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ORIGINAL ARTICLE

Chronic Hyperglycaemia Induced Alterations of Hepatic Stellate Cells Differ from the Effect of TGFB1, and Point toward Metabolic Stress

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Abstract

The deleterious effect of hyperglycemia on the biology of the liver is supported by clinical evidence. It can promote the development of fatty liver, liver fibrosis, even liver cancer as complication of diabetes mellitus. As liver fibrosis is the consequence of hepatic stellate cell (HSC) activation, the questions were addressed whether alterations induced by high glucose concentration are directly related to TGFB1 effect, or other mechanisms are activated. In order to obtain information on the response of HSC for high glucose, LX-2 cells (an immortalized human HSC cell lineage) were cultured in 15.3 mM glucose containing medium for 21 days. The effect of glucose was compared to that of TGFB1. Our data revealed that chronic exposure of high glucose concentration initiated profound alteration of LX-2 cells and the effect is different from those observed upon interaction with TGFB1. Whereas TGFB1 induced the production of extracellular matrix proteins, high glucose exposure resulted in decreased MMP2 activity, retardation of type I collagen in the endoplasmic reticulum, with decreased pS6 expression, pointing to development of endoplasmic stress and sequestration of $p21^{CIP1/WAF1}$ in the cytoplasm which can promote the proliferation of LX2 cells.

Keywords Hyperglycaemia · TGFB1 · Hepatic stellate cells · Fibrogenesis · Endoplasmic stress

Introduction

Unbalances in nutritional input and energy expenditure account for the dramatic increase of obesity and metabolic syndrome cases in the developed world leading to the rapid rise of type II diabetes [1, 2].

While most studies appreciate the well-known consequences of diabetes mellitus (DM), non-alcoholic fatty liver disease (NAFLD) receives significantly less attention [1]. NAFLD incorporates histologically and clinically different conditions including fatty liver (NAFL, steatosis hepatitis) and steatohepatitis (NASH) ± fibrosis, cirrhosis and even hepatocellular cancer. The prevalence of NAFLD is increased in patients with type 2 DM and NAFLD has a role in diabetes development [2].

Armed with the successful development of hepatitis C chemotherapy agents and the recognition of the association of type 2 DM and NAFLD and NASH, these two liver conditions are getting into the focus of medical research [3–5]. Several reports linked the effects of DM and the underlying elevated glucose levels on the damage of many organs, including the liver [2, 6–8]. Upon hepatocyte damage and necrosis the increased production of ROS activates Kupffer cells. In turn, this process feeds back with further increased ROS and cytokine production, including TGFB1, a pro-inflammatory cytokine that is known to be one of the major activators of hepatic stellate cells (HSC) [9]. In response, HSC alters its phenotype, loses its retinoid droplets, starts to proliferate, and synthesizes ECM proteins, mainly collagens [10–13].

Recently, we demonstrated that pancreatic stellate cells (PSC) growing in culture medium with high glucose concentration enhance the production of CXCL-12, which in turn binds to CXCR4 and CXCR7 receptors on pancreatic cancer cells and activates the MAP kinase pathway [14]. In the

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current study we investigated whether high glucose concentration in the circulation modifies the behavior of HSC and whether the effect of hyperglycemia is comparable with that we observed in PSCs. We also wanted to know what consequences of high glucose exposure brings regarding liver fibrosis.

In order to obtain information on the response of HSC for high glucose, LX-2 cells, the immortalized variant of HSCs, were exposed to 15.3 mM glucose containing medium for 21 days. The effect of glucose was compared to that of TGFB1.

Materials and Methods

Cell Culture

In all experiments we used LX-2 human hepatic stellate cell line (received as a kind gift from Dr. Scott L. Friedman). LX-2 had been previously immortalized by transfection with SV40 large T antigen, followed by selection of low serum level conditions and analysis for stellate cell markers [15]. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Sigma, St. Louis, MO) with 1000 mg/L (5.5 mM) glucose supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin (Sigma) at 37 °C atmosphere containing 5% CO_2 and passaged at 85–90% confluence using trypsin-EDTA solution (Sigma).

Growth Factors and Other Compounds

Transforming growth factor-β1 (TGFB1, Sigma) was added at 5 ng/mL final concentration to the cells. BSA (bovine serum albumin, Sigma, Part No.: A3294) was used for non-specific blocking of immunoblots.

