Protective effects of leukemia inhibitory factor against oxidative stress during high glucose-induced apoptosis in podocytes

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Abstract Leukemia inhibitory factor (LIF) is a pleiotropic glycoprotein belonging to the interleukin-6 family of cytokines. In kidney, LIF regulates nephrogenesis, involves in tubular regeneration, responds to pro- and anti-inflammatory stimuli, and so on. LIF also plays an essential role in protective mechanisms triggered by preconditioning-induced oxidative stress. Although LIF shows a wide range of biologic activities, effects of LIF on high glucose-induced oxidative stress in podocytes remain unclear. The aim of the study was to assess whether LIF can attenuate high glucose-induced apoptosis in podocytes. The result of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay indicated that LIF protected podocytes against high glucose-induced cytotoxicity. The flow cytometry assay showed that LIF attenuated high glucose-induced apoptosis in podocytes. Meanwhile, the result of flow cytometric assay gave the clear indication that LIF decreased high glucose-induced elevated level of reactive oxygen species (ROS). The measurement of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), malondialdehyde (MDA), and caspase-3 activity levels showed that LIF attenuated the high glucose-induced decreased level of SOD and elevated level of NADPH oxidase, MDA and caspase-3 activity. These results may provide potential therapy for diabetic nephropathy in the future.

Keywords Leukemia inhibitory factor · Podocytes · Apoptosis · ROS · SOD · MDA

Introduction

Diabetic nephropathy clinically manifests as progressively worsening albuminuria with a declining glomerular filtration rate (Mogensen 2003). The podocyte is a highly differentiated cell type that synthesizes components of the glomerular basement membrane, such as IV collagen (Heidet et al. 2000), and forms an interdigitating network of foot process, which are spanned by slit diaphragms. In diabetic nephropathy, the podocyte number is markedly reduced, the foot process width is significantly widened, and the slit diaphragm becomes narrower as the glomerular filtration rate declines (Bjorn et al. 1995; Steffes et al. 2001; Nakamura et al. 2000). Therefore, finding effective therapy of protecting podocytes from injury may be helpful for treating diabetic nephropathy.

Leukemia inhibitory factor (LIF) conforms to the gp130 signalling of interleukin-6 (IL-6) family cytokines. It is now almost 15 years since the purification and cloning of LIF and continues to be studied actively by disparate groups of biologists. LIF was shown to inhibit differentiation of myoblasts (Jo et al. 2005). Hunt et al. indicated that LIF also inhibited caspase-3 activation and DNA fragmentation of myoblasts caused by induction of apoptosis with staurosporine (Hunt et al. 2010). In addition, LIF suppressed doxorubicin-induced apoptosis by blocking the activation of caspase-3 and preventing the decrease in levels of Bcl-xL (Negoro et al. 2001). In kidney, LIF and transforming growth factor β 2 (TGF β 2)/fibroblast growth factor 2 (FGF2) cooperate to regulate nephrogenesis (Plisov et al. 2001). In addition, LIF promotes tubular regeneration after acute renal failure. Although LIF has been studied actively in various organs including kidney and shows its anti-apoptotic and regulating functions on various cells, there is little information about the effects of LIF on podocyte apoptosis in diabetic nephropathy. As far as we know,

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this is the first study that assesses the protective effects of LIF on podocytes *in vitro*.

There are various factors involved in the pathogenesis of diabetic nephropathy (Blazquez-Medela et al. 2010). One of them is the imbalance of pro- and anti-free radical processes and the formation of excessive free radicals in the kidney (Forbes et al. 2008). Several investigations have shown that in the presence of high glucose, overproduction of reactive oxygen species (ROS) in podocytes induces dysfunction and increases excretion of albumin with urine (Neale et al. 1993; Susztak et al. 2006; Ren et al. 2008). Renal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important source of oxidative stress, and its expression is enhanced in diabetic nephropathy. Tojo et al. showed that suppressing renal NADPH oxidase reduced renal ROS, proteinuria, and glomerulosclerosis (Tojo et al. 2007). Therefore, more and more recent studies focus on new drugs targeting to anti-oxidative stress during diabetic nephropathy (Eid et al. 2009; Jiao et al. 2011; Mao et al. 2011). LIF plays an essential role in neuroprotective mechanisms triggered by preconditioning-induced oxidative stress (Chollangi et al. 2009). However, little is known about the effects of LIF on oxidative stress in diabetic nephropathy. Therefore, we set out to examine the functions of LIF as well as to investigate whether LIF has the ability to protect podocytes from oxidative stress induced by high glucose.

