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A Potential Role of Fructose to Modulate Fibroblast Growth and Expression of Connective Tissue Growth Factor *In vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Author KM designed the study, wrote the protocol and wrote the first draft of the manuscript. Author YN performed cell culture and the experiments. Author MK managed the experimental process. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: Hyperglycemia has been posited to have an effect on the healing process at the cellular level, particularly on fibroblasts that produce extracellular matrix components including collagen. However, the mechanisms underlying this process have yet to be fully elucidated. In addition, excessive intake of fructose-containing drinks has recently been shown to induce hyperglycemia, and fructose induces metabolic changes that may influence normal wound healing. Therefore, we investigated the effect of fructose on the cellular proliferation of cultured fibroblasts.
Materials and Methods: The murine fibroblastic cell line NIH3T3 was incubated in the DMEM medium with or without the supplementation of either glucose (0.3, 1.4, or 2.8%) or fructose (1.4 or 2.8%) and was analyzed using microscopy, proliferation assays, and reverse transcription-polymerase chain reaction to determine the expression levels of type I collagen and connective

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tissue growth factor (CTGF).
Results: Our results demonstrate that *In vitro* culture in fructose-supplemented media suppressed fibroblast proliferation. The suppression of the expression levels of CTGF was also observed. **Conclusion:** These results suggest that fructose represents a dietary factor with a negative effect on wound healing via the modulation of CTGF expression.

Keywords: Glucose; fructose; fibroblasts; collagen; connective tissue growth factor.

1. INTRODUCTION

Wound healing consists of several complex stages including coagulation, inflammation, proliferation/tissue formation, and remodeling/maturation [1]. During the first two steps, injured tissue immediately reacts to stop bleeding through clot formation and the activation of cellular and soluble hemostatic components, including platelets and tissue factors. Cells adjacent to the wound then secrete chemical attractants, such as histamine and serotonin, leading to vasodilatation and the recruitment of neutrophils and macrophages. Migrating inflammatory cells then effectively "sterilize" the wound by phagocytosis and the secretion of proteases, e.g., collagenases, which digest necrotic tissue and cellular debris. Inflammatory cells also produce a plethora of cytokines and growth factors that control the wound healing process by stimulating various cell types.

In the proliferative phase, fibroblasts and endothelial cells infiltrate and proliferate in response to cytokines produced by macrophages. Fibroblasts are responsible for the production of components of the extracellular matrix, e.g., type I collagen, which form the granulation tissue of wound repair. This process is promoted by pro-fibrotic cytokines and growth factors, including transforming growth factor (TGF-β) and connective tissue growth factor (CTGF/CCN2). In the remodeling or maturation phase, fibroblasts stimulated with cytokines differentiate into myofibroblasts that express α-smooth muscle actin and contribute to wound contraction and remodeling [2].

Fibroblasts play an essential role during the complex phases of the wound healing process via the production of collagen. Factors that control collagen production by fibroblasts include TGF-β, interleukin (IL)-4, and histamine. Further, CTGF/CCN2 has also been demonstrated as a key regulator of fibrosis. CTGF is a 32–38 kDa protein that belongs to the CCN proteins family and is comprised of three structurally similar proteins (CYR61/CCN1, CTGF/CCN2, and NOV/CCN3) [3]. CTGF is predominantly expressed by fibroblasts and functions in stimulating the migration, proliferation, and collagen production of fibroblasts [4,5]. In addition, CTGF is also expressed by vascular endothelial cells, tumor cells, and chondrocytes, indicating the broad function of this fibrogenic growth factor [3].

Regarding wound healing, there are several factors that might inhibit the healing process, some of which may be improved by appropriate clinical intervention. For example, protein-energy malnutrition and deficiency of nutritional elements (e.g. zinc) are known to make the delayed healing.

Diabetes mellitus (DM) is generally accepted as a major risk factor for delayed or impaired wound healing. Besides an involvement of diabetic angiopathy and neuropathy, local hyperglycemia at wound sites may deteriorate the function of fibroblasts, inhibiting the appropriate formation of the granulation tissue [6]. Although fibroblasts require glucose for *in vitro* culture, a literature review [6] has reported that higher concentrations of glucose inhibit fibroblast proliferation, with increased oxidative stress believed to play a key role. More recently, Xuan et al. [7] have reported that high glucose concentrations inhibit fibroblast cell migration via the modulation of the c-Jun N-terminal kinase signaling pathway. However, the mechanisms underlying this effect have to be fully clarified, and the involvement of collagen-inducing factors, such as CTGF, remains unclear.

