalization of YB-1 into the cytoplasm was observed. In experimental models that lack profound mesangial cell proliferation (e.g., Puromycin-nephrosis, passive Heyman nephritis, spontaneous normotensive nephrosclerosis, hyperlipidemic diabetic nephropathy), YB-1 remained nuclear. This translocation coincided with upregulation of YB-1 protein levels within the mesangial compartment. Increased YB-1 expression and subcellular shuttling was dependent on PDGF-B signaling via the mitogen-activated protein kinase pathway because these alterations were prevented by specific PDGF aptamers and the mitogen-activated protein kinase pathway inhibitor U0126. Furthermore, PDGF-B strongly induced YB-1 expression in vitro. This induction was important because RNAi-dependent knockdown of YB-1 abolished the mitogenic PDGF-B effect. Taken together, YB-1 seems to represent a specific and necessary PDGF-B target in mesangioproliferative glomerular disease.


PDGF-B is a key mediator of mesangioproliferative glomerular disease (1). Among the known downstream signaling events that are activated by PDGF-B, the mitogen-activated protein kinase (MAPK) pathway seems to be of fundamental importance for nephritic diseases because the selective inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) leads to significant amelioration of the disease (2). A similar beneficial effect has been demonstrated by the specific inhibition of PDGF-B action using high-affinity aptamers that bind PDGF-B and abrogate mesangioproliferative disease (3). A recent report described that PDGF-B chain expression is regulated by the transcription factor denoted Y-box binding protein-1 (YB-1) in endothelial cells (4). This factor was originally identified because of its affinity for the Y-box sequence element within the MHC class II promoter (5) and belongs to the ancestral protein family of cold shock proteins with multifunctional properties (6). YB-1 acts as a transcriptional regulator of the genes coding for GM-CSF and IL-2 (7–9), matrix metalloproteinase-2 (MMP-2; gelatinase A) (10,11), and type I collagen (12,13). Depending on the cellular context, YB-1 may transactivate or -repress target gene transcription (9,10,14,15). In addition, YB-1 binds to mRNA in a nonspecific as well as a specific way, which has profound effects on mRNA translation (16) and mRNA half-lives, e.g., of the IL-2 and GM-CSF genes (7,17). Finally, by means of direct interaction with specific mRNA sequences, YB-1 may affect mRNA splicing, as has been demonstrated for the CD44 and E1A genes (18–20).

The functions described above can be accomplished only by a protein that is localized to both compartments, the nuclear and cytoplasmic. Hitherto, data on the subcellular YB-1 localization in nontumorous cells in vivo are scarce, and most information has been obtained in proliferating, immortalized cell lines. In the latter, YB-1 (also denoted p50) is predominantly localized in the cytoplasm and tightly bound to messenger RNA (21). After stimulation with cytotoxic agents, exposure to hyperthermia or ultraviolet radiation, nuclear translocation takes place (22,23). A nuclear staining pattern of YB-1 in several human cancer types has been associated with poor prognosis and metastasis formation (24–26).

In this study, we tested the hypothesis that PDGF-B may stimulate YB-1 expression and/or affects the subcellular localization. To this extent, the subcellular distribution of YB-1 in healthy kidney tissue and in various models of glomerular disease was determined. In addition to the observation of a relatively disease-specific nuclear export of YB-1 in mesangioproliferative renal disease, PDGF-B signaling via the MAPK...
ERK1/2 was identified as crucial for shuttling. Furthermore, YB-1 was found to be important for PDGF-B–dependent cell proliferation.

Materials and Methods

Cells and Culture Conditions

Primary human mesangial cells (BioWhittaker, Verviers, Belgium) were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, nonessential amino acids, 5 mg/L insulin, 3.4 µg/L sodium selenite, 2.8 mg/L transferrin, 100 µg/ml streptomycin, and 100 U/ml penicillin at 37°C in humidified 5% CO2 in air. Mouse podocytes (27) were grown at permissive temperature of 33°C in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 9 mM sodium bicarbonate, 10 U/ml mouse IFN-γ, 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified 5% CO2 in air. For cell differentiation, cells were incubated at nonpermissive temperature of 37°C in medium that lacked mouse IFN-γ. For stimulation experiments, podocytes that were cultured for 7 d in differentiation media were used.

Animal Models

All animal studies were approved by the local institutional review board. Ten male Wistar rats (Charles River Wiga GmbH, Sulzfeld, Germany) that weighed 140 to 180 g remained untreated and served as healthy control animals.

Mesangiproliferative Anti-Thy1.1 Nephritis

Anti-Thy1.1 nephritis was induced in 20 male Wistar rats (Charles River Wiga GmbH; 160 to 180 g) by intravenous injection of 1 mg/kg monoclonal anti-Thy1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK) as described (3). Animals remained untreated and were killed at days 1, 6, 7, 9, and 31 after disease induction (n = 4 at each time point).

Passive Heyman Nephritis

Passive Heyman nephritis (PHN) was induced in four male Sprague-Dawley rats (Charles River Wiga GmbH; 230 to 240 g) by intravenous injection of 0.8 ml of sheep anti-Fx1a antibody per rat (28). Animals were killed at day 8 after induction of the disease.