With the recent establishment of a mouse podocyte cell line that is conditionally immortalized, the study of fully differentiated podocytes in culture has become feasible (Mundel et al. 1997). The purpose of this study was to assess whether LIF can attenuate high glucose-induced apoptosis in podocytes, thereby providing an interesting view of the potential application of LIF in future therapy for diabetic nephropathy.

Materials and methods

Materials

RPMI 1640 culture medium was purchased from GIBCO Invitrogen. The fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and LIF were purchased from Sigma Chemical Co., St Louis, MO, USA. Reactive oxygen species (ROS) testing kit was purchased from Genmed Scientifics Inc., USA. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was from Bipece Biopharma Corporation, USA. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY, USA). Superoxide dismutase (SOD) assay kit and malondialdehyde (MDA) assay kit were purchased from the Nanking Jiancheng Bio-engineering Research Institute (Nanking, China). NADPH

oxidase ELISA kit and caspase-3 ELISA kit were purchased from R&D systems (USA).

Cell culture

Mouse podocytes from a conditionally immortalized cell line were cultured, as described previously (Mundel et al. 1997). Podocytes were cultured with the RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of 5% CO₂. To propagate podocytes, the culture medium was supplemented with 10 U/ml mouse recombinant-interferon (IFN) and cells were cultivated at 33°C to enhance the expression of the temperature-sensitive large T antigen (permissive conditions). To induce differentiation, podocytes were maintained at 37°C without IFN (non-permissive conditions) for 2 weeks.

For the different experiments, podocytes were cultured in normal glucose (5.6 mM) and high glucose (30 mM). Different concentrations of LIF (1 and 5 ng/ml) were prepared using RPMI 1640 medium.

Cell viability assay

The cells (1×10^4 /ml) were cultured with normal glucose (5.6 mM) and high glucose (30 mM) for 48 h. When the effects of LIF on podocytes were studied, various concentrations of LIF (1 and 5 ng/ml) were added for 1 h followed by the high glucose for 48 h. At the end of the exposure, 20 µl MTT was added to each well and further incubated for 4 h at 37°C. The medium was then removed carefully and 150 µl DMSO was added in and mixed with the cells thoroughly until formazan crystals were dissolved completely. This mixture was measured in an ELISA reader (Elx 800, Bio-TEK, USA) with a wavelength of 570 nm. The results were expressed as percentage of the control value from the normal cells without treatments of LIF and high glucose. Meanwhile, the concentrations of LIF used in assays of apoptosis, ROS, SOD, and MDA were based on the results of the MTT test.

Detection of apoptotic cells with flow cytometry

Apoptosis was assessed by annexin V-FITC and PI staining followed by the analysis with flow cytometry (Beckman-Coulter, USA). The methodology followed the procedure as described in the annexin V-FITC/PI detection kit. Cells were cultured with different concentrations of LIF (1 and 5 ng/ml) for 1 h before exposure to high glucose (30 mM) for 48 h. Eventually, the cells were re-suspended in a 400 µl 1×10^6 cells/ml, and the cells were stained with 5 µl annexin V-FITC and 5 µl PI for 15 min at room temperature in the dark. Then the cell suspension was ready for the analysis by the flow cytometry.

Measurement of ROS

The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA). The cells were incubated in a 6-well plate for 24 h for stabilization. Then different concentrations of LIF (1 and 5 ng/ml) were added for 1 h prior to the high glucose treatment. After exposure for 2 h, cells were washed with phosphate-buffered saline, and then they were re-suspended at a concentration of 1×10^6 cells/ml and were stained by the staining solution for 20 min. The cells were detected and analyzed by flow cytometry.