Treatment Schedule of LX-2 Cells

Cells were exposed to chronic high glucose concentration (CHG) and TGFB1 according to the following protocol: in control conditions cells were cultured as described above. For CHG treatment, cells were cultured in DMEM containing 15.3 mM glucose for 21 days, as our preliminary experiments showed the best response of ECM protein production at this time-frame. Subsequently, cells were starved for 24 h in FBS-free medium and thereafter for 48 h in FBS-free medium with or without the addition of TGFβ1 to compare the effect of the cytokine with CHG. Each sample was processed in duplicates and the experiments were repeated twice.

Cells grown in T75 flasks were collected and used for mRNA and protein analysis and the cell culture medium was collected for protein analysis. For immunocytochemistry the cells were grown on coverslips in 6-well plates. All analyses were repeated three times.

Real-Time RT PCR

Total RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized from 1 μg RNA using the M-MLV Reverse Transcriptase kit (Invitrogen by Life Technologies/Thermofisher Carlsbad, CA, USA) under the conditions recommended by the manufacturer. Real-time PCR was performed using the ABI Gene Expression TaqMan Assays and TaqMan Universal PCR Master Mix (Part No. 4324018) in an ABI Prism 7000 Sequence Detection System (all Applied Biosystems by Life Technologies/Thermofisher, Carlsbad, CA, USA) under conditions recommended by the manufacturer. Samples were run in triplicates in 20 μL total volume containing 50 ng cDNA [cycle conditions: denaturation: 95 °C (10 min) followed by 40 cycles: 95 °C (15 s), annealing + extension: 60 °C (1 min)]. Results were normalized to 18S rRNA (Part No. 4319413E). Cycle threshold (CT) values were recorded and relative gene expressions were calculated using the $2^{-\Delta\Delta CT}$ method.

Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of CXCL-12 in cell supernatant was performed by using a Solid Phase Sandwich ELISA kit (Quantikine R&D Systems, Minneapolis, MN, USA, Cat. No.: DSA00), according to the manufacturer's instructions. Each sample was tested in triplicates.

Fluorescent Immunostaining

For detection of intracellular collagen type-1 and $p21^{WAF1/CIP1}$, immunocytochemistry was performed. Cells were grown on coverslips and were fixed with ice-cold methanol. Non-specific protein-protein interactions were blocked with 5% BSA dissolved in phosphate-buffered saline (PBS) for 30 min. The cells were incubated with the primary antibody overnight at 4 °C. Alexa Fluor™ 488 was used as secondary antibody at a 1:200 dilution for 1 h. Cell nuclei were counterstained with 4′-6′-diamidino-phenylindole (DAPI, Sigma, Part No.: D9542). All washing steps were performed using PBS. Photographs were taken with a Nikon Eclipse E600 microscope operated by a Lucia Cytogenetics version 1.5.6 program. Details of antibodies and their appropriate dilutions are found in Supplementary Table 1.

Dot Blot

We performed dot blots to determine the ECM protein production of the cells. Aliquots of 200 μL cell supernatant/well were vacuum-filtered onto a PVDF membrane using a 96-well Minifold-Vacuum-Filtrations system SRC-96 (Schleicher&Schuell, Dassel, Germany). After washing the membrane with Tris-buffered saline (TBS), Ponceau staining was performed to detect the total protein content of the blotted material followed by blocking the peroxidase activity with 3% H_2O_2 for 10 min. After a repeated washing step, non-specific reactions were blocked with 5% BSA containing TBS. The filters were incubated with primary antibodies diluted in 1% BSA (TBS) overnight at 4 °C. Following a washing step, HRP-conjugated secondary antibodies in TBS were applied for 1 h. Positive signals were detected by using a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce/Thermo Scientific, Part No.: 34080), and visualized by Kodak 4000MM Digital Imaging System. Every dot blot analysis was repeated 3 times in triplicates. Details of the applied antibodies are detailed on Supplementary Table 2.

Western Blot (WB) Analysis

After cell lysis, protein concentration was measured by the Bradford method [16]. Twenty-five μg of denatured total proteins were loaded onto a 10% polyacrylamide gel and were run for 30 min at 200 V. Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA) for 1.5 h at 100 V. Ponceau staining was applied to determine the blotting efficacy. Membranes were blocked with 3% *w*/*v* non-fat dry milk (Sigma), or 5% BSA in case of phosphoproteins in TBS for 1 h, followed by incubation with the primary antibodies at 4 °C overnight. After the washing steps (0.05 *v*/v% Tween-20 in TBS), the appropriate secondary antibodies were applied for 1 h. Positive signals were detected by the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce/Thermo Scientific, Part No.: 34080) and visualized by a Kodak Image Station 4000MM Digital Imaging System. WB analyses were repeated 3 times. Applied antibodies are indicated in Supplementary Table 3.