Puromycin Nephrosis

Puromycin nephrosis (PAN) was induced in four male Sprague-Dawley rats (Charles River Wiga GmbH; 210 to 230 g) by intravenous injection of 150 mg/kg puromycin (Sigma, Deisenhofen, Germany) dissolved in normal saline as described (29). Renal tissues were obtained after the rats were killed on day 7.

Milan Normotensive Rats

Three Milan normotensive rats were obtained from the Hannover Medical School (Hannover, Germany). Renal tissues were obtained after the rats were killed at 40 wk of age (30).

Obese Zucker Rats

Three male obese (fa/fa) Zucker rats were obtained from Charles River Wiga. Renal tissues were obtained after the rats were killed at 60 wk of age (31).

Tissues were either fixed in methyl Carnoy’s solution, embedded in paraffin, and sectioned for immunohistochemistry or fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for in situ hybridization. The presence of morphologic features of the different renal diseases was examined in periodic acid-Schiff-stained sections. All tissue sections contained a minimum of 50 (usually >100) glomerular cross-sections.

PDGF-BB Infusion

Infusion of PDGF-BB in vivo was performed as described previously (32). Briefly, five normal male Wistar rats (Simonson, Gilroy, CA) that weighed 180 to 220 g received a 7-d intravenous infusion of 40 µg/d PDGF-BB, and five rats received a vehicle. Infusion was performed continuously through a catheter in the left internal jugular vein with micro-osmotic pumps. Renal biopsies were obtained from each rat on day 4 and upon killing on day 7.

Treatment of Nephritic Rats with PDGF-B–Specific Aptamers

Anti-Thy1.1 mesangiproliferative GN was induced in male Wistar rats as described above. Rats received twice-daily intravenous injections of 0.33 mg of PDGF-B–specific aptamers or scrambled aptamers, starting at day 3 after the induction of anti-Thy1.1 glomerulonephritis (GN) as described previously (3,33). Necropsies were performed in all rats at day 6. After immunostaining, the subcellular YB-1 localization was semiquantitatively graded into the categories “nuclear,” “strong nuclear/weak cytoplasmic,” “strong cytoplasmic/weak nuclear,” and “cytoplasmic” in >20 cross-sections of cortical glomeruli, each containing at least 20 discrete capillary segments, by a blinded observer.

Treatment of Nephritic Rats with MEK Inhibitor U0126

Anti-Thy1.1 mesangial proliferative GN was induced in male Wistar rats as described above. Rats received twice-daily intraperitoneal injections of 10 or 100 mg/kg body wt U0126 dissolved in DMSO or vehicle alone, starting on day 3 after the induction of anti-Thy1.1 GN as described (34). Necropsies were performed in all rats at day 6. The animals received the last dose of U0126 1 h before being killed.

Antibodies

Two affinity-purified rabbit polyclonal antibodies were raised against epitopes localized within the C-terminal domain of rat YB-1, as recently described (35). A third affinity-purified rabbit polyclonal antibody was raised against the N-terminal domain of human YB-1 and kindly donated by H.D. Royer (36). Specificity has previously been demonstrated for immunohistochemical procedures in paraffin-embedded tissue sections (36). Monoclonal anti-YB-1 antibody was obtained from Y.be.medic (Aachen, Germany). Monoclonal proliferating cell nuclear antigen (PCNA) antibody (clone Ab-1) was obtained from Dianova (Hamburg, Germany).

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry was performed on methyl Carnoy’s fixed, paraffin-embedded tissue sections following published protocols (28,37). Four-micron sections of tissue samples were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. The sections were incubated for 1 h at room temperature with the primary antibody diluted in PBS plus 1% BSA (Sigma, St. Louis, MO). After washes in PBS, the sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). An ABC Signal Amplification (ABC-Elite; Vector; Grünberg, Germany) was performed according to the instructions of the manufacturer. Finally, 3,3′ diaminobenzidine (with nickel chloride enhancement) was used as a chromogen. Sections were counterstained with methyl green, dehydrated, and coverslipped. Negative controls for the immunohistochemical pro-
Generation of Molecular Probes for In Situ Hybridization

The full-length 1.1-kb coding sequence of rat YB-1 was subcloned into pBluescript (Invitrogen, San Diego, CA), linearized with Hind III, and transcribed with T3 to obtain the antisense probe, or linearized with XbaI and transcribed with T7 for the sense probe. YB-1 mRNA was detected in tissue sections using in situ hybridization techniques following protocols that have been described previously (37). Riboprobes for in situ hybridization were generated from cDNA using 35S-UTP. Four-micron sections of formalin-fixed, paraffin-embedded tissue samples were deparaffinized and rehydrated through xylene and graded ethanols, washed with 0.5 × SSC (Life Technologies, Grand Island, NY), and digested with proteinase K (5 μg/ml, Sigma). Prehybridization was performed for 2 h by adding 100 μl of prehybridization buffer (0.3 M NaCl, 20 mM Tris [pH 8.0], 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, and 10 mM dithiothreitol). The hybridizations were started by adding 500,000 cpm of 35S-labeled riboprobe in 50 μl of prehybridization buffer and allowed to proceed overnight at 50°C. Sections were treated with RNase A (20 μg/ml, Sigma), followed by three high-stringency washes in 0.1 × SSC/0.5% Tween 20 (Sigma) for 45 min each at 50°C, and repeated 2 × SSC washes. After the tissue was dehydrated and air-dried, it was dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 3 wk. After developing, the sections were counterstained with hematoxylin and eosin, dehydrated, and coverslipped.

