Abstract. MicroRNA-21 (miR-21) has been found to promote cell proliferation and survival. It has also been shown to exhibit an increased expression in a number of forms of cardiovascular disease. However, the mechanisms underlying the involvement of miR-21 in atherosclerosis remain to be elucidated. In the present study, it was demonstrated that miR-21 was upregulated in a time-dependent manner in response to high-concentration glucose stimulation in Raw 264.7 macrophages. High concentrations of glucose induce macrophage apoptosis. miR-21-inhibited macrophages treated with a normal concentration of glucose exhibited increased levels of cell apoptosis and augmented levels of activated caspase-3, while cells treated with an miR-21 inhibitor and a high concentration of glucose, revealed significantly increased levels of apoptosis. In addition, inhibition of miR-21 increased mRNA and protein levels of programmed cell death 4 (PDCD4), which, by contrast, were reduced in miR-21-inhibited cells that had been treated with a high concentration of glucose. In conclusion, miR-21 is sensitive to high-concentration glucose treatment in macrophages, and appears to have a protective effect in macrophage apoptosis induced by high concentrations of glucose via PDCD4.
overexpression of miR-21, decreased neointima formation. The expression of miR-21 has been found to be significantly upregulated in atherosclerotic arteries (22). However, the mechanisms underlying miR-21 involvement in atherosclerosis remain to be elucidated. miR-21 has been observed to target and downregulate programmed cell death 4 (PDCD4) (23) and to promote cell proliferation. In addition, overexpression of miR-21 represses normal apoptotic signaling, and inhibition of miR-21 increases cell apoptosis (23,24). Therefore, cell apoptosis regulated by miR-21 may be a potential mechanism contributing to the development of atherosclerosis and miR-21 may be involved in macrophage apoptosis.

It was hypothesized that miR-21 is able to resist macrophage apoptosis induced by high glucose concentrations via PDCD4. In the present study, in order to investigate the effect of miR-21 on macrophage apoptosis, Raw 264.7 macrophages were stimulated with high glucose concentrations and transfected with an anti-miR-21 inhibitor. The levels of miR-21 expression, PDCD4 expression and cell apoptosis were subsequently measured.

Materials and methods

Cell culture and treatment. The Raw 264.7 murine monocytic cell line was routinely maintained in Dulbecco’s modified Eagle’s medium (5.5 mM D-glucose) supplemented with 10% fetal bovine serum and cultured at 37˚C in a humidified atmosphere containing 5% CO₂. Cells were passaged every 2-3 days in order to maintain exponential growth. Treatment of Raw 264.7 macrophages with 5.5 mM glucose (normal glucose, NG), 25 mM glucose (high glucose, HG) or 25 mM mannose (osmotic control, OC) were performed in serum-free media.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of miR-21 and PDCD4 mRNA expression. Total RNA was extracted from transfected cells using the mirVana miRNA isolation kit (Ambion, Inc., Austin, TX, USA) or TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). For analysis of the expression of miR-21, 10 ng total RNA was reverse transcribed into cDNA using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA). qPCR was performed using a standard TaqMan PCR protocol according to manufacturer’s instructions (Applied Biosystems). The relative miR-21 expression level was then calculated using the 2^ΔΔCT method (25) and normalized to the expression of U6. Relative levels of PDCD4 mRNA were examined using SYBR green quantitative reverse transcription-PCR (Applied Biosystems) and normalized to β-actin. The specific primer sequences were as follows: Forward: 5'-GGTGATGTGAAAGATCCAAA-3' and reverse: 5'-CATCCAGGGGCAAACATCA-3' for PDCD4 and forward: 5'-CAACCTTGATGATGAAGGCTTTGGT-3' and reverse: 5'-ACTTTTATTTGGCTCAAGTCAGAGC-3' for β-actin.

