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Effects of Short-term High Glucose on NIH/3T3 Fibroblast Proliferation, Apoptosis, and Collagen Type I Production

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Abstract

Objective: To investigate the mechanisms of in vitro high glucose induced model of liver fibrosis.

Material and methods: The effects of high glucose concentration on fibroblast proliferation were investigated by BrdU immunostaining. Apoptosis and necrosis levels were analyzed by flow cytometric assay. The content of collagen type I was measured by Collagen Estimation Assay through ELISA.

Results: A high glucose medium not only increased NIH/3T3 fibroblast proliferation, but also increased type I collagen synthesis, showing a similar condition to the fibrosis. Moreover, the high glucose caused an increased level of cellular apoptosis and necrosis.

Conclusions: High glucose modulates the fibrosis in NIH/3T3 fibroblast cells via inducing the production of type I collagen while maintains the homeostasis by inducing the apoptosis and necrosis of cells.

Keywords: High glucose, Collagen, NIH/3T3 fibroblast, Apoptosis

Kısa Süreli Yüksek Glikoz Uygulamasının NIH/3T3 Fibroblastlarında Proliferasyon, Apoptoz ve Kollajen Tip I Üretimi Üzerine Etkileri

Öz

Amaç: Yüksek glikoza bağlı karaciğer fibrozis mekanizmalarını in vitro model kullanarak araştırmak amaçlanmıştır.

Gereç ve yöntem: Yüksek glikoz konsantrasyonunun fibroblast proliferasyonu üzerindeki etkileri BrdU immünositokimya tekniği ile incelenmiştir. Apoptoz seviyeleri ve nekroz akım sitometri analizi ile tespit edildi. Kollajen tip I içeriği, ELISA ile ölçüldü.

Bulgular: Yüksek glikoz düzeyi sadece NIH/3T3 fibroblast proliferasyonunu arttırmadı, aynı zamanda fibrozise benzer bir durumun göstergesi olarak tip I kollajen sentezini de arttırdı. Ayrıca yüksek glikoz, apoptoz ve nekroz seviyelerinin artmasına da neden olmuştur.

Sonuç: Yüksek glikoz, NIH/3T3 fibroblast hücrelerinde fibrozisi, tip I kollajen üretimini indükleyerek modüle ederken, hücrelerin apoptoz ve nekrozunu indükleyerek homeostazı korur.

Anahtar Kelimeler: Yüksek glikoz, Kollajen, NIH/3T3 Fibroblast, Apoptoz

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Introduction

Hyperglycemia-induced inflammation and fibrosis have important roles in the pathogenesis of diabetic nephropathy and cardiomyopathy. Fibrosis is a significant pathological property of liver diseases, which involves in the abnormal accumulation of extracellular matrix (ECM) proteins, particularly the collagens (1,2). In fibrotic liver, ECM components are mainly produced by hepatic stellate cells (HSCs) and fibroblasts. During fibrogenesis, it is known that HSC undergoes a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation and ECM production, especially type I collagen synthesis (3). Type I collagen is the predominant component of ECM during liver fibrosis. Its production involve two processes: the intracellular synthesis including gene transcription, translation and modification to form procollagen, and the secretion of procollagen alpha chains to the outside of cell to form helix collagen by sorting and alignment etc. In the study, it was aimed to investigate whether the high glucose modulates type I collagen production through an intracellular synthetic process (4,5).

The fibrogenic cells have two major features: one is to be active in cell proliferation, which led to increase in cell number, another is strong fibrogenic ability per cell, which led to accumulation of ECM (6,7). The mouse NIH/3T3 fibroblasts also shared the features of active HSC presented, such as remarkable proliferation and substantial production of collagen, and stable cell line. Moreover, NIH/3T3 fibroblast is often used as a desirable cell model for investigation of antifibrotic drugs (8). In order to investigate the mechanism by which high glucose-induced liver fibrosis, we observed the effects of high glucose on NIH/3T3 fibroblast proliferation, apoptosis, necrosis, and collagen type I protein production.

Material Methods

Cell line and treatment

NIH/3T3 fibroblast cells obtained from the American Type culture Collection were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin at 37°C in 95% humidified air with 5% CO_2. Cells were subcultured on every 3rd day using trypsin for harvesting cells. NIH/3T3 fibroblast cells were grouped into two as a control (Ctrl, 5.5 mM/L glucose) group and high glucose group (HG, 25mM/L glucose).