Northern Blotting

Total RNA was extracted from the isolated glomeruli with the guanidinium isothiocyanate/phenol/chloroform method using standard procedures. The RNA content and the purity of the samples obtained was determined by ultraviolet (UV) spectrophotometry at 260 and 280 nm. For Northern analysis, RNA was denatured and 10 μg/lane was electrophoresed through a denaturing 1% agarose/formaldehyde gel. Integrity of the RNA was assessed by visualization of ethidium bromide–stained 28S and 18S rRNA bands. Separated RNA was transferred onto a nylon membrane (Hybond N; Amersham Pharmacia Biotech, Uppsala, Sweden). First-strand cDNA was synthesized from 1 μg total RNA sample, each 500 μM dNTP, 250 ng of random primer, 30 U of RNAsin, and 200 U of Maloney murine leukemia virus reverse transcriptase in a 50-μl reaction volume. Taqman PCR was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems, Weiterstadt, Germany). In each reaction, 0.75 μl of cDNA was amplified in a 25-μl volume using the qPCR Core Kit (Eurogentec, Seraing, Belgium). The PCR conditions were 50°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Taqman primer and probe sets for YB-1 were designed from sequences in the gene bank database using Primer Express software (Applied Biosystems): forward primer, 5′-CACCTTACTACATCGGAGACCT-3′; reverse primer, 5′-TGGTACGCACCCCTCCATC-3′; Taqman probe, 5′-ACCACGATTTCCACCCCTCTTGTCGAC-3′. For internal control, Taqman ribosomal RNA control reagents (Applied Biosystems) were used.

Knockdown of Endogenous YB-1 by Small Interfering RNA

Embryonic mouse fibroblasts (NIH3T3 cells) were grown to 50% confluence on 10-cm plates in RPMI 1640 with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were transfected with the empty vector pSuper or the pSuper vector harboring the sequence 5′-GGTCATCGGAGGTTTT5′ (OligoEngine, Seattle, WA) as a tail-to-tail tandem repeat of both bp 285 to 305 of the human YB-1 coding sequence (accession no. J03827). Stable transfections with liposomal preparation Fugene were performed in conjunction with G418 resistance plasmid pUHD15–Ineo (BD-Clontech, Heidelberg, Germany). Five micrograms of total plasmid DNA and 15 μl of Fugene solution were mixed in 500 μl of serum-free medium, incubated for 15 min at room temperature, and added dropwise to culture medium (10 ml/plate). After 24 h, the medium was exchanged and selection with G418 at a concentration of 400 μg/ml was started. Within 2 wk, single-cell clones were apparent and selectively picked. Screening for the presence of pSuper plasmid DNA was performed, and changes of YB-1 mRNA and protein levels were performed by real-time PCR and immunoblotting using a polyclonal anti–YB-1 antibody.

Proliferation Assay

Proliferation of YB-1 knockdown and control cells was measured by BrdU incorporation using the 5-bromo-2′-deoxyuridine (BrdU) colorimetric ELISA kit (Roche, Mannheim, Germany). Cells were plated on 96-well plates and growth-arrested with medium without FCS for 24 h followed by treatment with PDGF-B, U0126, or PDGF-B plus U0126 for 24 h. During the last 16 h, the cells were grown in the presence of BrdU and the incorporation thereof was measured by ELISA using an anti–BrdU mAb.

Stimulation of Human Mesangial Cells with PDGF-BB

Cells were grown until 80% confluence in a 75-cm² flask with RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, nonessential amino acids, 5 mg/L insulin, 3.4 μg/L sodium selenite, 2.8 mg/L transferrin, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in humidified air that contained 5% CO₂. Before stimulation, cells were growth-arrested with MCDB-Medium (Sigma) for 24 h. PDGF-BB was added in final concentration of 50 ng/ml, PDGF-AA was added in a final concentration of 100 ng/ml in RPMI medium, and total RNA was isolated 24 h after stimulation.

Stimulation of Podocytes with PDGF-BB

On day 7 of differentiation in the specified medium, cells were washed with PBS, and a differentiation medium without FCS was added for 24 h. Thereafter, the cells were stimulated with 50 ng/ml PDGF-BB and harvested after 1, 2, 4, 8, 24, and 48 h.

Real-Time PCR

Cells were grown until subconfluence in a 75-cm² flask. Total RNA was isolated using the Invisorb Spin Cell-RNA Mini Kit (Invitek, Berlin, Germany). First-strand cDNA was synthesized from 1 μg of each total RNA sample, each 500 μM dNTP, 250 ng of random primer, 30 U of RNAsin, and 200 U of Maloney murine leukemia virus reverse transcriptase in a 50-μl reaction volume. Taqman PCR was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems, Weiterstadt, Germany). In each reaction, 0.75 μl of cDNA was amplified in a 25-μl volume using the qPCR Core Kit (Eurogentec, Seraing, Belgium). The PCR conditions were 50°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Taqman primer and probe sets for YB-1 were designed from sequences in the gene bank database using Primer Express software (Applied Biosystems): forward primer, 5′-CACCCTTACTACATCGGAGACCT-3′; reverse primer, 5′-TGGTACGCACCCCTCCATC-3′; Taqman probe, 5′-ACCACGATTTCCACCCCTCTTGTCGAC-3′. For internal control, Taqman ribosomal RNA control reagents (Applied Biosystems) were used.