Results

miR-21 expression is upregulated by high levels of glucose. In order to examine whether the expression of miR-21 in macrophages was regulated by high concentrations of glucose, Raw 264.7 macrophages were treated with 5.5 mM glucose (NG), 25 mM glucose (HG) or 25 mM mannose (OC) for various time periods (0, 1, 3, 6, 12 and 24 h). TaqMan RT-qPCR demonstrated that the expression of miR-21 mRNA was significantly upregulated by HG, whereas it was reduced by OC (Fig. 1). Compared with the NG group, a non-significant difference was indicated to a statistically significant difference.

miR-21 regulates macrophage apoptosis induced by high levels of glucose. As cell apoptosis has previously been observed to increase with increased miR-21 expression (24), the complex role of miR-21 on high-glucose-induced macrophage apoptosis was investigated. Cells were transfected with
an anti-miR-21 inhibitor or a negative control, and incubated for an additional 48 h with NG or HG. Apoptosis of cells was detected by flow cytometry. miR-21 mRNA expression was significantly decreased following anti-miR-21 inhibitor transfection (Fig. 2). The results of the flow cytometric analysis demonstrated that apoptosis of macrophages transfected with the anti-miR-21 inhibitor was significantly higher than in those transfected with negative control and incubated with NG (Fig. 3A). In addition, miR-21-inhibited cells treated with HG exhibited a significant increase in apoptosis compared with HG-treated negative control cells, or cells treated with miR-21 inhibition alone (the NG group). The results indicated that miR-21 had a protective effect against high-concentration glucose-induced macrophage apoptosis.

Western blot analysis (Fig. 3B) indicated an increased level of activated caspase-3 protein in miR-21-inhibited cells compared with that in control cells, an increased level with HG-treated miR-21-inhibited cells compared with that in HG-treated controls, and an increased level with the miR-21 inhibitor alone (NG group) compared with that in cells treated with negative controls.

**PDCD4 expression in macrophages incubated with high levels of glucose.** Raw 264.7 macrophages were treated with NG, HG or OC for various time periods and the expression of PDCD4 mRNA was then determined. As shown in Fig. 4A, macrophages treated with HG revealed a markedly reduced expression of PDCD4 mRNA at 12 h, which was increased from 24 h compared with NG control cells. However, PDCD4 mRNA expression was significantly increased after 6 h in the OC group. PDCD4 protein level was also appeared to be reduced following HG treatment for 12 h (Fig. 4B).

**Inhibition of miR-21 promotes PDCD4 expression.** A previous study demonstrated that PDCD4 is a potential miR-21 target in various cell types (22). In order to verify PDCD4 as a target gene of miR-21 in high-glucose-incubated macrophages, the anti-miR-21 inhibitor was transfected into macrophages to inhibit miR-21 expression. Following NG treatment, the mRNA and protein expression of PDCD4 in macrophages transfected with anti-miR-21 inhibitor was higher than in those transfected with negative control (Fig. 5), which indicated that in macrophages, miR-21 inhibition promotes PDCD4 expression and that PDCD4 is the target gene of miR-21. Following HG treatment, PDCD4 expression in miR-21-inhibited macrophages remained higher than in cells transfected with negative control, but lower than that in miR-21-inhibited cells followed by treatment with NG, which indicates that high concentrations of glucose may reduce PDCD4 expression of macrophages via an miR-21 pathway.

**Discussion**

In the present study, it was demonstrated that miR-21 was sensitive to high glucose treatment in Raw 264.7 macrophages. The expression of miR-21 was significantly upregulated at 6 h, and a non-significant time-dependent increase was observed at the other time points in response to high glucose stimulation. In addition, miR-21 is able to resist macrophage apoptosis induced by high glucose via its action on the target gene, PDCD4.

miR-21, located on chromosome 17q23.2, exhibits a clear evolutionary conservation across a number of species and a markedly conserved role in gene regulation (26). miR-21 is reported to be universally expressed in mammalian organ systems, including the heart, spleen, colon and small intestine (27), whereas aberrant expression is observed in...
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Atherosclerosis, cardiac hypertrophy, heart failure and acute myocardial infarction (19). miR-21 expression has been revealed to be augmented by multiple stimuli, including lipopolysaccharides, shear stress, hydrogen peroxide, transforming growth factor-β, angiotensin II and ischemia/reperfusion injury (20,28-31). However, few studies

Figure 3. Effect of miR-21 inhibition on macrophage apoptosis. Raw 264.7 macrophages were transfected with an anti-miR-21 inhibitor or negative control for 24 h prior to incubation for an additional 48 h with NG (5.5 mM glucose) or HG (25 mM glucose). (A) Flow cytometry analysis of cell apoptosis by propidium iodide-Annexin V staining. (B) Western blot analysis of expression of activated caspase-3 protein. mir-21, microRNA-21; NG, normal glucose; HG, high glucose.

