Original Article

Regulation of Metal Metalloproteinase 9 Expression and Activity

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Our previous study demonstrates that matrix metalloproteinase 2 (MMP-2) is differentially regulated by the interplay between glucose and insulin. In this context, it was intriguing to examine the regulation of the other well-studied MMPs, MMP-9, in 3T3-L1 pre-adipocytes. Our results revealed that, as the scenario in MMP-2 regulation, glucose was a pre-requisite for determining pre-adipocytes' responses to external stimuli. Short-term and long-term insulin exposure exhibited opposite regulation to MMP-9 under high glucose-containing environment. The dominant MMP-9 regulatory role of long-term insulin under euglycemia was diminished when culture environment contained high glucose concentration. Our results suggest pre-adipocytes may participate in the process of increasing adiposity through mediating extracellular matrix alterations resulted from the changes of MMP-9 expression/activity.

Key words: insulin, glucose, obesity, pre-adipocytes, adipocytes, matrix metalloproteinase-9

INTRODUCTION

Obesity is a global health threat with and a risk factor leading to metabolic abnormalities such as type 2 diabetes mellitus (T2DM) [1]. In addition to the abundant adipocytes, adipose tissue contains other cells including pre-adipocytes, stromal-vascular cells, adipose-infiltrated macrophages, etc. Adipogenesis, defined as the differentiation of pre-adipocytes to mature adipocytes, requires dramatic and coordinated alterations in gene expression, cellular morphology and functions, etc. [2,3]. Extracellular matrix (ECM) must function to the renovation process for morphological changes of adipocyte differentiation, and the enlargement of adipose tissue as well. Therefore, ECM components are crucial for the growth of the fat mass during the progression of increasing obesity [4,5].

Matrix metalloproteinases (MMPs) are a family of enzymes responsible for connective tissue remodeling by degrading basement membrane and surrounding ECM components to meet the dynamic cellular needs. The proteolytic activities of adipocyte-derived MMP-9 are induced during adipogenesis [6], indicating MMP-9 is important in adipocyte differentiation. MMP-9 is strongly induced in the enlarged adipose tissue [7.8], suggesting MMPs could increase matrix plasticity and thereby facilitate adipose tissue remodeling and hypertrophy.

Enhanced production of collagen is a key event in the development of diabetic glomerular ECM abnormalities. ECM proteins are accumulated within the renal interstitium in progressive tubular atrophy, and MMPs are suggested to participate in the process of concurrent reduced degradation and the enlargement of mesangial area during the pathogenesis of diabetic nephropathy [9-11]. Therefore, the MMP-mediated ECM alterations are required both for the processes of adipose tissue expansion and the development of diabetic related complications.

Accumulating evidence had proved the role of MMPs in adipogenesis and diabetic complications. Nevertheless, regulation of MMPs in pre-adipocytes, the precursors which differentiate into mature adipocytes during fat mass increment, are scarce. Particularly, the

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responses of pre-adipocytes to the major mediator maintaining energy homeostasis are overlooked. We recently reported that glucose is a pre-requisite for determining pre-adipocytes' responses to external stimuli [12]. In particular, MMP-2 is differentially regulated by the interplay between glucose and insulin. Accordingly, it was reasonable to hypothesize that IL-4 and/or other metabolism-regulatory mediators also participate in the regulation of the other well-studied MMPs, MMP-9, in 3T3-L1 pre-adipocytes. Therefore, the specific aim of this study was to investigate and characterize putative regulation of pre-adipocyte MMP-9 by the crosstalk of various stimuli in the environment containing differential glucose concentrations.

METHODS

Cell culture and adipogenesis

3T3-L1 pre-adipocytic fibroblasts were maintained in DMEM supplemented with 10% calf serum. For adipogenesis [12-15], confluent cells were induced to differentiate by adding 0.5 mM 3-isobutyl-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin in 10% FBS for 2 days. Cells were fully differentiated into adipocytes in fresh DMEM with 5 μg/ml insulin for 6 more days. Low- (LG) and high-(HG) glucose environment was defined as the medium supplemented with 1 g/L and 4.5 g/L glucose respectively. For insulin and IL-4 treatment, cells were exposed to either 1 nM (CI) or 100 nM (AI) insulin with 100 nM IL-4 during the final 18 hr and 30 min of the 48 hr incubation in LG or HG.