Statistical Analyses

All values are expressed as means ± SD. Statistical significance (defined as P < 0.05) was evaluated using ANOVA and Bonferroni t test.
Results
Profound Changes in Subcellular Localization of YB-1 Characterize Course of Mesangioproliferative GN

In healthy, normal rat kidneys, YB-1 protein localized predominantly to the nuclear compartment of glomerular, tubular, and vascular cells (Figure 1A, d0). Manual counting of >20 cross-sections revealed that a fraction of approximately 25% of all immunopositive cells exhibited a weak perinuclear staining pattern (individual cell indicated by arrow in Figure 1A). YB-1 was detected in approximately 50% of the cells in all renal compartments with no cell type preference. No differences were observed between the 10 normal rat kidneys analyzed. The specificity of the immunohistochemical signal was verified by using three different antisera directed against different YB-1 epitopes. Identical staining patterns were obtained (compare Figure 1, A and B; and data not shown). Furthermore, substitution of the antiserum with species-matched, nonimmune serum resulted in the complete absence of a staining signal (Figure 1A, CON).

The anti-Thy1.1 nephritis model is an antibody-induced, complement-mediated, mesangioproliferative rat GN that closely resembles some features of human mesangioproliferative glomerular diseases. At day 1 after disease induction, a prominent mesangiolysis can be demonstrated ultrastructurally despite the presence of a normal light microscopic appearance. Induction of the anti-Thy1.1 GN resulted in a dramatic subcellular relocalization of YB-1. Within 1 d, YB-1 localized to the perinuclear region of cells within all glomeruli (Figure 1A, d1). In the course of this disease, the strongest cytoplasmic YB-1 signal coincided with the peak mesangial cell activation/proliferation between days 4 and 6 and was detected in a mesangial distribution pattern (Figure 1A, d6). Especially in areas of matrix turnover, e.g., microaneurysms, there was pronounced expression and cytoplasmic localization of YB-1 (Figure 1A, d7). Although the anti-Thy1.1 model is a glomerular disease model, the change in the subcellular distribution of YB-1 was not restricted to glomerular cells (Figure 1A, d1). With resolution of disease, a second dramatic change of the YB-1 distribution was apparent. At day 9, mesangial hypercellularity is reduced significantly and similar to healthy kidneys. At this stage, YB-1 was detectable primarily within nuclei of glomerular and tubular cells (Figure 1A, d9). In focal segments of persisting mesangial hypercellularity, YB-1 still localized to the cytoplasm. At day 31, a complete morphologic restitution was associated with a YB-1 localization that was restricted to nuclei (Figure 1A, d31). The detection of a changed YB-1 localization in the course of this nephritis was obvious with all antisera that were raised against different epitopes of YB-1 (compare Figure 1B).

Increased YB-1 mRNA Synthesis Occurs during Mesangial Cell Proliferation In Vivo

Given the profound changes in the YB-1 distribution in the course of the inflammatory glomerular disease, we next assessed whether this also coincides with increased abundance of YB-1 transcripts. By Northern blot analysis, a single band corresponding to the expected size of YB-1 mRNA, 1.8 kb, was observed as a single band on the blot corresponding to 1.8 kb. This band was observed in all samples tested, including healthy and diseased kidneys.

Figure 1. Localization of Y-box protein 1 (YB-1) in healthy rat kidney cells and after induction of anti-Thy1.1 mesangioproliferative glomerulonephritis (GN). (A) Immunohistochemistry for YB-1 was performed with kidney tissue from healthy rats and in the time course of anti-Thy1.1 nephritis (days 1, 6, 7, 9, and 31 after injection of anti-Thy1.1 antibody). Whereas a predominant nuclear YB-1 staining pattern except for individual cells (indicated by arrows) was observed with an antibody directed against the C-terminus in control tissue, a temporal shift of YB-1 to the cytoplasmic compartment was seen from day 1 on and included mesangial, glomerular endothelial, and epithelial cells. In areas of increased mesangial cell numbers and adjacent to microaneurysm formation, YB-1 expression was most pronounced. With resolution of the mesangioproliferative response from day 9 on, a reversal to a nuclear YB-1 localization that was indistinguishable from the staining pattern with healthy tissue was apparent. Healthy rat kidneys stained with nonimmune rabbit-IgG confirmed specificity of staining (CON). (B) By using an anti–YB-1 antibody directed against the C-terminus (36), a similar staining pattern was observed with nuclear YB-1 localization in all glomerular cells in control animals and exclusively cytoplasmic staining on day 6 after anti-Thy1.1 disease induction.
detected in glomerular RNA from healthy rats and from rats at day 6 after anti-Thy1.1 GN (Figure 2, A and B). Although there was a weak band in normal rat glomeruli indicating constitutive YB-1 mRNA synthesis, a significant, three-fold increase of YB-1 transcript abundance was apparent at day 6 after anti-Thy1.1 disease induction.