Figure 4. PDCD4 expression in macrophages incubated with high glucose. Raw 264.7 macrophages were treated with NG (5.5 mM glucose), HG (25 mM glucose) or OC (25 mM mannose) for 0, 6, 12, 24 and 48 h. (A) Reverse transcription-quantitative polymerase chain reaction analysis of PDCD4 mRNA expression in Raw 264.7 macrophages. *P<0.05, **P<0.01, compared with NG cells. (B) Western blot analysis of expression of PDCD4 protein in Raw 264.7 macrophages. NG, normal glucose; HG, high glucose; OC, osmotic control; PDCD4, programmed cell death 4.
Macrophages were transfected with an anti-miR-21 inhibitor to inhibit the expression of miR-21 in the present study. It was shown that inhibition of miR-21 in macrophages increased cell apoptosis and augmented activated caspase-3 expression, which suggests a role in increased macrophage apoptosis. Apoptosis was further increased in miR-21-inhibited macrophages treated with high glucose. These results suggest that miR-21 has an antiapoptotic effect on macrophage apoptosis induced by high concentrations of glucose, possibly via the caspase-3 pathway. Although high concentrations of glucose may induce macrophage apoptosis through multiple signaling pathways, miR-21 may be a key regulator of macrophage apoptosis.

PDCD4 has been identified as a gene that is upregulated during apoptosis (37). Recently, PDCD4 was found to be a principal target of miR-21 in numerous types of cells, including colon cancer cells, human glioblastoma cells, cardiomyocytes, HUVECs and human peripheral blood mononuclear cells (19,38-40). It remains to be elucidated whether PDCD4 is also a target gene of miR-21 in Raw 264.7 macrophages incubated with high glucose. In order to confirm this, Raw 264.7 macrophages were treated with a high concentration of glucose for various time periods, demonstrating that mRNA and protein levels of PDCD4 were downregulated by a high concentration of glucose, which suggested PDCD4 is a potential target of miR-21.

To further clarify the interaction between miR-21 and PDCD4 in high-concentration glucose-treated Raw 264.7 macrophages, miR-21 expression was modulated via the use of an anti-miR-21 inhibitor and the change in PDCD4 expression was observed. It was identified that PDCD4 expression was significantly increased following inhibition of miR-21 expression, which demonstrated that miR-21 suppresses PDCD4 expression. In addition, treatment of macrophages with an miR-21 inhibitor and a high concentration of glucose, markedly increased PDCD4 expression compared with that in cells treated with an miR-21 inhibitor and normal glucose. Previous studies have demonstrated that miR-21 binds to the 3′-untranslated region (UTR) of the PDCD4 gene at nucleotides 228-249 (41). Based upon this finding, it is hypothesized that PDCD4 is directly suppressed by miR-21 in macrophages and is involved in macrophage apoptosis induced by high concentrations of glucose. In the early stages of macrophage apoptosis, a high level of glucose primarily induces the expression of miR-21, which suppresses PDCD4 expression by binding to the 3′-UTR of PDCD4. In addition, the induction of miR-21 resists high-concentration glucose-independent macrophage apoptosis. However, in the late stages of apoptosis, high glucose levels cause a high level of cell apoptosis, accompanied by markedly increased PDCD4 expression, which suggests that upregulated PDCD4 may regulate miR-21 expression induced by high concentrations of glucose in a negative feedback manner.

Macrophage apoptosis has been observed to promote vulnerable atherosclerotic plaque progression. Since it has been identified that miR-21 is implicated in reducing macrophage apoptosis that is induced by high concentrations of glucose, and that miR-21 may inhibit high-concentration glucose-induced apoptosis, miR-21 may have a protective role in the development of vulnerable atherosclerotic plaques.
However, further studies are required in order to elucidate the underlying mechanisms. The preliminary findings from the present study suggest that miR-21 may be a novel therapeutic target in atherosclerosis.

In conclusion, a high concentration of glucose induces the expression of miR-21 in Raw 264.7 macrophages. miR-21 appears to have a protective effect against macrophage apoptosis induced by high concentrations of glucose via PDCD4. An investigation of the association between mannose and gene-mediated apoptosis may be beneficial in the future in order to expand this field of knowledge.

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