Fructose is a hexose sugar that has the same chemical formula ($C_6H_{12}O_6$) as glucose. Fructose is absorbed in the small intestine and is metabolized by specific mechanisms before entering glycolysis and gluconeogenesis pathways independently of insulin signaling. Recently, the intake of fructose-containing diets or drinks (e.g., polyethylene terephthalate (PET)-bottled beverage) has been increasing; however, such diets may induce glucose intolerance and increase insulin resistance [8,9]. Further, fructose

feeding has been reported to cause collagen abnormality [9]. However, its direct effect on fibroblasts *in vitro* has not been clarified.

In the present study, we evaluated the direct effects of fructose on fibroblast proliferation and the expression of collagen-related genes.

2. MATERIALS AND METHODS

2.1 Cell Culture

The murine skin-derived fibroblastic cell line NIH3T3 was kindly provided by Professor Toshiko Okabe of Sagami Women's University. NIH3T3 cells were cultured *in vitro* in high glucose (4,500 mg/L)-containing Dulbecco's Modified Eagle Medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% calf serum (CS) and 1% penicillin–streptomycin on 60 mm × 16 mm culture dishes under 5% CO_2 at 37℃. After reaching semi-confluence, cells were passaged once and further cultured in DMEM medium containing the same concentration of CS and antibiotics but with different concentrations of D-glucose ($C_6H_{12}O_6$, Wako Pure Chemical Industries, Osaka, Japan) or D-fructose ($C_6H_{12}O_6$, Wako Pure Chemical Industories) as indicated.

2.2 Microscopic Observation

Cells were cultured in a DMEM medium with different hexose concentrations (as indicated) for 24 h on 8-well chamber slides (Watson-FukaeKasei, Hyogo, Japan) and observed under a translucent microscope (EVOS® FL Cell Imaging System, LifeTechnologies Japan Ltd, Tokyo, Japan.).

2.3 Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured cells using Trizol® (ThermoFisher Scientific, K.K., Yokohama, Japan) according to the manufacturer's instructions and measured using a Nanodrop® spectrophotometer (ThermoFisher Scientific, K.K.). Reverse transcription and polymerase chain reactions were performed using 100 ng each of extracted RNA samples and TaKaRa Prime Script® RT-PCR Kits (Takara Bio, Shiga, Japan) using a My Cycler® thermal cycler (Bio-Rad Laboratories, Inc., CA, USA). PCR conditions were as follows: denaturation at 94℃ for 30 s, annealing at 59℃ for 30 s, and extension at 72℃ for 1 min for 30 cycles for collagen, and 94℃ for 30 s, annealing at 56℃ for 30 s, and extension at 72℃ for 1 min for 35 cycles for CTGF. After amplification, samples were subjected to electrophoresis using 1.5% agarose gels and a 100-bp DNA Step Ladder (Wako Pure Chemical Industries, Osaka, Japan).

The following primers were used:

Beta-actin (GenBank NM_007393); Forward ACCAACTGGGACGATATGGAGAAGA, Reverse TACGACCAGAGGCATACAGGGACAA (214 bp); Type I collagen (NC_000077.6), Forward TGTTCGTGGTTCTCAGGGTAG, Reverse CAGTGCGAGCAGGGTTCTTTC (254 bp); and CTGF (NM_010217.2), Forward GGAGCGTATAAAAGCCAGCG, Reverse TCACGCTCCGTACACAGTTC (475 bp).

2.4 MTS Assay (MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

Cells adjusted to a concentration of 5×10^5 cells/ml were cultured on 96-well plates for CellTiter 96™ AQueous One Solution Cell Proliferation Assays (Promega K.K., Tokyo, Japan) in DMEM medium containing a high concentration of glucose (4,500 mg/L) until semiconfluence. Cells were then washed and stimulated for 2 h by changing to conditioned media. Cellular proliferative responses were measured according to the absorbance change (450 and 620 nm) according to the manufacturer's instructions. Differences between culture conditions were assessed by one-Way ANOVA using Prism5™ software (GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

To investigate the potential effect of fructose on fibroblast proliferation, we cultured and stimulated NIH3T3 fibroblastic cells in DMEM medium containing different sugar concentrations. In general, NIH3T3 cells require high concentrations of glucose and are typically maintained in DMEM supplemented with "high glucose" (0.45% = 0.81 mM).