In situ hybridization studies confirmed the results that were obtained by Northern blot analysis. In healthy rat kidneys, there was no prominent YB-1 mRNA detection in the various compartments in any of the analyzed specimens (Figure 2C), and the hybridization pattern was indistinguishable from that obtained with a sense control probe. In the course of the anti-Thy1.1 GN, upregulated YB-1 mRNA expression was noted. Peak glomerular YB-1 mRNA expression was observed at days 4 and 6 (Figure 2C). Although all glomerular cell types seemed to be involved in the increased YB-1 mRNA synthesis, the strongest YB-1 mRNA hybridization signal localized to focal mesangial hypercellularity.

YB-1 Localization in Glomerular Disease Models

We further analyzed the YB-1 localization in different rat glomerular disease models, including immunologic injury to podocytes (PHN), toxic injury to podocytes (PAN), spontaneous normotensive nephrosclerosis (38), and diabetic Zucker rats. Despite the presence of significant glomerular injury in all of these models, YB-1 remained localized predominantly to cell nuclei within the glomerular, tubulointerstitial, and vascular compartment, comparable to healthy control animals (Figure 3). The number of cells with perinuclear YB-1 staining (indicated by arrows in Figure 3) was approximately 25% of the total immunopositive cells, as assessed by manual counting, and was not different from control animals.

In Vivo Infusion of PDGF-BB Results in Cytoplasmic Shuttling of YB-1

For the anti-Thy1.1 model, a strong induction of PDGF-B expression has been described, which distinguishes it from the other examined glomerular disease models. For addressing the question of whether PDGF-B alone, in the absence of antibody-mediated damage, induces a subcellular shuttling of YB-1, animals were infused continuously with PDGF-BB (40 μg of PDGF-BB/d versus vehicle alone). By immunohistochemistry, a similar cytoplasmic localization was apparent in the treated animals at both examined time points, days 4 and 7 (Figure 4). For vehicle-treated animals, the YB-1 staining pattern was predominant nuclear.

Figure 2. Upregulated YB-1 transcription localizes to the mesangial compartment in anti-Thy1.1 GN. (A) Northern blot analysis of pooled glomeruli was performed to quantify glomerular YB-1 mRNA expression in control animals (two animals per sample in lanes 1 and 2) and on day 6 after disease induction (lanes 3 and 4). (B) Densitometric analysis from two independent experiments and normalization for the loading amount by comparison with 18S RNA revealed a three-fold upregulation of YB-1 transcript numbers on day 6 after disease induction. (C) In situ hybridization with specific antisense probe (AS) revealed a scarce staining pattern nearly indistinguishable from background signal obtained with sense probe (S). In contrast, the antisense probe detected increased amounts of transcripts on day 6 after induction of anti-Thy1.1 nephritis.

Figure 3. Predominant nuclear staining pattern of YB-1 in experimental models of membranous, diabetic, and hypertensive nephropathy. Immunohistochemistry was performed in a variety of different rat glomerular disease models, including spontaneous normotensive nephrosclerosis (Milan; A), puromycin nephrosis (PAN; B), passive Heyman nephritis (PHN; C), and hyperlipidemic diabetic nephropathy (Zucker; D). Despite the presence of significant glomerular injury in all of the analyzed models, the YB-1 expression remained localized mostly to cell nuclei within the glomerular compartment. Individual cells with perinuclear YB-1 staining were detected and are marked by arrows. Changes comparable to the anti-Thy1.1 model were not apparent.
PDGF-B Signaling via ERK Enhances YB-1 Expression and Leads to Cytoplasmic Shuttling

For corroborating PDGF-BB as an upstream inducer of YB-1 shuttling and expression in vivo, PDGF-B signaling was blocked via application of specific aptamers. In a second set of experiments, the ERK1/2 signaling pathway downstream of the PDGF receptor was inhibited. For this, first, anti-Thy1.1 nephritis was induced and the rats subsequently received twice daily injections of scrambled or specific PDGF-B aptamers (Figure 5A). Treatment with PDGF-B aptamer antagonists not only significantly reduced the glomerular mitosis rate by 64%, and reported by Floege et al. (3), but also preserved a YB-1 expression pattern reminiscent of healthy rats (Figure 5B, d through f). By immunohistochemistry, YB-1 staining intensity decreased and predominantly nuclear/perinuclear localization was noted as compared with the predominantly cytoplasmic localization in the scrambled aptamer–treated group (3.4 ± 5.9% nuclear, 3.4 ± 7.6% strong nuclear/weak cytoplasmic, 20.7 ± 25.5% weak nuclear/strong cytoplasmic, 72.5 ± 38.5% cytoplasmic staining; Figure 5B, a through c). Quantification of the staining pattern revealed a predominantly nuclear localization in approximately 75% of all cells in the PDGF-B aptamer treatment group (44.9 ± 33.6% nuclear, 27.5 ± 11.6% strong nuclear/weak cytoplasmic, 15.1 ± 15.5% weak cytoplasmic/strong cytoplasmic, 12.5 ± 9.1% cytoplasmic staining; P < 0.05 compared with the scrambled aptamer group; Figure 5C).