NADPH oxidase ELISA assay

The generation of NADPH oxidase for cells was evaluated by the ELISA kit. The cells were incubated in a 6-well plate for 24 h for stabilization. Then different concentrations of LIF (1 and 5 ng/ml) were added for 1 h prior to the high glucose treatment. After exposure for 2 h, the level of NADPH oxidase was measured using ELISA kit according to the manufacturer's instructions. The NADPH oxidase level in control group was defined as 100% while the NADPH oxidase level in the rest groups was expressed as a percentage of the NADPH oxidase level of control group.

Total SOD assay

The generation of total SOD (T-SOD) for the cells was evaluated by the assay kit. The podocytes were incubated in a 6-well plate for 24 h for stabilization. Then different concentrations of LIF (1 and 5 ng/ml) were added for 1 h prior to the high glucose treatment. After exposure for 48 h, the level of T-SOD was measured using a UV-visible spectrophotometer (V-530UV/UISNIR Spectrophotometer, Jasco, Japan) at 550 nm according to the manufacturer's instructions. The percentage of T-SOD activity was obtained as follows:

% T – SOD activity

$$= (\text{the activity of treated group} / \text{the control group}) \times 100$$

MDA assay

The MDA level in podocytes was evaluated by MDA assay kit. The podocytes were incubated in a 6-well plate for 24 h for stabilization. Then different concentrations of LIF (1 and 5 ng/ml) were added for 1 h prior to the high glucose treatment. After exposure for 48 h, the level of MDA was measured according to the manufacturer's instructions. The MDA level in control group was defined as 100% while the MDA level in the rest groups was expressed as a percentage of the MDA level of the control group.

Measurement of caspase-3 activity

The activity of caspase-3 was determined with a caspase-3 ELISA kit. Cells were incubated in a 6-well plate for 24 h for stabilization. Then different concentrations of LIF (1 and 5 ng/ml) were added for 1 h prior to the high glucose treatment. After exposure for 48 h, the level of caspase-3 activity was measured using ELISA kit according to the manufacturer's instructions. The caspase-3 level in control group was defined as 100% while the caspase-3 level in the rest groups was expressed as a percentage of the caspase-3 level of the control group.

Statistical analysis

The results were expressed as mean $\pm$ SEM. The statistical significance was assessed by one-way analysis of variance and Tukey's multiple comparison post-test using the SPSS (11.5) software. The significant difference was taken as $P < 0.05$.

Results

LIF protected podocytes against high glucose-induced cytotoxicity

To determine effects of LIF on high glucose-induced cytotoxicity, podocytes were pretreated with different concentrations of LIF (1 and 5 ng/ml) for 1 h, and then high glucose (30 mM) was added for an additional 48 h incubation. The viability of cells was determined by MTT assay. As shown in Fig. 1, high

Fig. 1 Effects of LIF on high glucose-induced injury of podocytes. The podocytes were pretreated with different concentrations of LIF (1 and 5 ng/ml) for 1 h, and high glucose (30 mM) was added for an additional 48 h incubation. Results represent the means of three separate experiments, and error bars represent the standard error of the mean. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with high glucose group without LIF pretreatment

glucose decreased the cell viability ($P<0.05$) while LIF (1 and 5 ng/ml) alone did not cause the cell death. Pretreatment with LIF (1 and 5 ng/ml) significantly enhanced the cell viability compared with that of high glucose group ($P<0.05$).

LIF attenuated the high glucose-induced podocyte apoptosis

The apoptosis of podocytes was tested by flow cytometry. As shown in Fig. 2, the apoptotic rate of podocytes was increased

