Overexpression of glutamine:fructose-6-phosphate-amidotransferase induces transforming growth factor-β1 synthesis in NIH-3T3 fibroblasts

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Abstract Increased flux through the hexosamine biosynthetic pathway with glutamine:fructose-6-phosphate-amidotransferase (GFAT) as rate-limiting enzyme has been linked to the enhanced bioactivity of the prosclerotic cytokine transforming growth factor β1 (TGF-β1) in fibrotic complications, particularly in diabetic kidney disease. Here, we investigate in a stable transfection system the effect of overexpression of GFAT on TGF-β1 synthesis in NIH-3T3 fibroblasts. We demonstrate a 1.8-fold stimulation of TGF-β1 mRNA and a 1.9-fold increased protein expression, whereas TGF-β2 production was not upregulated. The 1.5-fold enhanced TGF-β1 promoter activity suggests a transcriptional regulation. The elevated TGF-β1 protein is biologically active since GFAT-overexpressing cells exhibit a 2-fold fibronectin production. The results indicate a GFAT-dependent induction of TGF-β1 synthesis.

Key words: Glutamine:fructose-6-phosphate-amidotransferase; Hexosamine biosynthetic pathway; Transforming growth factor β; Diabetic nephropathy; Stable overexpression; Fibroblast

1. Introduction

Glutamine:fructose-6-phosphate-amidotransferase (GFAT) is the rate-limiting enzyme of the hexosamine biosynthetic pathway, catalyzing the conversion of fructose-6-phosphate to glucosamine-6-phosphate (GlcN-6-P) with glutamine as amino donor [1–3]. The product GlcN-6-P is very rapidly further converted and activated to uridine-5-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), a precursor for the biosynthesis of the carbohydrate moiety of glycoproteins, glycolipids and proteoglycans. GFAT controls the flux of glucose into the hexosamine pathway [4], and thus the availability of precursors for *N*- and *O*-linked glycosylation of proteins. Accordingly, GFAT expression is highly regulated, e.g. in yeast [5] and in human tissues [6]. Recent studies indicate that GFAT, besides its key role in the synthesis of amino sugar precursors, may have regulatory functions. These include the findings that the hexosamine pathway may serve as a nutrient-sensing pathway [7] and that it may mediate glucose-induced insulin-resistance [2,4,8,9]. Furthermore, this pathway has been linked to the development of diabetes-associated macrovascular complications [10–12] and glomerulosclerosis [13,14]. The hyperglycemia-induced overexpression of the prosclerotic cytokine transforming growth factor β1 (TGF-β1) has been shown to be involved in the pathogenesis of diabetic renal fibrosis [15–17]. TGF-β1 acts as a key mediator with its fibrogenic potential to stimulate matrix synthesis and to inhibit matrix degradation [18–22] and by interaction with the renin–angiotensin system [23]. It was reported previously that the high-glucose-induced overexpression of TGF-β1 in renal mesangial cells is mediated by the hexosamine pathway, since increased flux through this pathway induced by ambient high glucose or glucosamine causes enhanced TGF-β1 production [13]. Furthermore, inhibition of GFAT expression by antisense oligonucleotides or application of the GFAT inhibitor azaserine prevented the expression of biologically active TGF-β1 [13]. These results suggest a role of the hexosamine pathway in the regulation of TGF-β1 expression.

To assess the role of increased GFAT expression in TGF-β1-mediated tissue fibrosis we investigated in a stable transfection system the effect of overexpression of GFAT on TGF-β1 expression in NIH-3T3 fibroblasts. We demonstrate that increase of GFAT protein enhances TGF-β1 mRNA and TGF-β1 protein production and that the stimulatory effect may be mediated by activation of TGF-β1 promoter activity.

2. Materials and methods

2.1. Preparation of GFAT expression vector

A full-length GFAT cDNA was generated by reverse transcriptase-polymerase chain reaction using RNA of human B-lymphocytes as template with the primer 5′-CGGATCCCGCATCATGTGTGG-TATATTT-3′ and 5′-GGAATTCCGTATAGATTCCTCACTCT-3′ which contain *Bam*HI or *Eco*RI linker. The PCR-product was cloned into the expression vector pJ6Ω (American Type Culture Collection, Rockville, USA) and verified by sequencing. The GFAT cDNA was identical to human GFAT (GenBank accession number M90516).