RNA extraction and RT-PCR

Total RNA was isolated using TRIZOL reagent, followed by being reversely transcribed into cDNA. The first-strand cDNA was amplified using specific PCR primer sets (MMP-9: 5'-TGTACCGCTATGGTTACAC-3' and 5'-CCGCGACACCAAACTGGAT-3'; GAPDH: 5'-TATGACAACTCCCTCAAGAT-3' and 5'-AGATCCACAACGGATACATT-3'). All RT-PCR reactions were carried out with BIO-RAD PCR iCYCLER instrument, and the amplified products were identified by gel electrophoresis.

Analysis of MMP-9 activities

MMP-9 activities were detected by gelatin zymography as described [12, 16]. Briefly, cell lysates were loaded into each well on 10% gelatin zymography gels. The gels were rinsed twice in 2.5% Triton X-100 after electrophoresis, followed by overnight incubation in substrate buffer containing 50 mM Tris-HCl and 5 mM $CaCl_2$. The activated gels were stained by Coomassie blue R-250.

Results AND DISCUSSION

MMP-9 is involved in adipogenesis and diabetic complications [9-11]. Antibodies against MMP-9 and MMP inhibitor inhibit adipocytes differentiation and adipose tissue development [6, 17]. MMPs in arterial vasculature from diabetic subjects are suppressed [18, 19]. Nevertheless, Garvey et al. reported that ventricle MMP-9 activity is increased in mice with diabetes [20]. In human subjects, elevated serum MMP-9 is increased in diabetic patients complicated with coronary artery disease, and linked to atherosclerosis [21]. Our previous study also revealed that MMP-9 expression and activities are up-regulated in T1DM patients [16]. The above reports indicate that MMP-9 participates in the process of increasing adiposity and diabetic complications. Nevertheless, reports focusing on the regulation of MMPs in pre-adipocytes are scarce. In this context, this study aimed at examining regulation of MMP-9 expression and activities by IL-4 and/or insulin in pre-adipocytes under different glucose-containing environments.

Regulation of MMP-9 mRNA by IL-4 in pre-adipocytes

Cells were cultured in HG media, then MMP-9 mRNA was analyzed after IL-4 and/or insulin treatment (Fig. 1). MMP-9 mRNA was not significantly altered by IL-4 (lanes 1-2), while insulin slightly up-regulated MMP-9 levels (lanes 3-4). It was intriguing to investigate if physiological LG would regulate MMP-9 expression in response to IL-4 and/or insulin. Cells were first maintained in HG, and MMP-9 mRNA was analyzed after 48 hrs of LG exposure with either IL-4 or insulin treatment at the final 30 min. The results showed that MMP-9 remained consistent under IL-4 and/or insulin treatment (lanes 7-10).

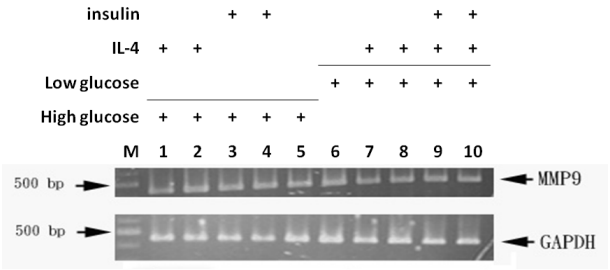

Fig.1 Regulation of MMP-9 mRNA in pre-adipocytes Lanes 1~6: Cells were maintained in HG medium, then MMP-9 mRNA was examined by RT-PCR after the cells were exposed to either IL-4 or insulin. Lanes 7~10: Cells were first maintained in HG media, and MMP-9 mRNA was examined after the cells were shifted to LG for 48 hrs with exposure to either IL-4 or insulin. IL-4: 100 nM IL-4 treatment for 30 min; insulin: 100 nM insulin treatment for 30 min

Regulation of MMP-9 mRNA by insulin in pre-adipocytes

Whether pre-adipocytes responded differentially to different insulin concentrations was next analyzed. The effects of combined IL-4/insulin treatment on MMP-9 under the environment mimicking diabetic hyperglycemia with insulin treatment were also investigated (Fig. 2).