Gelatinase Assay

To assess the activity of MMP-2 and MMP-9 matrix metalloproteinases, gelatinase assay was performed using the collected cell supernatants**.** Twenty μg of total protein was loaded onto a 7.5% polyacrylamide gel containing gelatin in 150 μg/mL concentration. The samples were run for 30 min at 200 V, the gel was washed for 30 min in 2.5% TritonX-100 solution, then incubated at 37 °C for 20 h in digesting buffer (50 mM Tris 7.5 pH; 10 mM $CaCl_2$; 20 mL non-buffered 1 M Tris, 8 mL 0.5 M $CaCl_2$ dissolved in H_2O to 400 mL). After digestion gels, were fixated (30% methanol, 10% acetic acid) for 15 min and stained with Coomassie Brilliant Blue R-250 for 30 min. Positive signals were visualized by a Kodak Image Station 4000MM Digital Imaging System. The gelatinase analysis was repeated three times.

Statistical Analysis

Shapiro-Wilks test was used to assess normality. Two-tailed Student's t-test with independent variables was used to compare means (normal distributions). ANOVA with Scheffe post-hoc test was used for multiple comparisons. Statistica (release 7) software was used for these calculations.

Results

The Response of LX-2 Cells to High Glucose Concentration Exposure is Different from that of to TGFB1

We measured the amount of collagen type-1, type-3 and type-4, as well as laminin-α1, thrombospondin-1 (TSP-1), tissue inhibitor of metalloproteinases-1 (TIMP1) and MMP1 in culture media of LX-2 cells using dot blot. Identical amounts of media were used to prepare a zymogram to detect MMP-2 and MMP-9 activity. As indicated in Table 1, no statistically significant increase was detected in the levels of collagens in the medium of cells exposed to high CHG. A dose of 5 ng/mL TGFB1 increased the production of all three collagen types almost equally in both low and high glucose environment. In the meantime LX-2 cells exposed to CHG sequestered abundant amounts of type-1 collagen in their cytoplasm, as indicated by immunocytochemistry (Fig. 1). On contrary, the immunostaining of collagen appears to be located extracellular in both control and TGFB1 exposed samples.

Compared to the control, the increase of laminin-α1 chain was similar in all groups, whereas elevated levels of TSP-1 and TIMP1 was found in cell samples exposed to TGFB1. In the culture medium of CHG, and CHG with TGFB1 treated cells we found lower MMP-2 activities. (Table 1 and Fig. 2).

Different Signaling Pathways are Activated in CHG Compared to TGFB1 Treated LX-2 Cells

Exposure of TGFB1 upregulated pSMAD2 and pSMAD3 expression both alone and in combination with CHG treatment. High glucose environment alone did not activate pSMADs, (Fig. 3 and Table 2). TGFB1 exposure resulted in the phosphorylation of Akt on Ser473 residue with subsequent phosphorylation and inactivation of GSK3-α and β and pS6 activation. Furthermore, TGFB1 alone enhanced the

Table 1 Effect of CHG and TGFB1 on the amount of matrix proteins in LX-2 culture medium

	Glucose 5.5 mM (%)	Glucose 15.3 mM (%)	Glucose 5.5 mM +TGF-β1 (%)	Glucose 15.3 mM +TGF-β1 (%)
Collagen type-1	100	121	165***	162***
Collagen type-3	100	156	164	166
Collagen type-4	100	103	197	132
MMP-1	100	153***	72	95
Laminin-α1	100	212*	239***	214
TSP-1	100	51	230*	113
TIMP-1	100	105	175*	179*

Chronic exposure to CHG alone significantly increased the production MMP1 and laminin-α1 chain, but no significant elevation of collagens is detected as documented by dot blot. Exposure of TGFB1 with or without CHG resulted significant elevation of type I collagen, and TIMP1, whereas increase in laminin-α1 and thrombospondin-1 reached significant level after TGFB1administration alone. Interestingly, although the alteration did not prove significant, CHG decreased the level of TSP-1 in all setting. *** indicates $p > 0.001$, ** indicates $p > 0.005$ and * indicates $p > 0.01$. Results are expressed as percent of untreated control. Collagen type I significantly increases in case of TGFB1 and glucose+ TGFB1 groups