2.2. Culture and transfection of NIH-3T3 fibroblasts

Fibroblasts were cultured with DMEM medium containing 25 mM glucose, 10% fetal calf serum, 1 mM sodium pyruvate, 4 mM glutamine, non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Eggenstein, Germany). For experiments DMEM medium contains 5.5 mM glucose and fetal calf serum was substituted with 2% Ultroser (Gibco, Eggenstein, Germany). For determination of TGF-β1 protein in the cell culture supernatant, serum-

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAT, glutamine:fructose-6-phosphate-amidotransferase; GlcN-6-P, glucosamine-6-phosphate; SDS, sodium dodecyl sulfate; Sp1, stimulatory protein 1; SSC, sodium chloride sodium citrate; TGF-β1, transforming growth factor β1; UDP-GlcNAc, uridine-5-diphosphate-*N*-acetylglucosamine

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free medium was used. Fibroblasts were transfected with Superfect (Qiagen, Hilden, Germany) according to the instructions of the supplier. 1×10^5 cells in a 6-cm dish received 4.5 μg pJ6ΩGFAT or pJ6Ω as control and 0.5 μg pSVneo encoding neomycin resistance. Transfected cells were selected for their resistance to the antibiotic G418 (0.5 μg/ml). G418-resistant clones were screened for expression of GFAT by Western blotting. For determination of TGF-β1 promoter activity 1.5×10^5 GFAT transfectants or controls were seeded in 6-well plates and transfected with 1.75 μg pGL3TGF-β1 containing the human TGF-β1 promoter region −453/+11 [24] fused to the firefly (*Photinus pyralis*) luciferase gene (provided from A. Pfeiffer, Bochum, Germany) and 0.25 μg pRL-CMV (Promega, Madison, WI, USA) for standardization of transfection efficiencies. Transfection was performed with Superfect. Transfected cells were harvested after 24 h and reporter gene activities were measured.

2.3. Western blot

Cellular extracts of GFAT transfectants or controls were applied to sodium dodecyl sulfate (SDS) gel electrophoresis and Western blotting was performed as recently described [13].

2.4. Determination of UDP-GlcNAc

Cell extracts were prepared and UDP-GlcNAc was determined by capillary electrophoresis as described previously [25].

2.5. Determination of glucose, lactate and DNA concentrations

In supernatants glucose and lactate were measured with Ektachem Vitros systems (Ortho-clinical-diagnostics, Neckargmünd, Germany). DNA in total cell extracts was measured by fluorometry with bisbenzimidazole [18].

2.6. Northern blotting

RNA was prepared from cultured GFAT transfectants and controls with the RNeasy kit (Qiagen, Hilden, Germany). Total RNA (25 μg) was separated on a formaldehyde-containing agarose gel and transferred to a Nylon membrane. RNA probes for TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are described previously [13] and digoxigenin-labelled by in vitro transcription. Hybridization was performed overnight in 5×sodium chloride sodium citrate (SSC), 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 2% blocking reagent (Roche, Mannheim, Germany) at 68°C, and the filters were then washed with 2×SSC, 0.1% at room temperature and 0.5×SSC, 0.1% SDS at 68°C. For detection the DIG Luminescent Detection kit (Roche, Mannheim, Germany) was used.