Similar changes in YB-1 expression and localization were observed after blockage of the ERK signaling pathway by daily injections of the MEK inhibitor U0126 from days 3 to 6 after disease induction (Figure 6A). Treatment with U0126 resulted in a significant reduction of mesangial cell proliferation (2), and the YB-1 expression was similar to healthy controls with mostly nuclear localization in glomeruli of nephritic rats that were treated with U0126 (Figure 6B, d through f). These changes were observed to the same extent with both MEK inhibitor concentrations, that is 10 and 100 mg/kg body wt. For comparison, YB-1 staining in nephritic animals that were treated with DMSO vehicle are shown in Figure 6B, a through c. Counting of
stained cells again revealed a predominantly nuclear pattern in U0126-treated animals (19.4 ± 41.1% nuclear, 34.1 ± 20.2% strong nuclear/weak cytoplasmic, 30.9 ± 17.7% weak nuclear/strong cytoplasmic, 15.6 ± 21.3% cytoplasmic staining; P < 0.05 compared with the vehicle-treated group; Figure 6C). The changed subcellular localization of YB-1 in PDGF-B– or MAPK inhibitor–treated in comparison with untreated nephritic animals correlated to a significant reduction of mesangial cell proliferation as shown by PCNA staining in serially cut sections (25.4 ± 4.4 PCNA-positive cells per glomeruli in aptamer and 27.5 ± 12.4 positive cells/glomeruli in U0126-treated rats versus 43.8 ± 8.3 positive cells/glomeruli in untreated nephritic rats; Figure 7).

**PDGF-B Induces YB-1 Expression in Primary Human Mesangial Cell Cultures**

In vitro studies have previously demonstrated that YB-1 is the main regulator of PDGF-B chain expression in endothelial cells stimulated with thrombin (4). Given the above in vivo results, which additionally identify YB-1 as a downstream target of PDGF-B, an autostimulatory loop might exist in the case of YB-1 and PDGF-B. To test for this hypothesis, we incubated primary human mesangial cell cultures with PDGF-BB at 50 ng/ml, at which proliferation rates were maximum, and YB-1 protein as well as mRNA levels were determined. A significant, five-fold induction of YB-1 mRNA levels was detectable after stimulation with PDGF-BB but not PDGF-AA for 48 h (Figure 8A, top). In accordance with the upregulated transcript number, YB-1 protein levels were elevated after PDGF-BB stimulation, starting at 2 h after incubation, that lasted for at least 8 h (Figure 8, B and C). By immunoblotting using a monospecific anti–YB-1 antibody, a major band with molecular size of 50 kD and a minor band at 53 kD were detected in nonstimulated cells (denoted 4 and 3 in Figure 8B). Upon PDGF-B incubation, additional bands of relative molecular sizes 35, 60, and 66 kD appeared (denoted 1, 2, and 5). Cross-reactivity of the secondary antibody was excluded by omission of the primary antibody (lane 8). Other cell types, such as podocytes, also express YB-1 (10); however, the increase of YB-1 transcript number after stimulation with PDGF-BB was not present (Figure 8A, bottom) indicating a cell-specific effect.

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**Figure 6.** Mitogen-activated protein kinase (MAPK) inhibitor treatment prevents YB-1 upregulation and nucleocytoplasmic shuttling. (A) Anti-Thy1.1 nephritis was induced in animals, which were thereafter treated with daily injections of vehicle (a through c) or MEK inhibitor U0126 (d through f) from days 3 to 7. (B) Immunohistochemistry for YB-1 reveals mostly nuclear staining and no apparent upregulation compared with healthy controls in the MEK inhibitor U0126 treatment group. Representative results obtained with three different animals are depicted. (C) Manual count of YB-1 staining pattern.

**Figure 7.** Treatment of nephritic rats with PDGF-B–specific aptamers or MAPK inhibitor prevents YB-1 nucleocytoplasmic shuttling and mesangial cell proliferation. Proliferating cell nuclear antigen (PCNA) immunohistochemical staining was performed in non-nephritic rats (A), nephritic rats (B), rats treated with PDGF-B–specific aptamer (C), and rats treated with MAPK inhibitor U0126 (D). (E) PCNA-positive cells were counted in 50 glomeruli per animal by a blinded observer. Treatment of nephritic animals with aptamers or U0126 results in a significant decrease of PCNA-positive cells. *P < 0.05 versus nonnephritic rats; #P < 0.05 versus nephritic rats.
Depletion of Endogenous YB-1 Leads to Reduced Mesangial Cell Proliferation and Abolishes Mitogenic PDGF-B Effect In Vitro

To assess the relevance of YB-1 for the PDGF-B–dependent mitogenic response, we established a model system with depleted YB-1 expression. To exclude nonspecific effects of plasmid DNA insertion, we tested three different individual clonal cell lines. Efficacy of YB-1 knockdown was assessed by quantitative reverse transcription–PCR, demonstrating a 70 to 80% decrease of YB-1 transcript numbers (Figure 9A), and by immunoblotting with a 90% reduction of YB-1 protein levels (Figure 9B). The YB-1 siRNA clones exhibited a decreased basal proliferation rate, as assessed by BrdU incorporation (Figure 9C). The cell viability was assessed by trypan blue staining. In both cell lines, with and without YB-1 knockdown, >98% of all cells were viable, indicating that apoptosis and cell death was not responsible for the decreased proliferation rate of the YB-1 siRNA clones (data not shown). The mitogenic effect of PDGF-BB at 50 ng/ml was clearly present in control clones, whereas it was almost absent in YB-1 siRNA clones (Figure 9C). The mitogenic effect of PDGF-BB was attributed to activation of the MEK signaling pathway, as preincubation with MEK inhibitor U0126 abrogated the mitogenic effect in control cells (Figure 9C).