Cell proliferation assay

Analysis with 5-Bromo-2'-Deoxyuridine (Br-dU) (Sigma-Aldrich, Schnelldorf, Germany) was performed according to our previous protocol. HRP/AEC (ABC) Detection IHC Kit (Abcam, ab93705, USA) was used for immunocytochemical staining. A mouse monoclonal Br-dU Antibody (Santa Cruz Biotechnology Inc., sc-20045) was used as the primary antibody (1:200, overnight). Aminoethylcarbazole (AEC) (Invitrogen, Calsbad, USA) was used as the chromogen. BrdU was detected by visual colorimetric staining. Br-dU-labeled cells were assessed by two researchers and Br-dU index (number of positively stained cells in total number of cells counted) was calculated by evaluating at least 100 viable and nonviable cells. The ratios of Br-dU positive cell nuclei and the total numbers of cells were calculated for all doses.

Collagen Estimation Assay

The protein level of collagen Type I was determined using a commercially available ELISA kit for collagen type I (Shanghai Sunred Biological Technology Co., Ltd., China) according to the manufacturer's guideline.

Apoptosis Assay

Apoptosis was measured using an Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, USA) and analyzed by flow cytometry. Stained cells were acquired by using BD FACS Calibur system and obtained data were analyzed by using CellQuest Prosoftware. Briefly, NIH/3T3 fibroblasts were treated with different concentrations of high glucose (5.5 mM and 25 mM) for 24 h. Apoptotic index was calculated by means of all five repeated experiments and given as percentage.

Statistical analysis

Statistical analyses of the data were performed using GraphPad InStat Software (Version 3.06). Results are presented as means ± standard error mean (SEM) and compared by unpaired t test

with Welch correction. *P<0.05 was accepted as statistically significant.

Results

Effects of high glucose concentration on proliferation in NIH/3T3 fibroblasts

To determine whether high glucose concentration affect the proliferation of fibroblasts, we added 5.5 mM and 25 mM concentrations of glucose to the culture medium. As determined by the BrdU staining assay, 25 mM of glucose significantly increased the proliferation of NIH/3T3 fibroblast, as compared with 5.5 mM/L glucose (p<0.05) (See Figure1-2).

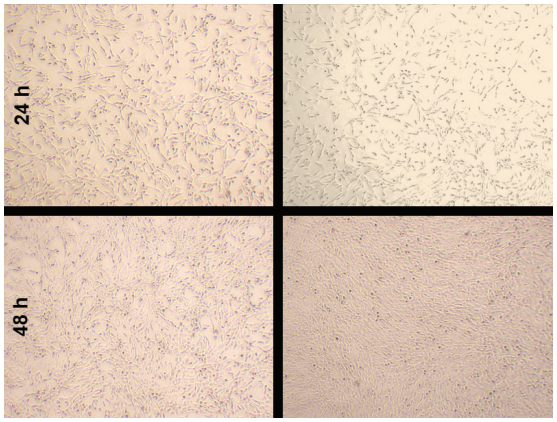

Figure 1. Representative live micrographs of 3T3 fibroblast cells incubated in normal (5.5 mM) and high glucose (25 mM) for 24 and 48 hours (h). Original magnification, × 40

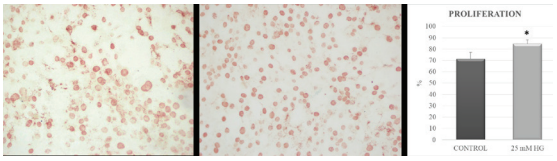

Figure 2. Representative micrographs of anti-BrdU immunohistochemical staining of NIH/3T3 fibroblast cells incubated in two dosage of glucose (5.5 mM and 25 mM HG). Original magnification, X40. Statistical analysis of distribution of BrdU expression in NIH/3T3 fibroblast cells. *p<0.05 vs control.* Bars represent mean ± SEM.

Effects of high glucose concentration on collagen type I production in NIH/3T3 fibroblasts

Confluent NIH/3T3 fibroblasts treated with high glucose (25mM) for 24 h showed increased production of collagen type I. The amount of collagen I protein was 17.67 ± 3.37 ng/ml for the control group and 18.02 ± 0.86 ng/ml for the high glucose group without a statistically significant difference (p=0.64) (See Figure 3).