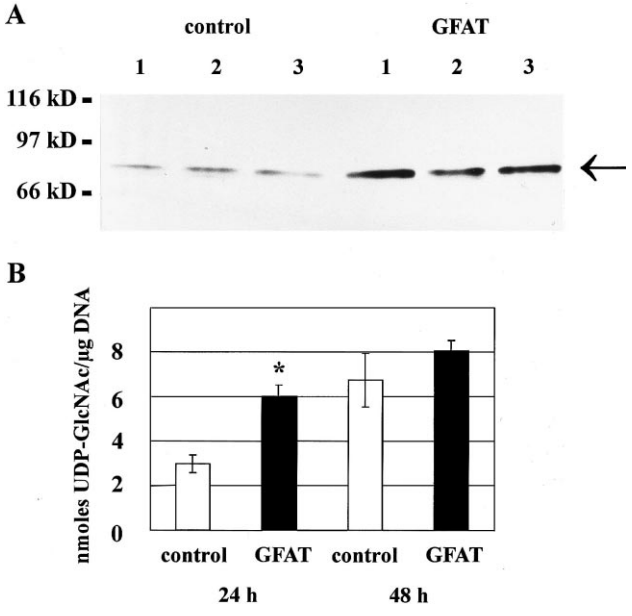

Fig. 1. Overexpression of GFAT in NIH-3T3 fibroblasts. A: GFAT transfectants and controls were generated as described in Section 2 and GFAT expression was determined by Western blotting. Three control and GFAT clones were tested. The band of the 80-kDa protein of GFAT is marked by an arrow. B: In cell extracts of controls and GFAT transfectants the concentration of UDP-GlcNAc after 24 and 48 h cell culture was measured by capillary electrophoresis. Bar graphs show nmol UDP-GlcNAc normalized to DNA concentration of the cells. The error bars mark ±S.E.M. $^*P<0.05$ vs. control (24 h).

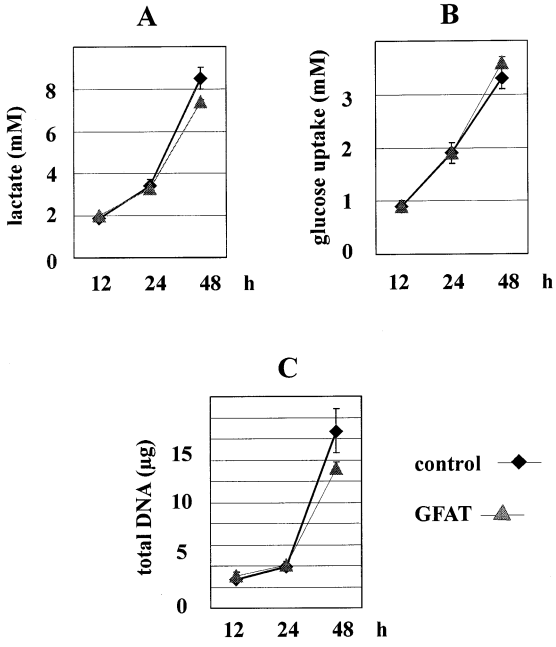

Fig. 2. Lactate production, glucose uptake, and cell proliferation of controls and GFAT transfectants during 48 h cell culture. Supernatants of controls and GFAT transfectants were collected after 12, 24 and 48 h of cell growth and lactate (A) and glucose (B) were measured as described in Section 2. Total cell extracts were prepared after 12, 24 and 48 h of growing, and DNA was determined (C).

2.7. ELISA for TGF-β1, TGF-β2 and fibronectin

For quantification of the proteins cell culture supernatants were collected and stored at −80°C. Determinations of total TGF-β1 and -β2 protein were performed with the Quantikine Immunoassays (R&D Systems, Minneapolis, MN, USA). Fibronectin was measured by the Quantimatrix™ human fibronectin ELISA kit from Chemicon International (Temecula, CA, USA).

2.8. Reporter gene assays

Transfected cells were washed once with phosphate-buffered saline, incubated with 150 μl lysis buffer from the β-galactosidase assay chemiluminescent (Roche, Mannheim, Germany) for 30 min and harvested. Cotransfected firefly and seapansy luciferase activities were assayed with the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Chemiluminescence was determined with a Magic Lite Analyzer (Ciba Corning, Fernwald, Germany). All transfection experiments were repeated at least three times.

2.9. Statistical analysis

Results presented are derived from at least three independent experiments. Means ± S.E.M. were calculated and groups of data were compared using Student's *t*-test. Statistical significance was set at $P<0.05$.

