Growth arrest–specific protein 1 is a novel endogenous inhibitor of glomerular cell activation and proliferation

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Growth arrest–specific protein-1 (GAS1) is a GPI-anchored protein which is highly expressed in embryonic mouse fibroblasts and inhibits their proliferation. Glomerular mesangial cells release soluble GAS1 protein into the supernatant *in vitro*. Growth arrest led to GAS1 overexpression and increased release. Secretion involved disintegrin and metalloproteinase 10 and 17 as signified by inhibition experiments. Recombinant soluble GAS1 protein inhibited the proliferation of mesangial cells. Conversely, the induction of mesangial cell proliferation by PDGF-BB or -DD led to downregulation of GAS1 mRNA. Specific ligands of the PDGF α-receptor, PDGF-AA and -CC, had no effect. The GAS1 protein was localized in podocytes in kidneys from healthy rats. During the time course of mesangioproliferative glomerulonephritis in anti-Thy1.1-treated rats, glomerular GAS1 expression decreased prior to the onset of mesangial cell proliferation and increased at later stages during glomerular recovery. Finally, a plasmid expressing soluble GAS1 fused to an Fc fragment was systemically overexpressed in rats with mesangioproliferative glomerulonephritis. This ameliorated renal damage was indicated by decreased albuminuria and serum creatinine. Gas1/Fc-transfected rats also exhibited a reduction of the glomerular mesangial cell activation and proliferation. Thus, GAS1 is a novel endogenous inhibitor of glomerular mesangial cell proliferation and may be a novel therapeutic target in mesangioproliferative glomerular diseases.

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A number of common renal diseases, such as diabetic nephropathy and various types of glomerulonephritis (GN), were characterized by glomerular mesangial cell (MC) proliferation and/or matrix accumulation. Such common pathological features may represent particularly useful therapeutic targets. In fact, numerous factors promoting the development of mesangioproliferative changes have been identified and specific interventions have been developed against these factors, e.g., the platelet-derived growth factor (PDGF) isoforms PDGF-B and -D in mesangioproliferative GN.[1–3] In contrast, very little is known about endogenous inhibitors of glomerular disease. We identified the nephroblastoma overexpressed gene (*CCN3/NOV*) as the first such endogenous inhibitor, regulated by PDGF-B and -D.[4]

The present study identifies a second inhibitor, namely growth arrest–specific gene (GAS)-1. GAS1 is a glycosyl-phosphatidyl-inositol (GPI)–anchored protein, which is overexpressed in growth-arrested embryonic mouse fibroblasts (NIH3T3) and inhibits their proliferation by blocking the cell cycle G0/S transition.[5,6] Its functionality depends on p53 activity.[7,8] GAS1 shows high structural homology to the glial cell–derived neurotrophic factor (GDNF) family receptor-α.[9] GAS1 binds to the receptor tyrosine kinase Ret, reduces Ret tyrosine phosphorylation, and modifies its downstream signaling.[9,10] In addition, GAS1 acts as a co-receptor in the Sonic hedgehog (SHH) signaling pathway by binding to the Hedgehog protein and amplifying the signal.[11] During embryonic development, GAS1 is expressed in the kidney,[12] but the function of GAS1 in the adult kidney is so far completely unknown.

Given the downregulation of GAS1 by the two potent mediators of mesangioproliferative disease PDGF-B and -D, we tested the hypothesis that GAS1 functions as a glomerular autocrine and paracrine growth regulator and that it can be therapeutically manipulated.

RESULTS

A cDNA array identifies GAS1 downregulation in PDGF-BB and -DD-stimulated primary human MCs

Genes that are downregulated by the two potent mitogens PDGF-BB and -DD in MCs were identified using an Affymetrix human genome chip U133A. One of the most potently suppressed genes was *GAS1*. Its expression decreased by 38% in PDGF-BB- and 40% in PDGF-DD-stimulated primary human MCs compared with unstimulated cells (data not shown).

To confirm the array data, growth-arrested human MCs were stimulated with the different PDGF isoforms, and GAS1 mRNA expression was measured using real-time reverse transcriptase PCR (real-time RT-PCR). PDGF-BB and -DD, i.e., ligands of the PDGF β-receptor and potent mitogens for MCs *in vitro* and *in vivo*, led to a suppression of *GAS1* mRNA expression, whereas the two specific ligands of the PDGF receptor-α, PDGF-AA and -CC, had no effect (Figure 1).