Discussion

This is the first report that identifies a tightly regulated expression of the transcription factor YB-1 in the course of experimental mesangioproliferative GN. In addition to an increased expression, shuttling of YB-1 between the nuclear and the cytoplasmic compartment occurs as the consequence of PDGF-BB action in the course of mesangioproliferative GN.

Figure 8. PDGF-BB stimulates YB-1 expression in mesangial cells in vitro. (A) Mesangial cell cultures were stimulated with PDGF-AA or -BB for a prolonged time period. A significant, five-fold induction of YB-1 message was detected by quantitative reverse transcription–PCR analysis (n = 3 from two independent experiments; top). In contrast, there was no upregulation of YB-1 transcript number after stimulation with PDGF-BB in differentiated mouse podocytes (bottom). (B) Mesangial cell cultures were stimulated with PDGF-BB for the indicated periods, and immunoblotting of whole-cell lysates was performed using a monoclonal anti–YB-1 antibody (lanes 1 to 7). Upregulation of YB-1 protein levels and a marked change of the banding pattern with the appearance of novel bands was detected within 2 h of PDGF-BB incubation. As control, the primary antibody was omitted in lane 8. (C) Densitometric quantification of band intensities detected by immunoblotting in B.

Figure 9. Depletion of endogenous YB-1 leads to reduced mesangial cell proliferation and abolishes the mitogenic PDGF-B effect in vitro. (A and B) A knockdown of YB-1 in mesangial cells was achieved by means of small interfering RNA technology, which led to a 90% decrease of YB-1 transcript (A) and protein (B) levels. (C) In mesangial cells with YB-1 knockdown, BrdU incorporation was reduced by approximately 70%, indicating markedly reduced DNA synthesis. Whereas in control cells PDGF-B had a profound stimulatory effect on DNA-synthesis, this effect was abrogated in YB-1–depleted cells. Results are means from three independent single-cell clones that were confirmed in three independent experiments.
PDGF-B Signaling via ERK1/2 Is Sufficient and Required for YB-1 Shuttling In Vivo

Previous reports have outlined the pivotal role of PDGF-B in mesangioproliferative disease (3), both as a mitogen for mesangial cells and as a coordinator of extracellular matrix synthesis acting downstream of TGF-β (39). PDGF signaling includes PDGF receptor tyrosine kinase activation, resulting in autophosphorylation (40). The receptor thereafter interacts with several cytoplasmic proteins that contain SH2 domains. Second messengers and effectors for PDGF-B signaling include inositol-(1,4,5)-triphosphate, diacylglycerol, intracellular calcium release, and protein kinase C activation (40). Homodimeric α- and β-receptor complexes induce partially overlapping but also distinctly different effects on target cells as a result of differential interactions with various SH2-domain proteins. For the heterodimeric αβ-receptor complex, autophosphorylation on different tyrosine residues explains the differences in comparison with homodimeric receptors. In our experiments, the selective YB-1 upregulation by PDGF-B but not PDGF-A might be explained by these differences.

For assessing the relevance of PDGF-B signaling for YB-1 expression and nuclear export, PDGF-B was infused in nonnephritic rats and PDGF-BB antagonistic aptamers were used. In vivo infusion of PDGF-BB resulted in a predominant cytoplasmic localization of YB-1. These findings rule out that the antibody-dependent cell damage is required for the shuttling process. In addition, blockade of PDGF-B actions in vivo resulted in a marked downregulation of YB-1 expression concomitant with a reversal to a predominantly nuclear localization. Application of U0126 in the anti-Thy1.1 model largely abolished the nucleocytoplasmic YB-1 shift and ablated its upregulated expression. It had previously demonstrated that both treatments result in a significant decrease of mesangial cell proliferation and therefore prevents the development of mesangioproliferative disease (1,36). Thus, our results suggest that YB-1 is a novel mediator in mesangioproliferative disease. The peak of YB-1 expression in the course of the disease between days 4 and 6 coincides with the second peak of PDGF-B expression, as determined by Ostendorf et al. (41) on day 4. This hypothesis was supported further by in vitro results with YB-1 knockout cells. In this model system, cell replication and basal proliferation was already reduced by approximately 80%. More important, the proliferative response to PDGF-B was almost completely abrogated.

**YB-1 May Be Involved in an Autoinhibitory and Autostimulatory Loop**

In a recent study by Fukada et al. (42), YB-1 was identified as a transactivator of the prototypic protein tyrosine phosphatase 1B (PTP1B) gene transcription by directly interacting with a response element in the proximal promoter. PTP1B has an impact on insulin and cytokine signaling by dephosphorylating the insulin receptor and interfering with the phosphorylation status of membrane-bound receptors (43). In Rat-1 cells, depletion of endogenous YB-1 protein by means of antisense constructs led to a markedly increased MAPK signaling. A close correlation of YB-1 and PTPB1 expression levels was observed in all cell lines tested. These findings suggest that YB-1 expression levels affect several cytokine signaling events, namely the Ras-Raf-MAP-MAPK signaling cascade. In conjunction with our observation that YB-1 expression and subcellular localization is regulated by ERK1/2 signaling, one may speculate that YB-1 uses a negative regulatory feedback loop to allow for high-level MAPK signaling in the healthy condition and by itself downregulates MAPK signaling with upregulation of its own expression (Figure 10). Analogous feedback regulation has been described for TGF-β signaling, as TGF-β upregulates Smad7 expression and interferes with its own signaling cascade (44).