3. Results

A stable transfection system was used to evaluate the role of the hexosamine biosynthetic pathway in regulating expression of the TGF-β1. The full-length cDNA of the rate-limiting enzyme of this pathway, GFAT, was inserted into the expres-

sion vector pJ6Ω downstream of the rat β-actin promoter. The GFAT gene construct was introduced into NIH-3T3 fibroblasts by Superfect and G418-resistant clones were evaluated for GFAT protein expression by Western blotting and activity of the hexosamine pathway by determination of intracellular UDP-GlcNAc. The expression of GFAT protein in stable transfectants was increased 2.6-fold in three different clones compared to three different controls (Fig. 1A). The concentration of the end-product of the hexosamine biosynthetic pathway, UDP-GlcNAc, was 6.02 ± 0.5 nmol/μg DNA in the GFAT-overexpressing fibroblasts after 24 h culture, while in controls only 2.97 ± 0.41 nmol/μg DNA were found (Fig. 1B). After 48 h control cells contained 6.73 ± 1.2 nmol UDP-GlcNAc/μg DNA and GFAT transfectants 8.04 ± 0.5 nmol UDP-GlcNAc/μg DNA (Fig. 1B). Since glucose uptake and lactate production and thus flux through glycolysis were unchanged in the GFAT-overexpressing fibroblasts (Fig. 2A,B), the enhanced flux through the hexosamine biosynthetic pathway indicates an increase in enzymatically active GFAT protein. GFAT overexpression had only marginal effects on cell proliferation (Fig. 2C).

We studied then whether the expression of the TGF-β1 mRNA and protein was altered by overexpression of GFAT. After 24 h cell culture TGF-β1 mRNA was increased in GFAT clones, while in control cells only marginal amounts of TGF-β1 mRNA were detected (Fig. 3A). Evaluation of three independent experiments showed a 1.8-fold increase of TGF-β1 mRNA in GFAT transfectants compared to controls (Fig. 3B). In accordance with the elevated TGF-β1 mRNA-levels after 24 h an increase of secreted TGF-β1 protein was observed after 24 h, which was enhanced 1.9-fold after 48 h in GFAT-overexpressing fibroblasts (Fig. 4A). To confirm the specificity of GFAT-dependent upregulation of TGF-β1 expression, the concentration of secreted TGF-β2 protein was measured. The time course of TGF-β2 production reached the maximum after 24 h, after 48 h no further elevation of TGF-β2 was found (data not shown). No significant increase in TGF-β2 protein concentration was observed in GFAT-transfected cells after 12 and 24 h compared to controls (Fig. 4B).

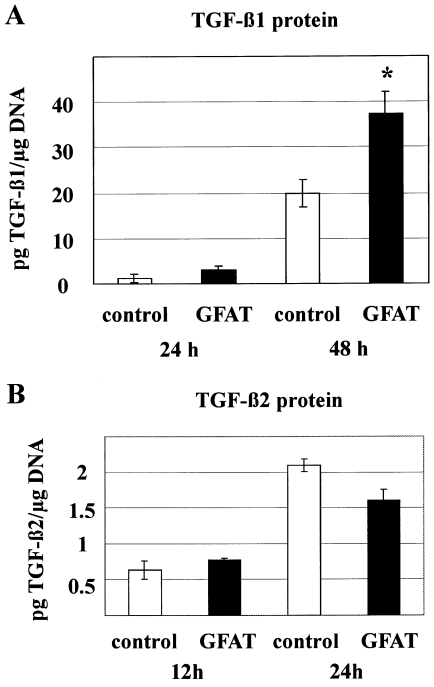

Fig. 4. Effect of GFAT overexpression on TGF-β1 and -β2 protein production. Supernatants of control and GFAT transfectants are collected after the indicated time of cell culture and total TGF-β1 and -β2 protein was measured by ELISA. A: Concentration of TGF-β1 after 24 and 48 h. Bar graphs show pg TGF-β1 protein normalized to DNA content of the cells. The error bars mark ± S.E.M. $*P < 0.05$ vs. control. B: Concentration of TGF-β2 after 12 and 24 h. The earlier points of time were shown since after 48 h no further production of TGF-β2 was found. Bar graphs show pg TGF-β2 protein normalized to the DNA content of the cells.