phosphorylation of p38 (Fig. 3, Table 2) In contrast, significant decrease in the amount of pp38, pAkt ^{Ser473} was detected in CHG exposed cells. This went parallel with low pS6 expression (Fig. 3, Table 2). These changes were consistent with the PDGFR alpha downregulation in both CHG treated groups, and upregulated after TGFB1 exposure. No significant change in ERK1/2 levels was observed upon either TGFB1 or CHG exposure (Fig. 3, Table 2). Elevated amount of PKCα was detected both after TGFB1 and combined TGFB1-CHG administration, and in a less extent after CHG exposure. On the other hand CHG was more effective in upregulating the NFkB protein (Fig. 3, Table 2). Unexpectedly, CHG alone, as well as in combination with TGFB1 significantly enhanced the expression of $p21^{WAF1}$, whereas only modest effect was detected after solo TGFB1 exposure (Fig. 4). Immunostaining of the cells revealed that the $p21^{WAF1}$ protein in high glucose exposed groups was confined to the cytoplasm of LX-2 cells, whereas it was detected in the nuclei of control and TGFB1-exposed cells (Fig. 4). Furthermore, immunocytochemistry revealed that combined exposure of TGFB1 and high glucose stimulated the proliferation of LX-2 cells.

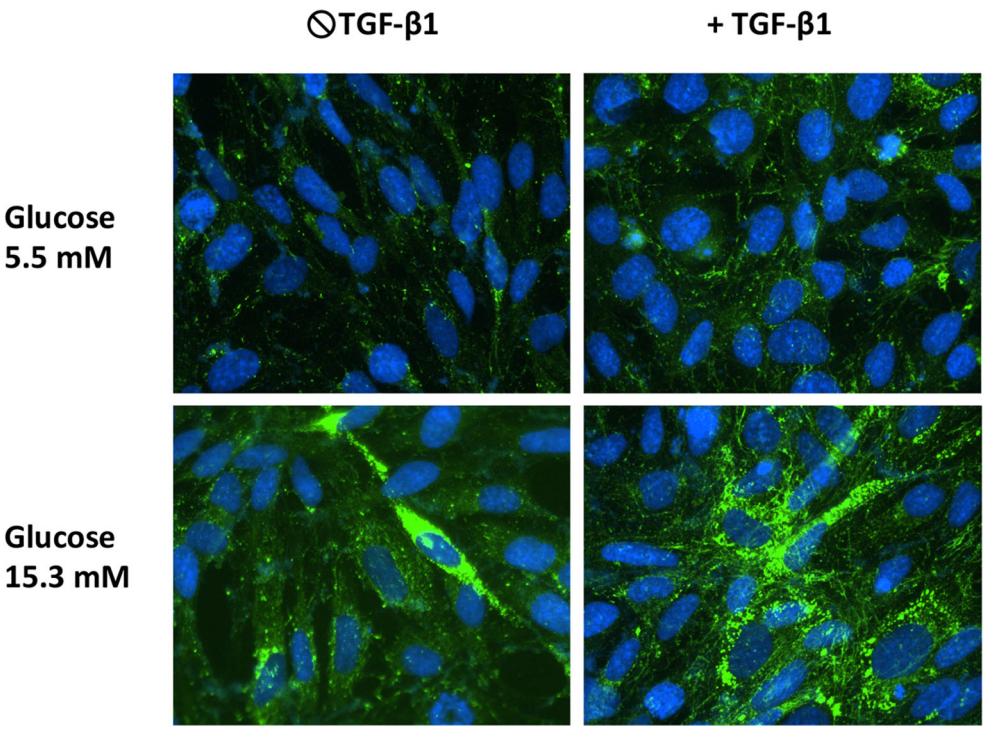

Fig. 1 Immunocytochemistry on LX-2 cells showing collagen type-1 deposition. As expected, TGFB1 exposition increases type-1 collagen expression in the pericellular area of LX-2 cells. On contrary, cells exposed to CHG sequestered material immunopositive for collagen type-1 in their cytoplasm indicating deterioration of collagen transport or unfolded protein response as an effect of high glucose exposure. Original magnification ×100