**Subcellular Shuttling of YB-1 in Inflammatory Glomerular Disease**

In healthy kidney tissue, YB-1 is localized exclusively in the nuclear compartment. To confirm the staining pattern and exclude the detection of a subfragment of YB-1 protein, as has been reported by Stenina et al. (4,45), this observation was verified using three different primary antibodies directed against domains located in both the YB-1 N- and C-terminus. The staining pattern changed dramatically during the course of an experimental model of mesangioproliferative GN: A predominant cytoplasmic localization that reversed back to nuclear in parallel to resolution of disease at approximately day 31 was observed. This observation contrasts with data obtained by IFN-γ (13), results in enhanced PTPB1 transcription and autoinhibition of YB-1 activation.

![Figure 10. Model for autostimulatory and -inhibitory YB-1 regulation. Previous findings by Fukada et al. (42) demonstrate that YB-1 is a trans-activator of prototypic protein tyrosine phosphatase 1B (PTP1B) gene transcription, which inhibits phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). With binding of PDGF-B, intracellular signaling of ERK1/2 among others is stimulated, resulting in nuclear export of YB-1. As a consequence, the stimulatory effect of YB-1 on the PTPB1 promoter is abrogated, leading to enhanced ERK1/2 phosphorylation and signaling. Contrary to this autostimulatory loop, nuclear shuttling of YB-1, e.g., by IFN-γ (13), results in enhanced PTPB1 transcription and autoinhibition of YB-1 activation.](image-url)
with proliferating primary as well as tumor cell cultures, in which YB-1 localizes predominantly to the cytoplasm and translocates to the nucleus in response to cellular stress such as DNA-damaging agents, UV irradiation, virus infection, and hypothermia (23,46–48). In mesangial cells in vitro, YB-1 is localized to the cytoplasm, which corroborates with the notion that these cells are activated by the cell culture conditions and are actively cycling cells. Nuclear localization of YB-1 has also been associated with a poor prognosis in several types of human cancer (25,26,36), whereas in other cases, overexpression of YB-1 is linked with an unfavorable outcome (24,49). These observations suggest that the mechanisms that target the subcellular localization of YB-1 are different in nontransformed compared with tumor cells.

Given that YB-1 shuttles to the cytoplasm in mesangioproliferative GN, the question is arises why YB-1 may orchestrate gene expression in the nucleus, because YB-1 trans-activates proliferation-associated genes such as thymidine kinase and DNA polymerase α (14). The time course of the cytoplasmic localization closely correlated with the mesangioproliferative phase of the disease, which peaks between days 3 and 6 (33). This close correlation may suggest that YB-1 acts predominantly as regulator of translational processes at this stage of disease, e.g., by affecting the half-life of cytokine messenger RNA (7,17), or by nonspecifically binding to the 5′ RNA cap structure (16). Notably, YB-1 regulates the translation of its own mRNA (50), which may explain differences in YB-1 protein and mRNA synthesis after stimulation with PDGF-B. However, because of limitations of the immunohistochemical detection method, it cannot be excluded that a minor YB-1 fraction is still present in the nucleus and participates in processes such as gene transcription or splicing (19). This enigma furthermore arises with transient transfection procedures performed with YB-1 expression plasmids. In proliferating cells in vitro, YB-1 protein is localized in the cytoplasmic compartment. Therefore, the nuclear export may be accompanied by release of trans-repression (12,51,52), e.g., of the collagen gene (12).

Until now, four mechanisms that determine the YB-1 shuffling from the cytoplasmic to the nuclear compartment have been described. (1) Zhang et al. (53) observed nuclear YB-1 shuffling in a subset of cisplatin-treated or adenosvirally infected cells in the presence of wild-type p53 but not mutated p53. This shuffling occurred quantitatively at higher p53 concentrations and counteracted the proapoptotic effect of p53. (2) Splicing factor SRp30c, a partner protein for YB-1, may “piggy-pack” YB-1 to the nuclear compartment (19). (3) Nuclear shuffling of YB-1 after genotoxic stress with cisplatin and UV light is prevented by protein kinase C inhibitor, possibly involving an anchorage protein that releases YB-1 for nuclear shuttling (23). (4) Finally, a casein kinase II–dependent shuffling mechanism is operative in the IFN-γ effect on YB-1 (13,54). None of these mechanisms, however, explains the converse shuffling event from the nucleus to the cytoplasmic compartment in our model of mesangioproliferative nephritis. On the basis of our data with immunoblotting, we speculate that this process involves posttranscriptional YB-1 protein modifications. Such modifications were apparent after PDGF-B stimulation of mesangial cells in vitro—that is, the appearance of additional bands. The mouse homologue of YB-1, MSY2, has been reported to be associated with a kinase activity (55) that regulates binding affinity to mRNA and it has been described that YB-1 is highly phosphorylated in vivo. Such phosphorylation events may “activate” nuclear export and/or import signals that have not been mapped hitherto.

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