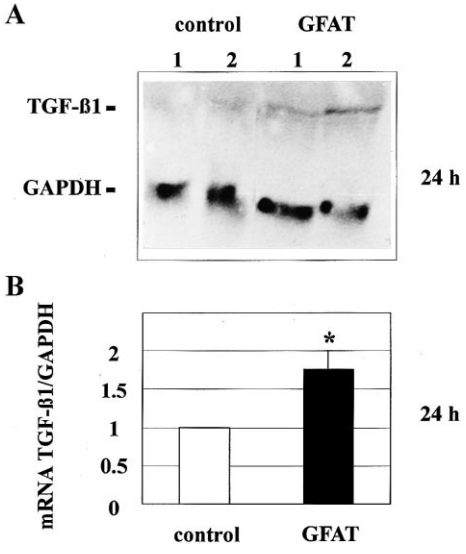

Fig. 3. Effect of GFAT overexpression on TGF-β1 mRNA expression. Controls and GFAT transfectants were cultured for 24 h (A, B). Total RNA (25 μg) was separated on agarose–formaldehyde gels and transferred onto nylon membranes for hybridization with digoxigenin-labelled TGF-β1 or GAPDH probes. A: Representative Northern blot with two different clones of controls and GFAT transfectants harvested after 24 h cell culture. B: Densities of TGF-β1 mRNA signals were normalized relative to those of GAPDH mRNA signals. Ratio of control cells was set as 1. Results are expressed as mean ± S.E.M. of three separate experiments. $*P < 0.05$ vs. control.

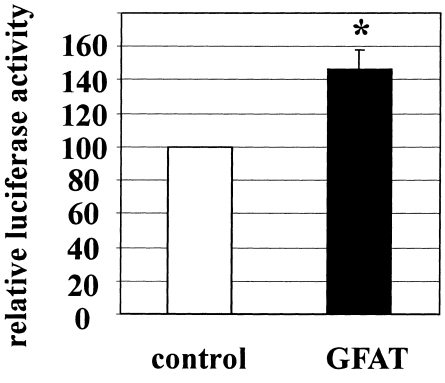

Fig. 5. Effect of GFAT overexpression on TGF-β1 promoter activity. Transfection of controls and GFAT transfectants with pGL3TGF-β1, containing the TGF-β1 promoter region −453 to +11, and reporter gene assays were performed as described in Section 2. Bar graphs show relative luciferase activities normalized to cotransfected control vector pRL-CMV. Promoter activity of control cells was set as 100%. The error bar marks ± S.E.M. $*P < 0.05$ vs. control.

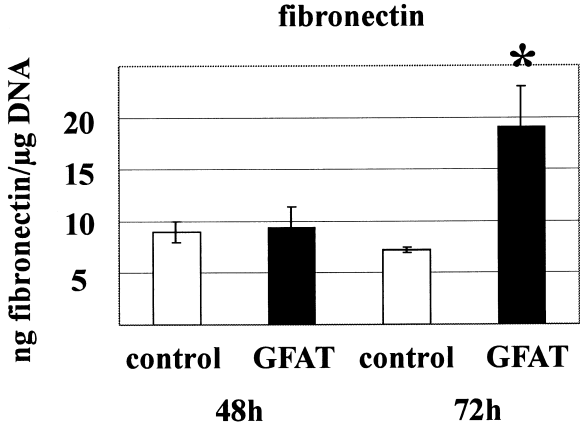

Fig. 6. Effect of GFAT overexpression on fibronectin protein production. Supernatants of controls and GFAT transfectants were collected after 48 and 72 h of cell culture and fibronectin was measured by ELISA. Bar graphs show ng fibronectin protein normalized to the DNA content of the cells. The error bars mark ± S.E.M. *$P < 0.05$ vs. control.