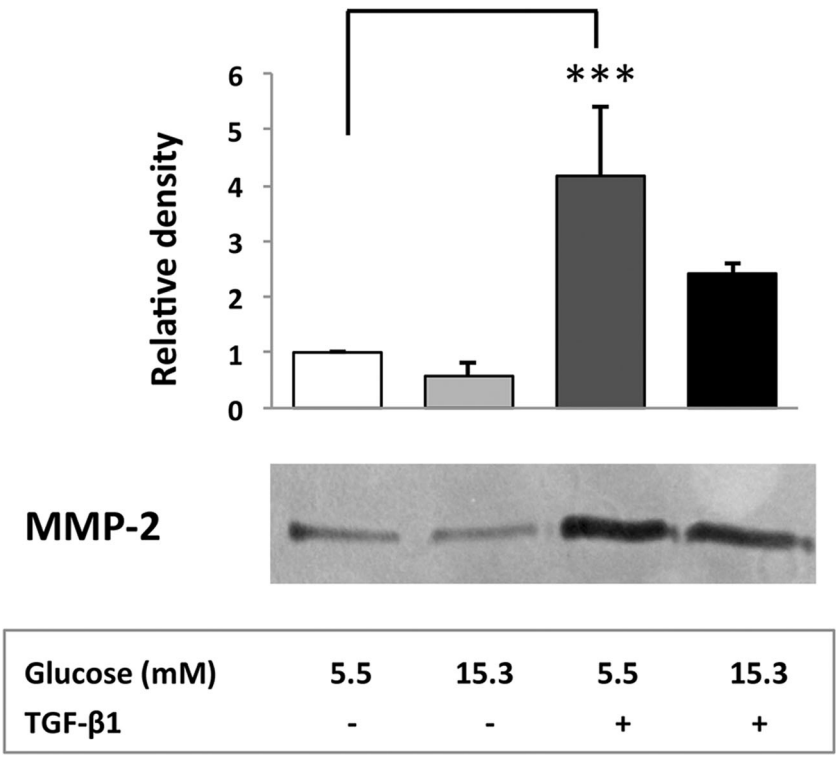

Fig. 2 Gelatinase zymogram showing decreased activity of MMP2 in the culture medium of CHG treated LX-2 cells. Gelatinase assay revealed the inhibitory effect of CHG on the activity of MMP-2. The decrease did not reach significant level. In contrast, TGFB1 exposure enhanced the activity of the protease