GAS1 is released into the supernatant by MCs *in vitro*

GAS1 was described as a GPI-anchored protein. Because various other GPI-anchored proteins are shed from the plasma membrane into the supernatant,[13–15] we searched for GAS1 in the supernatant of rat MCs transfected with an expression vector encoding for the full-length GAS1 protein or a control vector (mock transfected). As primary human MCs could not be successfully transfected, rat MCs were used for all subsequent experiments. By using a GAS1-specific enzyme-linked immunosorbent assay (ELISA), released GAS1 protein was detected in the supernatant of mock-transfected and, at higher levels, GAS1-transfected cells (Figure 2a). To assess the role of MC proliferation in GAS1 release, reversible growth arrest was induced by incubating the cells in serum-free MCDB medium for 24 h. Incubation in MCDB induced growth arrest without irreversible damage of MCs.[4] Growth arrest increased GAS1 mRNA and protein expression in the cells (Figure 2b and c and Supplementary Figure S1 online), as well as in supernatants of MCs (Figure 2d and e).

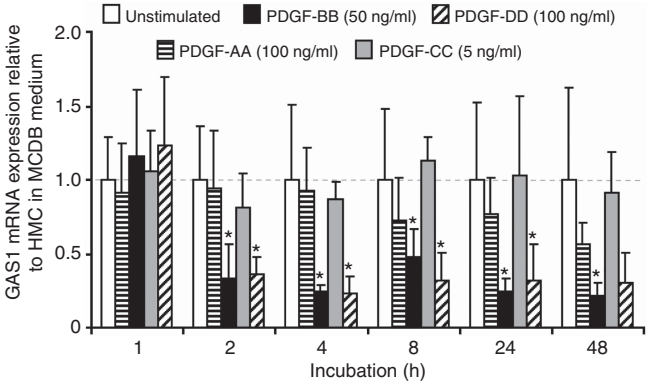

Figure 1 | Platelet-derived growth factor-BB (PDGF-BB) and -DD regulate *growth arrest-specific protein-1 (GAS1)* expression in mesangial cells (MCs). PDGF-BB and -DD, but not PDGF-AA and -CC, induce a downregulation of *GAS1* mRNA as determined by real-time reverse transcriptase PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Before the experiment, human MCs (HMCs) had been cultured for 72 h in MCDB medium to upregulate GAS1 expression. Data are means ± s.d. of four independent experiments. *Indicates $P < 0.05$ versus unstimulated cells.

GAS1 shedding is mediated by the proteases disintegrin and metalloproteinase 10 and 17

To identify the proteases involved in the shedding of cell-bound GAS1, growth-arrested, as well as proliferating, MCs were incubated with inhibitors of the disintegrins and metalloproteinases (ADAM) 10 and 17. Both proteases have been found to mediate shedding of various surface molecules including GPI-linked prion protein. Their contribution toward shedding can be investigated by the inhibitors GW280264x (blocking ADAM10 and 17) and GI254023x (blocking ADAM10 but not ADAM17).[16,17] Both inhibitors reduced GAS1 release in growth-arrested and proliferating rat MCs (Figure 3a), suggesting that ADAM10 contributes to shedding in unstimulated cells. Shedding of other membrane-bound proteins can also be enhanced by protein kinase C activation with phorbol esters such as phorbol 12-myristate 13-acetate (PMA) or by Ca^{2+} ionophores such as ionomycin.[18,19] In growth-arrested MCs, stimulation with PMA but not with ionomycin increased soluble GAS1 protein concentrations (Figure 3a). Incubation of growth-arrested, as well as PMA-stimulated, MCs with the inhibitors GI254023x and GW280264x decreased GAS1 levels in the supernatant (Figure 3a). In contrast, in proliferating MCs cultivated with fetal calf serum (FCS), PMA-stimulated GAS1 shedding was inhibited by GW280264x but not by GI254023x (Figure 3a), suggesting that ADAM17 but not ADAM10 was relevant for the stimulated shedding in these cells. ADAM protease activity assays confirmed increased α-secretase activity, especially in PMA-stimulated proliferating and growth-arrested MCs (Figure 3b). Finally, mRNA expression of *ADAM10* and *17* was dependent on the proliferation status of MCs, as both were overexpressed in growth-arrested, compared to proliferating, MCs (Figure 3c).

Shed GAS1 protein inhibits the growth of proliferating MCs

To assess the functional role of released GAS1 protein in MCs, we incubated rat MCs in MCDB medium and then induced proliferation with 2% FCS, followed by the addition of recombinant GAS1 protein without a GPI anchor. Proliferation of MCs induced by 2% FCS was significantly reduced by 5 ng/ml recombinant GAS1 protein (Figure 4a). In primary human MCs, the proliferation of growing cells was completely inhibited by 0.1 μg/ml recombinant GAS1 protein, whereas no effect of recombinant GAS1 protein could be detected in growth-arrested cells (Supplementary Figure S2 online). In primary human MCs stimulated with PDGF-BB, only high doses of recombinant GAS1 protein inhibited the growth (Supplementary Figure 2 online). The overexpression of a soluble GAS1/Fc fusion protein in proliferating rat MCs downregulated endogenous *PDGF-B*