In addition, the activity of the TGF-β1 promoter region −453 to +11 containing the regulatory elements for basal promoter function [24], phorbol-ester-responsiveness [26], autoinduction [27], and high-glucose-responsiveness [28] was investigated in GFAT transfectants. In fibroblasts overexpressing GFAT, the activity of the cotransfected TGF-β1 promoter construct was 146% compared to control cells (Fig. 5). These results suggest that the GFAT-induced increase in TGF-β1 mRNA and protein levels is regulated by transcriptional activation of the TGF-β1 gene. To evaluate if the increased TGF-β1 expression is biologically active the production of the TGF-β1-responsive gene fibronectin was studied. We found a 2-fold increased production of fibronectin after 72 h but not after 48 h (Fig. 6) indicating (i) that enhanced TGF-β1 expression precedes fibronectin production and (ii) that the GFAT-induced TGF-β1 protein is biologically active.

4. Discussion

In a previous report it was demonstrated that increased flux through the hexosamine biosynthetic pathway by high glucose or glucosamine led to increased concentrations of the prosclerotic TGF-β1 [13], a cytokine which has been implicated in the development of diabetic late complications, e.g. diabetic nephropathy and vasculopathies [15–23]. In the present study we established a stable transfection system in NIH-3T3 fibroblasts to clarify whether overexpression of the rate-limiting enzyme of the hexosamine biosynthetic pathway, GFAT, independent of the presence of high glucose concentrations, induces the synthesis of TGF-β1. The 2.6-fold overexpression of GFAT led to an elevated concentration of UDP-GlcNAc, the end-product of the hexosamine biosynthetic pathway, after 24 h. The lower increase after 48 h may be explained by the negative feedback inhibition of GFAT enzyme activity by UDP-GlcNAc [1,3]. In this stable transfection system we obtained clear evidence for an involvement of the hexosamine biosynthetic pathway in the up-regulation of TGF-β1 synthesis. We demonstrated an increase of TGF-β1 mRNA and protein levels as well as a stimulated TGF-β1 promoter activity in fibroblasts overexpressing GFAT. This induction appeared to be specific since TGF-β2 production was not affected. Furthermore we found an enhanced TGF-β1 bioactivity since the expression of the TGF-β1 responsive gene fibronectin was elevated in the GFAT-transfected cells. These results indicate that an increased flux through the hexosamine biosynthetic pathway induced by high glucose or by an increase of GFAT protein in normal glucose concentrations stimulate TGF-β1 synthesis and action. The latter observation may be important since an increased amount of GFAT protein was detected in renal sections of diabetic patients [6] and in skeletal muscle and in cultured skeletal muscle cells obtained from diabetic patients increased enzyme activity was measured [29,30]. The activation of the TGF-β1 promoter activity gives rise to a transcriptional regulation of the enhanced TGF-β1 synthesis by the hexosamine biosynthetic pathway, comparable with the hexosamine pathway-dependent enhanced gene expression of the epithelial growth factor-like transforming growth factor α in smooth muscle cells [10–12]. Since the UDP-sugars generated by this pathway are precursors for glycosylated proteins, the regulation of transcription factors by altered glycosylation must be considered [31]. Suggested mechanisms include altered proteolytic degradation [32] or changes in the *trans*-acting activity of transcription factors [31], e.g. by alternative phosphorylation or glycosylation of the same residues [33]. Since the activity of the ubiquitous transcription factor stimulatory protein 1 (Sp1), which is involved in the TGF-β1 promoter activation [24], is regulated by *O*-glycosylation [32,34], the increased TGF-β1 gene expression in GFAT-overexpressing NIH-3T3 cells may be mediated by altered glycosylation of Sp1.

In conclusion, the results of the present study demonstrate the importance of the hexosamine biosynthetic pathway in the specific upregulation of the prosclerotic cytokine TGF-β1 thereby suggesting that an increase in the rate-limiting enzyme GFAT is involved in the development of tissue fibrosis, e.g. in the pathogenesis of vascular complications in diabetes.

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