First, we checked cellular morphology using microscopic observation. Fibroblasts did not reach confluence when cultured in non-glucose-

containing DMEM medium (Fig. 1, a), indicating a general requirement of glucose for culture. However, proliferating cells with a spindle morphology, i.e., a fibroblastic appearance, were confirmed in cultures containing 0.3% (0.51 mM), 1.4% (2.53 mM), and 2.8% (5.07 mM) glucose (Fig. 1, b–d).

Next, fructose instead of glucose was added to fibroblast cultures. When fructose concentrations were comparable to glucose (i.e., 1.4%), no significant change in the cell shape was observed, although proliferation was suppressed (Fig. 1e). However, when fibroblasts were cultured in high fructose-containing media (2.8% = 5.07 mM) instead of glucose for 24 h, the number of cells was decreased and cells obtained rounded morphology, indicating increased cellular viability and proliferation (Fig. 1f).

The potential stimulatory effect of hexoses was investigated using MTS assays. Cells cultured in high-glucose media until semi-confluence were transferred to conditioned media supplemented with different concentrations of glucose or fructose before the measurement of cellular responses. More specifically, according to the manufacturer's instructions, cells were exposed to the different concentration of hexose for 2 h and then the proliferative response was measured. As summarized in Fig. 2, fibroblasts proliferated in the presence of 0.3%–1.4% glucose, whereas higher glucose concentrations (2.8%) were less effective. In contrast, cells incubated with fructose demonstrated no acute increases in proliferation. A significant difference in the proliferative response was observed between culture conditions ($P = 0.001$, $F = 10.32$), indicating that the exposure to fructose suppressed the proliferation of NIH3T3 cells.