Fig. 2 Effects of LIF on high glucose-induced apoptosis in podocytes. Podocytes were pretreated with different concentrations of LIF (1 and 5 ng/ml) for 1 h before exposure to the high glucose (30 mM) for 48 h. The flow cytometry assay was carried out for the detection of apoptotic cells. **a** control, **b** 1 ng/ml LIF+normal glucose (5.6 mM), **c** 5 ng/ml LIF+normal glucose (5.6 mM), **d** high glucose (30 mM) without LIF

pretreatment, **e** 1 ng/ml LIF+high glucose, **f** 5 ng/ml LIF+high glucose, and **g** the corresponding linear diagram of flow cytometry. Data were presented as mean $\pm$ SEM of three independent experiments. * $P<0.05$ compared with the control group; # $P<0.05$ compared with high glucose group without LIF pretreatment

to $22.25 \pm 2.82\%$ from $4.73 \pm 1.97\%$ in control group after exposure to high glucose (30 mM) for 48 h ($P < 0.05$) while LIF (1 and 5 ng/ml) alone did not cause the cell apoptosis. Pretreatment with 1 and 5 ng/ml LIF reduced the cell apoptosis to $12.75 \pm 3.14\%$ and $11.78 \pm 2.16\%$, respectively. Results indicated that LIF attenuated the high glucose-induced apoptosis in podocytes.

LIF decreased high glucose-induced intracellular accumulation of ROS

The effects of LIF on the intracellular ROS level of podocytes were measured using the ROS test kit. As shown in

Fig. 3, when podocytes were exposed to the high glucose (30 mM) for 2 h, the intracellular ROS level increased obviously ($P < 0.01$). Preconditioning with 1 and 5 ng/ml LIF significantly reduced the ROS level from $31.35 \pm 3.46\%$ in high glucose group to $11.19 \pm 2.37\%$ and $14.92 \pm 3.85\%$ ($P < 0.01$).

LIF decreased the high glucose-induced intracellular accumulation of NADPH oxidase

The effects of LIF on the intracellular NADPH oxidase level of podocytes were measured using the ELISA kit. As shown in Fig. 4, when podocytes were exposed to the high glucose

Fig. 3 Effects of LIF on high glucose-induced intracellular accumulation of ROS. Podocytes were maintained in different concentrations of LIF (1 and 5 ng/ml) for 1 h, and then high glucose (30 mM) for an additional incubation of 2 h. **a** control, **b** high glucose (30 mM) without LIF pretreatment, **c** 1 ng/ml LIF+high glucose, **d** 5 ng/ml LIF+high

glucose, and **e** the corresponding linear diagram of flow cytometry. Data were presented as mean $\pm$ SEM of three independent experiments. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with high glucose group without LIF pretreatment

Fig. 4 Effects of LIF on high glucose-induced intracellular accumulation of NADPH oxidase. Podocytes were maintained in different concentrations of LIF (1 and 5 ng/ml) for 1 h, and then high glucose (30 mM) for an additional incubation of 2 h. Data were presented as mean±SEM of three independent experiments. ** $P<0.01$ compared with the control group; ## $P<0.01$ compared with high glucose group without LIF pretreatment

(30 mM) for 2 h, the intracellular NADPH oxidase level increased obviously ($P<0.01$). Preconditioning with 1 and 5 ng/ml LIF significantly reduced the NADPH oxidase level from $240.13\pm14.50\%$ in high glucose group to $123.34\pm15.28\%$ and $150.45\pm19.37\%$ ($P<0.01$).

LIF attenuated high glucose-induced the decrease of T-SOD in podocytes

As shown in Fig. 5, the cellular level of T-SOD was significantly decreased when cells were cultured in media containing high glucose (30 mM) for 48 h ($P<0.05$). When pretreatment with 1 and 5 ng/ml LIF, the T-SOD level increased from $86.35\pm1.25\%$ in high glucose group to $98.11\pm1.31\%$ and $95.72\pm0.97\%$, respectively ($P<0.05$).

LIF decreased high glucose-induced the elevated MDA level in podocytes

As shown in Fig. 6, the level of MDA was significantly increased when cells were exposed to high glucose (30 mM) for 48 h ($P<0.05$) while pretreatment with 1 and 5 ng/ml LIF decreased the MDA level from $163.26\pm9.03\%$ to $117.23\pm6.34\%$ and $121.85\pm4.68\%$ ($P<0.05$).