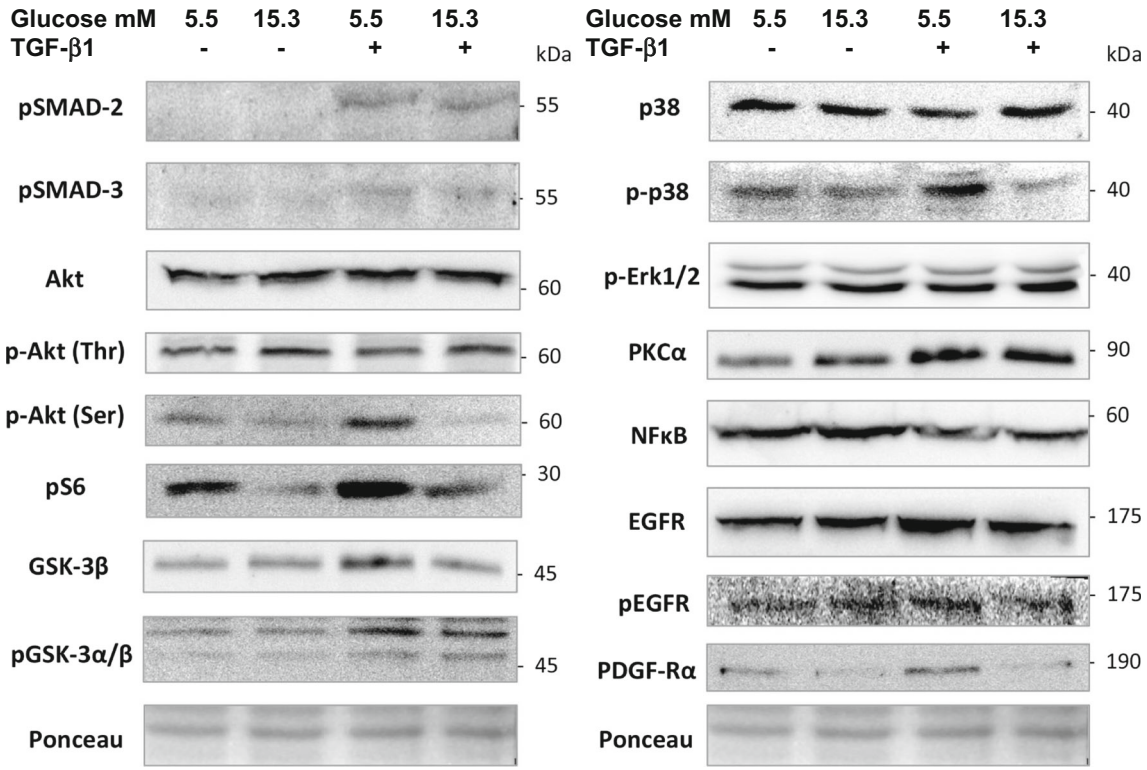

Fig. 3 Western blot analysis of key molecules affected by the exposure of CHG, TGFB1 or both in LX2 cells. TGFB1 treatment alone enhanced the amount of phospho-Smad2 and 3, phospho-AKT (Ser), pS6 kinase, phospho-GSKβ, phospho-p38 and PKCα all known as target of the growth factor. All these changes are implicated in the growth factor-induced fibrosis. CHG treatment resulted in weak phosphorylation of Akt ^{Thr308}, but considerable decrease in PDGFRα, phosphorylation of Akt ^{Ser473}, p38, pS6 and upregulation of NFκB. CHG did not activate EGFR, and PDGFR receptors, a phenomenon seen after TGFB1 exposure

Table 2 Statistical analysis of the changes in signaling molecules brought about by the exposure of CHG and TGFB1

	Glucose 5.5 mM (%)	Glucose 15.3 mM (%)	Glucose 5.5 mM +TGF-β1 (%)	Glucose 15.3 mM +TGF-β1 (%)
pSMAD-2	100	71	268**	250**
pSMAD-3	100	102	377**	352**
Akt	100	128	133	126
Akt (Thr)	100	132	102	112
Akt (Ser)	100	62***	229***	34***
GSK-3β	100	114	207**	112
pGSK-3α/β	100	115	200**	157*
pS6	100	44***	164***	83**
p38	100	91	78	108
p-p38	100	72***	143***	59***
pERK1/2	100/100	121/76	171/115	165/57
PKCα	100	123*	170***	175***
NFκB	100	160**	89	121
EGFR	100	108	126	100
pEGFR	100	102	194	139
PDGFR-α	100	44***	180***	22***

Asterix indicate significant changes: ***$p > 0{,}001$, **$p > 0{,}005$, *$p > 0{,}01$. Results are expressed as percent of control

Combined Exposure to Chronic Hyperglycemia and TGFB1 Induced a Modest Increase in CXCL-12 Production by LX-2 Cells

In order to assess for potential similarly to pancreatic stellate cells in which chronic exposure to high glucose concentration elevates the expression of CXCL-12, we measured the concentration of the chemokine from the cell culture medium. In contrast to pancreatic stellate cells, hepatic LX2 cells did neither respond to either high glucose concentration nor to TGFB1 exposure with increased CXCL-12 production. Only combined treatment of the cells resulted in modest (30%) elevation of the chemokine. In contrast, SP-1, a major transcription factor in the initiation of CXCL-12 transcription, did not show any change in the protein expression level (data are not shown).