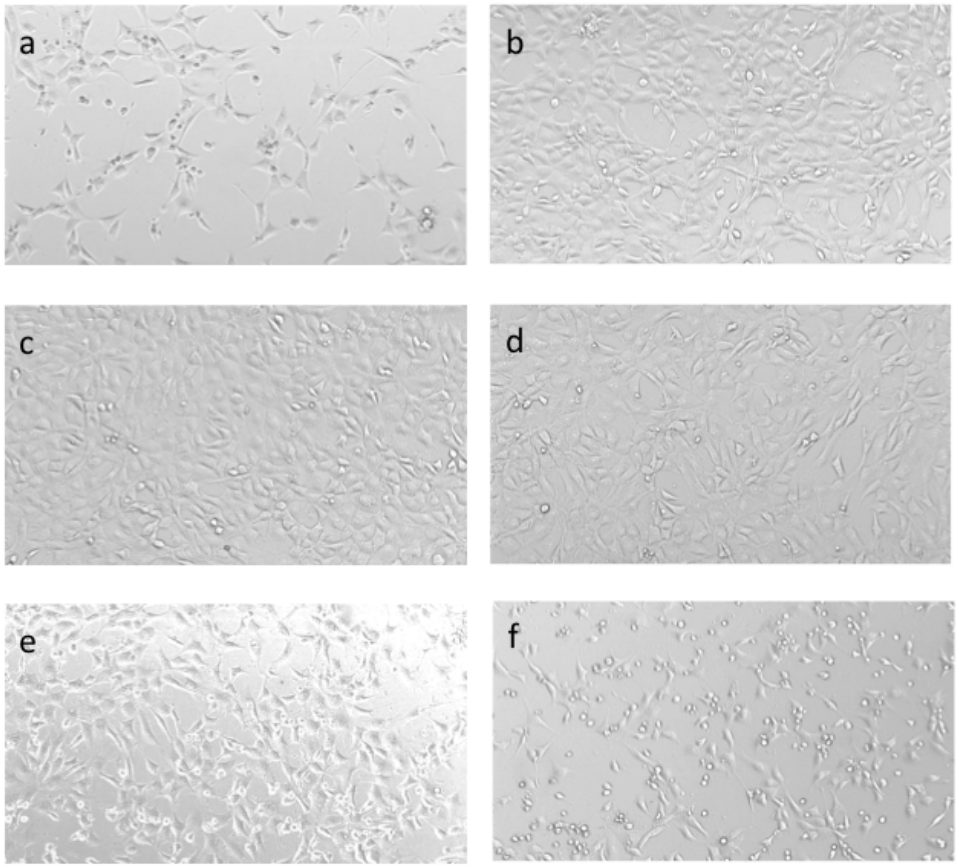

Fig. 1. Microscopic observation of fibroblasts cultured in the presence of different concentrations of hexose

NIH3T3 cells were exposed to various concentrations of hexose for 24 h and observed using a microscope (x40). a: no sugar; b: glucose 0.3%; c: glucose 1.4%; d: glucose 2.8%; e: fructose 1.4%; f: fructose 2.8%. Representative figures are shown from three independent experiments with similar results

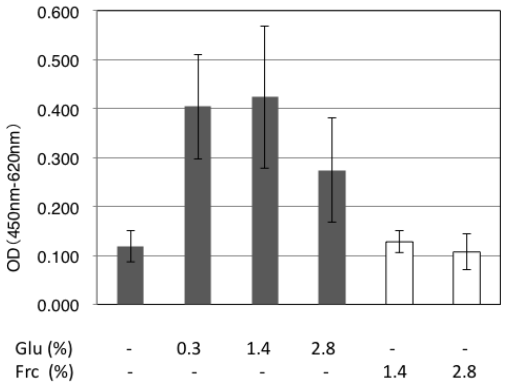

Fig. 2. RT-PCR analyses of collagen and CTGF gene expression levels

NIH 3T3 cells were incubated for 24 h in various hexose conditions and then subjected to RT-PCR analyses to determine type I collagen and CTGF mRNA expression levels. Representative figures from five (for type I collagen) and three (for CTGF) assays are shown. Glu, glucose; Frc, fructose

To examine the effect of hexoses on the expression of fibrosis-related genes, cultured cells were subjected to RT-PCR analysis. As shown in Fig. 3, fibroblasts cultured in the presence of glucose expressed both type I collagen and CTGF. Expression levels of type I collagen were highest in cells cultured in the presence of 1.4% glucose, followed by 0.3 and 2.8% glucose. In addition, CTGF expression was observed in all tested samples. In contrast, fibroblasts cultured in media containing fructose for 24 h demonstrated a diminished expression of type I collagen and CTGF, indicating that the presence of fructose abolished the production of ECM components via the inhibition of CTGF expression.

The results of the present study demonstrate that glucose maintains, whereas fructose inhibits, the proliferation of fibroblasts, and the expression of both type I collagen and CTGF is suppressed by the presence of fructose.

Glucose concentration is an important factor in stimulating the growth of fibroblasts. Previous studies have investigated the effect of high glucose on cellular proliferation *in vitro*. Results of these studies, however, vary according to the experimental condition used, including the degree of glucose exposure and the origin of fibroblast cell lines. For example, high glucose may induce cellular proliferation and the expression of type I procollagen in cardiac fibroblasts [10], whereas impaired proliferation was demonstrated in dermal fibroblasts cultured under high glucose conditions [11]. At lower glucose concentrations, Han et al. [12] reported that normal human dermal fibroblasts proliferated in a dose-dependent manner at glucose concentrations up to 2.3 mM but in a non-dose-dependent manner at concentrations between 5.5 and 25.5 mM. Of note, the glucose concentrations used in these studies may be much higher (for example, 25 mM = 450 mg/dL or 30 mM = 520 mg/dL [10,11]) than baseline or physiological levels (approximately 4 mM = 72 mg/dL to 6 mM = 106 mg/dL); thus, more detailed and comparative studies are required to clarify the physiological function of fructose *in vivo* [6].