LIF attenuated high glucose-induced caspase-3 activation in podocytes

As shown in Fig. 7, high glucose treatment for 48 h induced an increase in caspase-3 activity compared with that of control cells ($P<0.01$) while pretreatment with 1 and 5 ng/ml LIF

Fig. 5 Effects of LIF on high glucose-induced the decrease of T-SOD level. Podocytes were pretreated with different concentrations of LIF (1 and 5 ng/ml) for 1 h before exposure to high glucose (30 mM) for 48 h. Data were presented as mean±SEM of three independent experiments. * $P<0.05$ compared with the control group; # $P<0.05$ compared with high glucose group without LIF pretreatment

decreased the level of caspase-3 activity from $206.36\pm21.83\%$ to $137.60\pm11.45\%$ and $138.67\pm17.19\%$ ($P<0.01$).

Discussion

Diabetic nephropathy is now the most common cause of end-stage renal disease worldwide (Schieppati and Remuzzi 2005). Emerging evidence suggests that type 1 and type 2 diabetes lead to changes in the structure of podocyte foot

Fig. 6 Effects of LIF on high glucose-induced the elevation of MDA level. The podocytes were pretreated with different concentrations of LIF (1 and 5 ng/ml) for 1 h, and high glucose (30 mM) was added for an additional 48 h incubation. Data were presented as mean±SEM of three independent experiments. * $P<0.05$ compared with the control group; # $P<0.05$ compared with high glucose group without LIF pretreatment

Fig. 7 Effects of LIF on high glucose-induced the elevation of caspase-3 activity. Podocytes were maintained in different concentrations of LIF (1 and 5 ng/ml) for 1 h, and then high glucose (30 mM) for an additional incubation of 48 h. Data were presented as mean $\pm$ SEM of three independent experiments. ** P <0.01 compared with the control group; ## P <0.01 compared with high glucose group without LIF pretreatment

processes, loss of podocytes, reduction in slit diaphragm proteins, and failure of the glomerular filtration barrier (Pagtalunan et al. 1997; Steffes et al. 2001; Doublier et al. 2003), which in turn triggers a cascade of events that can finally lead to the end-stage renal failure. Therefore, developing new drugs that inhibit podocyte apoptosis may be a good way to slow the development of diabetic nephropathy.

LIF is a pleiotropic cytokine belonging to the IL-6 family of cytokines (Gearing et al. 1987), which is expressed in multiple tissues and involved in many biological processes. In the highly inflammatory, oxidative and apoptotic milieu of regenerating and dystrophic muscle (Sandri and Carraro 1999; Tidball 2005), levels of LIF are up-regulated (Kurek et al. 1996). LIF may play a role as a trauma factor preventing myoblast cell death. There exists plenty of evidence to suggest that LIF promotes survival of various cell types (Cheema et al. 1994a; Cheema et al. 1994b; White et al. 2001; Wysoczynski et al. 2007; Negoro et al. 2001; Murphy et al. 1993; Oberle et al. 2006; Whitlon et al. 2006). Moreover, Sergei et al. also suggested that LIF and TGF β 2/FGF2 cooperate to regulate nephrogenesis through a common Wnt-dependent mechanism (Plisov et al. 2001). There are a large number of studies focused on the protective or regulating effects of LIF on kidney and other tissue or cell types, but to the best of our knowledge, little is known about the effects of LIF on podocyte apoptosis in diabetic nephropathy. Therefore, we examined the influence of LIF on high glucose-induced apoptosis in podocytes through oxidative stress in cultured mouse podocytes. In this study, the viability of podocytes incubated with high glucose and different concentrations of LIF was investigated. It was found that when

exposed to high glucose, the cell viability was decreased while LIF attenuated this influence. The viability increased from $78.32\pm 1.59\%$ in high glucose-treated group to $90.27\pm 2.01\%$ and $88.73\pm 2.36\%$ in LIF (1 and 5 ng/ml) pretreatment group. Induction of apoptosis may be account for the anti-proliferation effect. The detection of the cell apoptosis by flow cytometry showed that pretreatment with LIF inhibited high glucose-induced apoptosis in podocytes (Fig. 2).