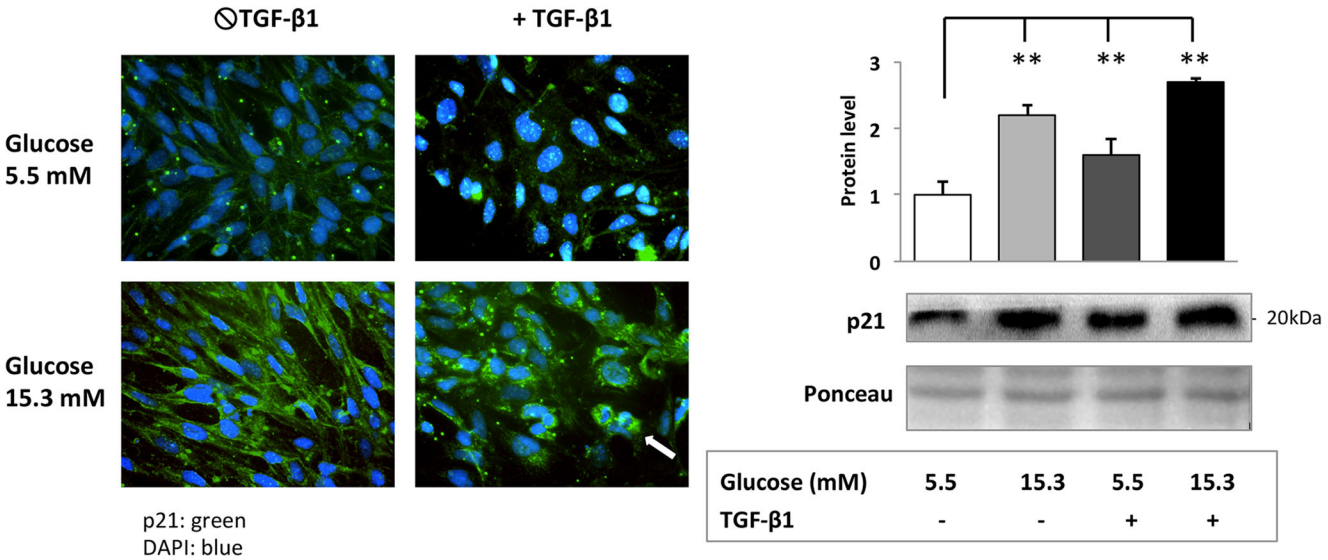

Fig. 4 CHG sequesters p21^{WAF1} in the cytoplasm of LX-2 cells. Upregulation of p21^{WAF1} was seen in all sample conditions according to Western-blot (right panel). Immunocytochemistry indicates that p21^{WAF1} was sequestered in the cytoplasm of LX-2 cells upon exposure to CHG. In contrary, p21^{WAF1} was localized in the cell nucleus after TGFB1 exposure. The changes were significant in all groups, $p > 0.05$

Discussion

The implication of hepatic stellate cells in the physiology and the pathology of the liver has been widely studied [17]. Recent reports describe its role in the development, regeneration, fibrosis and cancer of the liver [18]. One of its most studied functions is the active participation in liver regeneration, serving for the integrity of the organ [19]. However, this regenerative capacity turns to a harmful process when chronic injury maintains a permanently activated state of HSCs [17, 20, 21].

Liver, as a first guard against injuries caused by toxic agents derived from the intestine, has to cope with the everyday challenge of environment, as well as with the metabolic alterations associated with obesity and diabetes [2, 22, 23]. DM and NAFLD are particularly closely related and such, steatosis, steatohepatitis, fibrosis and even cirrhosis or hepatocellular cancer might develop over time in the liver. The role of HSC is particularly evident in cirrhosis [23].

In the current study we established an in vitro system to assess the direct effect of chronic hyperglycemia on LX-2 cells, an immortalized human Ito cell line. We addressed the first question whether the response of LX2 cells to high glucose levels is comparable with that of pancreatic stellate cells. Our second question was whether high glucose concentration alone is capable of activating Ito cells and inducing the synthesis of matrix proteins, or this effect requires the influence of TGFB1. To this end LX-2 cells were exposed to 15.3 mM glucose or TGFB1 alone, or to subsequent exposure of the two compounds.

While CXCL12 chemokine CXCR4 receptor binding promotes profibrogenic phenotype of LX-2 cells in vitro [24], the inhibition of the CXCR4 receptor results in an increased hepatic inflammatory response [25]. This might reflect that the regulation/effect of the CXCL12/CXCR4 axis in stellate cells is possibly different in the liver and in the pancreas. In support of this hypothesis, in contrast to the pancreas [14] in our presented system CHG alone did not stimulate stellate cells to increase the production of CXCL12.

Moreover, our results indicated, that the effect of high glucose and TGFB1 differs from each other.

The medium of LX-2 cells exposed to CHG contained less type IV collagen than that observed after TGFB1 addition, and this occurred together with low MMP2 activity. Interestingly, opposing results were presented in tendon cells where high glucose concentrations could up-regulate the mRNA expression and enzyme activity of MMP-9 [26]. All these findings might reflect the different tissue-specific effects of hyperglycemia on the liver compared to the musculoskeletal system.

The levels of collagen type I secreted to the medium of CHG-exposed cells did not increase significantly, which could be explained partly by high MMP-1 expression. In the meantime, the cytoplasm of CHG-treated LX-2 cells was loaded with material immunopositive for type I collagen, indicating the impairment of collagen transport from the cytoplasm to the ECM pointing to the development of metabolic stress.

In contrast to the effect of CHG, LX2 cells exposed to TGFB1 secreted more collagen type I to the medium, the MMP-1 expression was low but the MMP-2 activity was increased. Indeed, TGFB1 can differentially enhance the expression of the MMPs by activating the p38 MAPK pathway [27]. The considerable increase of TSP1 protein points to an upregulatory circle, as TSP1 was reported to be implicated in the activation of latent TGFB1 whereupon active TGFB1 stimulates the production of TSP1 [28, 29].