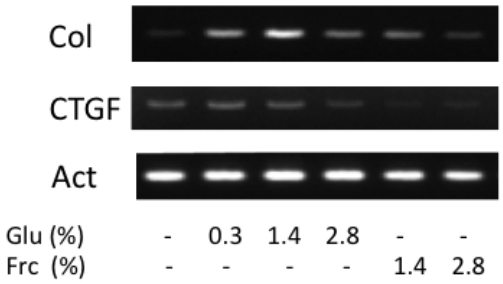

Fig. 3. MTS assay

NIH3T3 cells after a 24-h incubation in high-glucose-containing ("normal") media were subsequently exposed to various hexose condition for 2 h, and the proliferative responses were measured using the MTS assay. Average values of absorbance at 450–620 nm obtained from two distinct assays are shown. Col, type I collagen; CTGF, connective tissue growth factor; Act, beta actin; Glu, glucose; Frc, fructose

In the present study, we used glucose and fructose concentrations of up to 2.8% (approximately 5.07 mM = 90 mg/dL). This concentration may be considered to be more physiological; therefore, the impact of exposure to fructose on cellular proliferation and collagen expression may be more significant than at higher fructose concentrations. However, because even the lowest concentration of fructose was found to affect cell growth, the findings of the present study should be confirmed using fibroblasts from different origins, particularly human. In this regard, there have been previous studies by Delhotal et al. [13,14] in which human dermal fibroblasts were cultured *in vitro* in media containing fructose. In these studies, the authors reported that the consumption of fructose by fibroblasts was lower than for glucose and that cells grown in fructose-containing media had much lower glycogen

stores compared to cells cultured in glucose-containing media.

Collagen is critical to the whole process of wound healing, and its expression is regulated by pro-fibrotic factors, such as TGFβ and CTGF, secreted by various cell types. In high glucose conditions or hyperglycemia, collagen composition is known to be impaired because of the formation of glycated products resulting from the Maillard reaction. This reaction may also be induced by fructose [9]. The results of the present study demonstrate that fructose, but not glucose, suppresses fibroblast growth and CTGF expression in NIH3T3 fibroblastic cells. The suppression of CTGF expression may be due to the decreased cellular viability; however, this result would be the first to demonstrate the *in vitro* effect of fructose on CTGF expression in fibroblasts. In our preliminary study, 1.3 mM of mannitol (as an osmotic control) did not show significant effect on CTGF expression in RT-PCR (data not shown).

On the other hand, Xuan et al. [7] demonstrated that high glucose impaired fibroblast migration via suppression involving basic fibroblast growth factor (bFGF) expression in fibroblasts. Since bFGF is considered to be also a critical factor in wound healing process [15,16], the regulations of expressions of these growth factors may be key to understand the potential role of the sugars in wound healing. Further, the distinct effects of glucose and fructose on fibroblasts may have clinical implications as fructose may be recommended as an alternative to glucose because fructose does not directly increase blood sugar level in the same manner as glucose.

Liu X et al. [17] reported that high concentrations of glucose (25 mM) promoted CTGF expression in vascular smooth muscle cells, suggesting the involvement of CTGF in the pathogenesis of diabetic macrovascular complications. Although the glucose concentration used the previous study was much higher than that in the present study, it is hypothesized that dietary sugar may effect collagen and CTGF expression at a cellular level *in vivo* via a mechanism distinct from glycation. In this regard, it may be interesting to investigate the potential involvement of expression of glucose transporters (GLUT). For example, GLUT-5 is not an insulin-regulated GLUT which expression is limited, and therefore it might have implications in fructose-induced cellular metabolism, e.g. in liver but not in cutaneous tissue [18]. As we have not checked the expression and its regulation of GLUT-5 in fibroblasts including NIH-3T3, the issue should be further clarified.

Of note, although the potential role of the profibrogenic cytokine CTGF in wound healing has been suggested, its direct involvement in the healing process is controversial since Liu S et al. [19] claimed that CTGF/CCN2 gene knockout did not affect the kinetics of cutaneous tissue repair in mice. Nevertheless, in a more recent study by Henshaw et al. [4] the topical administration of CTGF reportedly improved wound healing in an experimental animal model of diabetes, thus, further exploration would be needed. Taken together, these results suggest that the modulation of CTGF expression represents a future therapeutic target for diabetic complications as well as for fibrotic process, including wound repair [20].

4. CONCLUSION

Our results demonstrate that fructose affects the expression of type I collagen and CTGF in fibroblasts *in vitro*. These findings may have implications in future investigation regarding the role of fructose in fibroblast functions during wound healing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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