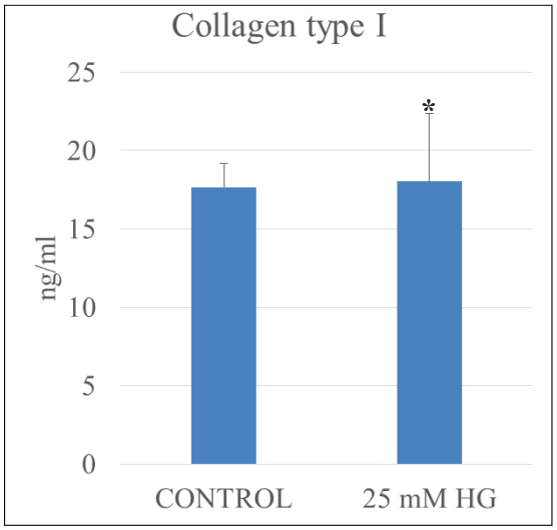

Figure 3. Effects of 25 mM high glucose treatment on collagen I protein level (ng/ml) of NIH/3T3 fibroblasts. Values are the mean ± SD of protein levels from three trials. *$p < 0.05$ vs control group.*

Effects of high glucose concentration on apoptosis/necrosis of NIH/3T3 fibroblasts

Figure 3 presents the evidence for apoptosis and necrosis. The high glucose-induced apoptosis was examined by the annexin V method. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. This allows for live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with annexin V). To check this, NIH/3T3 cells were treated for 24 hours with two concentrations of glucose and then stained with annexin V–FITC. The late apoptosis and necrosis percentage of fibroblasts was increased considerably in 25 mM HG compared with control group (p<0.05) (See Figure 4).

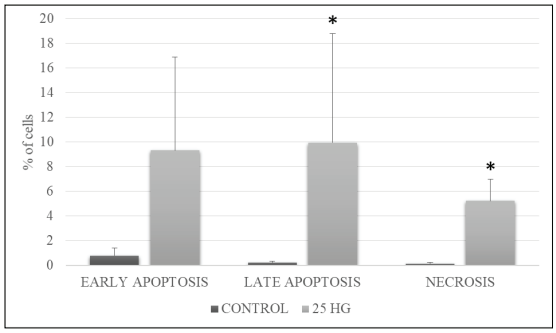

Figure 4. Effects of 25 mM high glucose treatment on early, late apoptosis, and necrosis in NIH/3T3 fibroblasts. Values are the mean ± SD of percentages from three trials. **p < 0.05 vs control group*.

Discussion

Tissue fibrosis is considered as an abnormal wound healing process, during which the balance between collagen synthesis and degradation is broken. Fibrogenesis in different tissues has similar pathological features, including the inflammatory response, myofibroblast activation, and subsequent excessive ECM deposition (9).

The main structural protein of the composition of ECM is collagen type I. Hyperglycemia promotes the fibrosis by increasing synthesis of ECM components, mainly the collagen type I. Several studies of cardiomyopathy have demonstrated an accumulation of collagen, including collagen type I (1,10,11). In the present study, we investigated the effects of high extracellular glucose on the production of collagen type I by NIH/3T3 fibroblast and found that in vitro production of the collagen type I was up-regulated by high glucose probably based on the increase in cellular proliferation. This is consistent with findings in other cell types such as cultured rat and human renal fibroblasts and human peritoneal fibroblasts (12-14). However, Benazzoug et al. reported that the high glucose without alteration of collagen type I production in human skin fibroblasts (15).

A complex molecular mechanism playing various important roles in regulating the fibroblast apoptosis, have been shown to be closely related to the occurrence of fibrosis. Apoptotic cells act as the drivers of fibrotic process, it may act directly on fibroblasts, enhancing the cellular proliferation and profibrotic phenotypes. High levels of apoptosis are either initiators or perpetuators of the fibrotic response seen in lung fibrosis, liver fibrosis, and chronic myocardium fibrosis accompanied by deposition of extracellular matrix, synthesis of collagen, and fibroblast proliferation (16-18). High glucose also induced the process of apoptosis in fibroblasts, in which oxidative stress is the key inducement. The major mechanisms of oxidative stress-induced apoptosis may be based on (19) the increased level of ROS leading to nuclear factor kappa B (NF-κB) activation. The light-chain-enhancer of NF-κB in activated B cells was shown to be involved in the control of a large number of cellular processes, such as immune and inflammatory responses, cellular growth and development, and apoptosis (20-22). In the present study, we observed that the high glucose content increased the proliferation index in a liver fibroblast model. Moreover, our findings showed that the apoptosis and necrosis rate of NIH/3T3 fibroblasts was increased obviously when cultured with high glucose medium.