The oxidative stress has been implicated in the pathogenesis of diabetes complications, including podocyte apoptosis (Gorin et al. 2005; Kim et al. 2006; Li and Shah 2003). The high glucose stimulates hypertrophy of podocytes through ROS-dependent activation of ERK1/2 and Akt/PKB pathways (Gorin et al. 2005; Kim et al. 2006). Eid et al. also demonstrated that high glucose-induced the apoptosis of cultured podocytes through the generation of ROS via sequential upregulation of CYP4A and the NADPH oxidases Nox1 and Nox4 (Eid et al. 2009). As far as we know, there is no study on the role of ROS in the protective effects of LIF. Therefore, the changed level of ROS was determined using ROS-sensitive DCFH-DA dyes (Fig. 3). It was found that there was an elevated ROS level in cells of high glucose group compared with that of control group (P <0.01). Pretreatment with LIF decreased ROS level in podocytes compared with that of high glucose group (P <0.01).

Renal NADPH oxidase is an important source of oxidative stress and its expression is enhanced in the glomerulus and distal tubules of diabetic nephropathy. The present results showed that the activity of NADPH oxidase was increased in high glucose group compared with that in control group (Fig. 4; P <0.01), which were in agreement with the previous results (Eid et al. 2009; Piwkowska et al. 2010). Although multiple enzymes were involved in the oxidative stress in different tissues or cells, a large number of studies have showed that NADPH oxidase derived superoxide is central to high glucose-induced oxidative stress in diabetic nephropathy (Griendling et al. 2000; Paravicini and Touyz 2008). Consistent with the reduction in ROS generation, Pretreatment with LIF reduced NADPH oxidase activity compared with that of high glucose group (Fig. 4; P <0.01).

Oxidative stress, where there is an imbalance between ROS and the cell's antioxidant capacity (Ott et al. 2007). The enzymatic defense of cells against ROS involves the cooperative action of several enzymes, including SOD (Kuwabara et al. 2008), an enzyme that changes superoxide into H_2O_2 , or by catalase and glutathione peroxidase (GPx), which decompose H_2O_2 into H_2O (Paes et al. 2001). In the present study, high glucose group showed the reduction of T-SOD activity (Fig. 5; P <0.05) while pretreatment with LIF attenuated the inhibitory effects of high glucose on T-SOD activity in podocytes.

Oxidation of lipid stimulation can produce the end-products of lipid peroxidation, such as MDA (Termini

2000). To evaluate the consequences of high glucose-induced oxidative stress and the protective role of LIF, the formation of MDA was determined as another marker of oxidative stress. As shown in Fig. 6, when treated with high glucose, the products of MDA significantly increased compared with that of control ($P < 0.05$). In contrast, podocytes pretreated with LIF (1 and 5 ng/ml) had significantly lower level of MDA compared with that of high glucose group ($P < 0.05$).

During apoptosis, caspase-3 is one of the key executors of apoptosis in response to various stimuli (Luthi and Martin 2007). It has been previously shown that LIF inhibits caspase-3 activation and apoptosis of various cells (Negoro et al. 2001; Hunt et al. 2010). LIF can promote oligodendrocyte survival through inhibition of caspase-3 activation after spinal cord injury (Kerr and Patterson 2005). To further analyze whether one of the cytoprotective effects of LIF was mediated by decreased caspase-3 activity, we quantified the enzyme activity in high glucose-treated podocytes. As shown in Fig. 7, when treated with high glucose, the activity of caspase-3 significantly increased compared with that of control ($P < 0.01$). In contrast, podocytes pretreated with LIF (1 and 5 ng/ml) had significantly lower level of caspase-3 activity compared with that of high glucose group ($P < 0.01$). The present study showed that LIF had the ability to protect high glucose-induced podocyte apoptosis through inhibition of ROS generation and caspase-3 activation. However, we thought the protective effect of LIF was not only specific to the high glucose injury, it may also protect cells from other apoptosis inducers though anti-oxidative stress.

In conclusion, the present study indicated that LIF was able to attenuate podocyte apoptosis from high glucose-induced cell oxidative stress, which may add a novel targeted therapy for diabetic nephropathy in the future.

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