To clarify events potentially implicated in these results, we studied signaling pathways that are involved in HSC-mediated fibrogenesis. Addition of TGFB1 to the medium in our model activated all known signaling pathways of the growth factor. As a major effect related to fibrogenesis, we detected elevated phospho-SMAD2 and phospho-SMAD3 expression [30]. The activation of Akt on Ser 473 residue increased after TGFB1 exposure, whereas CHG alone or in combination with TGFB1 dramatically decreased the Ser 473 phosphorylation. Phosphorylation of threonine is accomplished by PIK3C PDK axis, whereas serine 473 is phosphorylated by the mTORC2 complex which also mediates the effect of TGFB1 [31]. Indeed the phosphorylation of threonine was only at a moderate level, while TGFB1 exposure resulted in strong phosphorylation of Ser473. Double phosphorylated Akt is more active in terms of phosphorylation together with the fibrogenic involvement of inactivation of GSK-3β [32]. Moreover, as a consequence of TGFB1 exposure we could detect the phosphorylation of p38, known to be targeted by TGFB1. This finding indicates that in addition of the SMAD pathway, the growth factor exerts a more generalized action resulting in the synthesis of matrix proteins [33].

Neither GSK inactivation, nor MAPK activation could be found upon chronic hyperglycemia exposure in HSCs. As MAPK activity is needed for the increased synthesis of MMP-2 and MMP-9, the modest activity of MMP-2 can be explained by the lack of the stimulation of its synthesis [34]. Low activity of MMP-2 has also been described for high glucose-exposed mesangial cells suggesting the role of TGFB1 in MMP downregulation [35] that is in contrast with the significant role of TGFB1 in the upregulation of MMP-2 and -9 [27].

Although it is published that Akt protein upregulates type I collagen synthesis [36], and it is also implicated in the stimulation of collagen type IV and laminin production [37], the modest Akt activation on Thr308 points toward the impairment of matrix synthesis, caused by the dysfunction of the Akt-mTOR signaling pathway. As opposed to diabetes-induced glomerulosclerosis [38], the information is scarce on the signaling pathways that HSC selects in order to initiate the increased synthesis of particular matrix proteins. However, the synchronous downregulation of PDGFR, Akt Ser473, pp38 and pS6 together with the obvious disturbance of

collagen type I secretion indicates their cooperation as an effect of high glucose exposure.

Transfection of Ito cells with a dominant negative construct of PIK3C inhibits the deposition of ECM proteins providing evidence for the PIK3C-Akt pathway in the maintenance of hepatic fibrosis [39]. How does Akt phosphorylation activate the transcription of matrix proteins? Akt activates or inactivates transcription factors by phosphorylation. At least two of these, namely, Sp1 and NFkB play important roles in the regulation of collagen type I and type IV polypeptide chain synthesis [40–42]. The importance of NFkB in our system is underlined by the increased expression of the transcription factor after CHG treatment.

Another possibility is that high glucose concentration-induced imbalance of collagen metabolism is partly a consequence of impaired secretion of procollagen I from the LX-2 cells. Immunoreaction on Fig. 1 demonstrates that cytoplasm of LX-2 cells is loaded with material positive for collagen type I. Hyperglycemia decreases the MMP2 activity of mesangial cells [35] which we could witness in our system, as well. Taken together, although we cannot dissect the actions of high glucose and TGFB1 under in vivo conditions, our in vitro experiments indicate that chronic exposure of high glucose concentration initiates profound alteration of LX-2 cells and the effect is different from those observed upon interaction with TGFB1.

Another important consequence of CHG is the upregulation of $p21^{WAF1}$. However, the protein is also sequestered in the cytoplasm, which was not the case in the control and TGFB1 exposed cells. Cytoplasmic p21 can interact with procaspase-3 and the complex behaves as a cell survival signal [43]. Cytoplasmic localization of the molecule requires its phosphorylation by Akt [44]. This action stabilizes p21 providing the cell a surviving, proliferative phenotype [45]. Indeed, we observed an increase in the number of dividing cells, especially in cultures co-treated with TGFB1 and CHG, where both serine and threonine residues of Akt were found to be phosphorylated.

In conclusion, our results indicate that the effect of CHG on LX-2 cells differs from that of TGFB1. Out of all characterized TK signaling pathways, the inactivation of Akt and p38, downregulation of pS6 and upregulation of NFkB could be detected. Although NFkB is known to act as a transcription factor of collagen synthesis, in our case it is more probable that its upregulation is related to endoplasmic stress initiated by glucose overload [46]. Decreased procollagen transport and degradation may be an indicator of unfolded protein response, which is a further task to evaluate.

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