Pre-adipocytes were first cultured in HG-containing media, then MMP-9 mRNA was analyzed after the cells received concomitant IL-4 stimulus with either short-term (100 nM for 30 min, AI) or long-term (1 nM for 18 hr, CI) insulin treatment. Notably, CI had a dominant role in regulating MMP-9 (lane 4) but AI did not cause prominent alteration to IL-4-induced MMP-9 expression (lanes 5-6).

Regulation of MMP-9 actvities by IL-4/insulin in pre-adipocytes

Cells were cultured in HG supplemented with IL-4 and/or insulin, then MMP-9 activities were analyzed by gelatin zymography (Fig. 3). Either IL-4 or AI treatment alone regulated MMP-9 activities (lanes 1&3). Notably, MMP-9 activities were significantly promoted by CI treatment (lane 2). CI-induced MMP-9 activities remained unchanged (lane 4) while CI partially neutralized IL-4-induced MMP-9 mRNA expression (Fig. 2, lane 4), suggesting relative stronger regulatory activity of CI on MMP-9 than IL-4.

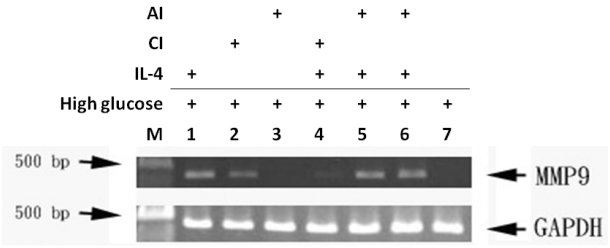

Fig.2 Regulation of MMP-9 mRNA in pre-adipocytes under high glucose environment. Cells were maintained in HG, then MMP-9 mRNA was analyzed by RT-PCR after the cells were exposed to either AI or CI with IL-4

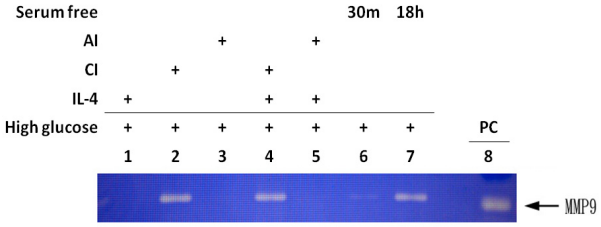

Fig.3 Regulation of MMP-9 activities in pre-adipocytes Cells were maintained in HG, then MMP-9 activities were analyzed by gelatin zymography in the presence of IL-4 and/or insulin. Lanes 6 & 7: MMP-9 activities after serum-free condition for 2 hr and 18 hr, respectively. Lane 8: positive control for MMP-9

The possible regulation of IL-4 and/or insulin to MMP-9 activities in different glucose-containing environments was subsequently examined. Cells were maintained in LG, and MMP-9 activities were examined after being treated with either AI or CI and IL-4 in LG or by shifting to HG for further 48 hrs (Fig. 4). MMP-9 activities remained changed in HG (lane 2) but were decreased in CI (lane 3). AI did not exhibit regulatory activities to MMP-9 in LG (lane 4). Nevertheless, regulatory capacity of CI to MMP-9 activities in LG was attenuated by HG (lane 5), indicating glucose is a dominant environmental factor mediating MMP-9 activities.

The above observations reveal that CI enhances MMP-9 activities in HG but the regulatory pattern of CI to MMP-9 mRNA is influenced by environmental glucose. The suppressed activity of CI to MMP-9 activities in LG is attenuated by hyperglycemia. These results demonstrate that glucose is the dominant factor determining cellular responses to environmental stimuli.