In summary, high glucose modulates the fibrosis in NIH/3T3 fibroblast cells via inducing the production of type I collagen while maintains the homeostasis by inducing the apoptosis and necrosis of cells. Thus, increased rates of apoptosis, necrosis, and collagen type I production may be determining factors in a progression of fibrosis.

Acknowledgements

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REFERENCES

1. Tang M, Zhang W, Lin H, et al. High glucose promotes the production of collagen types I and III by cardiac fibroblasts through a pathway dependent on extracellular-signal-regulated kinase 1/2. Mol Cell Biochem 2007;301:109-14.

2. Friedman SL. The cellular basis of hepatic fibrosis: mechanisms and treatment strategies. New Eng J Med 1993;328:1828-1836.

3. Olaso E, Friedman SL. Molecular regulation of hepatic fibrogenesis. J Hepatol 1998;29:836-847.

4. Hynes RO. The extracellular matrix: not just pretty fibrils. Science 2010;326:1216-19.

5. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209-218.

6. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008;134:1655-1669.

7. Dranoff JA, Wells RG. Portal fibroblasts: Underappreciated mediators of biliary fibrosis. Hepatology 2010;51:1438-1444.

8. Liu CH, Hu YY, Wang XL, et al. Effects of salvianolic acid-A on NIH/3T3 fibroblast proliferation, collagen synthesis and gene expression. World J Gastroenterol 2000;6:361-364.

9. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med 2012;18,1028-1040.

10. Dostal DE, Rothblum KN, Conrad KM, et al. Detection of angiotensin I and II in cultured rat cardiac myocytes and fibroblasts. Am J Physiol Cell Physiol 1992;263:C851-C863.

11. Eghbali M. Cardiac fibroblasts: function, regulation of gene expression, and phenotypic modulation. Basic Res Cardiol 1992;87:183-189.

12. Higuchi C, Tanihata Y, Nishimura H, et al. Effects of glucose and plasminogen activator inhibitor-1 on collagen metabolism in the peritoneum. Ther Apher Dial 2005;9:173-178.

13. Saed GM, Diamond MP. Effect of glucose on the expression of type I collagen and transforming growth factor-beta1 in cultured human peritoneal fibroblasts. Fertil Steril 2003;79:158-163.

14. Lam S, van der Geest RN, Verhagen NA, et al. Connective tissue growth factor and igf-I are produced by human renal fibroblasts and cooperate in the induction of collagen production by high glucose. Diabetes 2003;52:2975-2983

15. Benazzoug Y, Borchiellini C, Labat-Robert J, et al. Effect of high-glucose concentrations on the expression of collagens and fibronectin by fibroblasts in culture. Exp Gerontol 1998;33:445-455.

16. Chen CC, Lau LF. Deadly liaisons: fatal attraction between CCN matricellular proteins and the tumor necrosis factor family of cytokines. Journal of Cell Communication and Signaling 2010;4:63-69.

17. Johnson A, DiPietro LA. Apoptosis and angiogenesis: an evolving mechanism for fibrosis. The FASEB Journal 2013;27:3893-3901.

18. Liu WL, Wang XX, Mei Z, et al. BNIP3L promotes cardiac fibrosis in cardiac fibroblasts through [Ca^{2+}]i TGF-βSmad2/3 pathway. Scientific Reports 2017;7:1906.

19. Chu Q, Yang K, Wang A. Research progress on oxidative stress and apoptosis. Journal of Hygiene Research 2003;323:276-279.

20. Kanigur Sultuybek G, Soydas T, Yenmis G. NF-κB as the mediator of metformin's effect on ageing and ageing-related diseases. Clin Exp Pharmacol Physiol 2019;46:413-422.

21. Soydas T, Yaprak Sarac E, Cinar S et al. The protective effects of metformin in an in vitro model of aging 3T3 fibroblast under the high glucose conditions. J Physiol Biochem. 2018;74:273-281.

22. Moiseeva O, Deschênes-Simard X, St-Germain E, et al. Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-kB activation. Aging Cell. 2013;12:489-498.