Taken the above results together, CI significantly down-regulates MMP activities (Fig. 4) while AI does

not exhibit regulatory effects (Fig. 3) under eulgycemic condition. On the contrary, CI significantly enhances MMP-9 activities and IL-4 slightly promotes MMP-9 mRNA expression in hyperglycemia (Fig. 2). Therefore, chronic insulin exposure shows differential regulation to MMP-9 activity according to the environmental glucose concentrations.

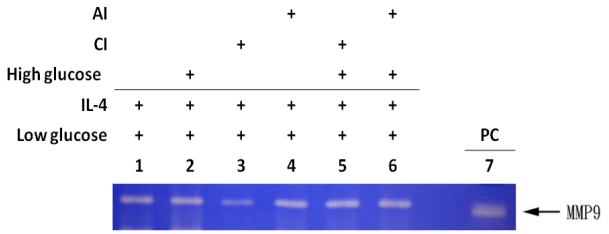

Fig.4 Regulation of MMP-9 activity in pre-adipocytes under different glucose environment
Cells were maintained in LG, and MMP-9 activities were analyzed by gelatin zymography after cells were exposed to AI or CI either in LG or by shifting to HG for further 48 hrs with IL-4 exposure. Lane 7: positive control

Regulation of MMP-9 mRNA by IL-4/insulin in mature adipocytes

It was intriguing to compare the regulatory pattern of IL-4 and/or insulin to MMP-9 between undifferentiated pre-adipocytes and mature adipocytes (Fig. 5). After fully differentiated into mature adipocytes, cells were treated by the conditions as above mentioned. The results showed that MMP-9 was slightly up-regulated by IL-4 (lane 1). CI positively regulated MMP-9 (lane 2), but AI only moderately mediate MMP-9 in HG (lane 3). Both CI and AI did not significantly influence IL-4-induced MMP-9 (lane 4&5). The above data indicate that effects of IL-4 and AI on MMP-9 in pre-adipocytes and mature adipocytes are very similar. In addition, as the scenario in pre-adipocytes, CI exhibits opposite regulatory activities to MMP-9 according to the environmental glucose levels.

Taking the above results and our previous report [12] together, pre-adipocytes and adipocytes show similar scenario regarding MMPs regulatory pattern in response to external stimuli. Notably, glucose take the dominant role in determining cellular responses. As MMPs expression abnormality is identified in the lesion of diabetic complications with thickening ECM as one of the pathological characteristics, mature adipocytes and pre-adipocytes in adipose tissue are likely to participate in ECM alterations both in pre-diabetic status and the development of diabetic complications. The present study uncovers observations regarding pre-adipocytes behaviors and their possible roles in the process of increased adiposity and diabetic complications which have never been examined. We hope this information provides new insights for better understating of metabolic and diabetic pathogenesis and pathophysiology.

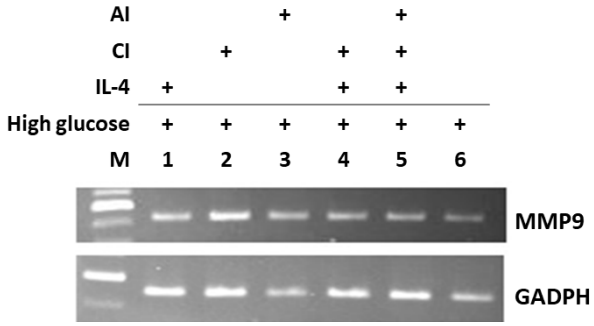

Fig.5 Regulation of MMP-9 activity in adipocytes 3T3-L1 pre-adipocytes were allowed to differentiate into mature adipocytes as described in Methods, then MMP-9 mRNA in the adipocytes exposed to IL-4, AI, CI or combined treatment in HG, respectively, was examined. IL-4: 100 nM IL-4 treatment for 30 min, AI: 100 nM insulin treatment for 30 min

Conflicts of Interests

There is no duality of interest associated with this manuscript.

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