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Mammalian Ste20-like protein kinase 3 plays a role in hypoxia-induced apoptosis of trophoblast cell line 3A-sub-E

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ABSTRACT

Mammalian Ste20-like protein kinase 3 (Mst3) is a key player in inducing apoptosis in a variety of cell types and has recently been shown to participate in the signaling pathway of hypoxia-induced apoptosis of human trophoblast cell line 3A-sub-E (3A). It is believed that oxidative stress may occur during hypoxia and induce the expression of Mst3 in 3A cells via the activation of c-Jun N-terminal protein kinase 1 (JNK1). This hypothesis was demonstrated by the suppressive effect of DL-α-lipoic acid, a reactive oxygen species scavenger, in hypoxia-induced responses of 3A cells such as Mst3 expression, nitrotyrosine formation, JNK1 activation and apoptosis. Similar results were also observed in trophoblasts of human placental explants in both immunohistochemical studies and immunoblot analyses. These suggested that the activation of Mst3 might trigger the apoptotic process in trophoblasts by activating caspase 3 and possibly other apoptotic pathways. The role of nitric oxide synthase (NOS) and NADPH oxidase (NOX) in hypoxia-induced Mst3 up-regulation was also demonstrated by the inhibitory effect of N^G-nitro-L-arginine and apocynin, which inhibits NOS and NOX, respectively. Oxidative stress was postulated to be induced by NOS and NOX in 3A cells during hypoxia. In conclusion, hypoxia induces oxidative stress in human trophoblasts by activating NOS and NOX. Subsequently, Mst3 is up-regulated and plays an important role in hypoxia-induced apoptosis of human trophoblasts.

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1. Introduction

Hypoxia is a condition of relatively low oxygen supply with respect to tissue metabolic demand (Neubauer, 2001). Cells under hypoxic stress may exhibit an adaptive response that increases the rates of metabolism and angiogenesis. Successful placentation and embryo development at the early stages of pregnancy occurs in a predominantly low oxygen environment with a partial pressure of oxygen (pO_2) of <20 mmHg (<2.6%) (Heazell et al., 2008; Khong et al., 1987; Rodesch et al., 1992). Organogenesis of the vascular system, placenta, lung and skeleton during embryonic and fetal development is also facilitated by hypoxic stress (Dunwoodie, 2008; Shinkai et al., 2005; Simon and Keith, 2008; Yue and Tomanek, 1999). However, hypoxia occurring during acute and chronic vascular disease, pulmonary disease, brain injury and cancer results in cell death (Harris, 2002; Shimizu et al., 1996). Various studies shown that the cell death induced by hypoxia can occur via apoptosis (Shimizu et al., 1996).

Apoptosis is an intrinsic mechanism that progressively eliminates excessively generated cells, the improperly developed cells, aged cells and cells with sustained genetic damages (Cohen and Duke, 1992; Golstein et al., 1991). A dysregulation of apoptotic cell death may be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases, neurodegenerative disorders, and viral infection (Raff et al., 1993; Sarraf and Bowen, 1988; Vaux et al., 1994; Williams, 1991). Apoptosis also plays roles in the placental development, remodeling and aging (Huppertz and Kingdom, 2004; Rama and Rao, 2003; Smith et al., 1997). Hypoxia was shown to induce apoptosis in trophoblasts (Hung et al., 2001; Levy et al., 2000; Chen et al., 2010). Trophoblasts isolated from the placenta in pregnancies complicated by intrauterine fetal growth retardation (IUGR) and preeclampsia exhibit enhanced apoptosis in response to hypoxia compared with trophoblasts from uncomplicated pregnancies (Crocker et al., 2003; Hung et al., 2002). It is believed that hypoxia-induced trophoblast apoptosis further promotes the pathogenic progression of IUGR and preeclampsia. Several cellular factors, such as p53 (Crocker, 2007; Heazell et al., 2008; Levy et al., 2000), Bcl-2 family members (Hu

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and Zhou, 2006; Humphrey et al., 2008), NF-κB (Cindrova-Davies et al., 2007) and c-Jun N-terminal protein kinase (JNK)/p38 MAPK cascades (Cindrova-Davies et al., 2007; Humphrey et al., 2008; Jessmon et al., 2010), were shown to participate in the signaling of hypoxic responses in trophoblasts. However, the molecular mechanism leading to hypoxic injury of human placenta has not been fully defined.

Recently, mammalian Ste20-like protein kinase 3 (Mst3) was shown to mediate the apoptosis of human placenta from normal term delivery and the trophoblast cell line (Wu et al., 2008). Mst3 is a member of the GCK-III subfamily of human Ste20-like serine/threonine protein kinases with a molecular mass of around 50 kDa (Huang et al., 2002; Lee et al., 2004). It was shown to participate in the apoptosis of many cell lines upon the treatment with Fas-ligand or staurosporine (Lee et al., 2004). In human placenta, however, oxidative stress was shown to be responsible for the upregulation of Mst3 (Wu et al., 2008). Oxidative stress is induced by the overproduction of reactive oxygen species (ROS), is postulated to play a role in cell apoptosis (Hung et al., 2001; Heazell et al., 2007; Jauniaux et al., 2000; Redman and Sargent, 2000). ROS, including superoxide radicals (O_2^-) and its reduction products hydrogen peroxide (H_2O_2) and the hydroxyl radical, have been shown to induce cellular damage through membrane lipid peroxidization, DNA modification and protein denaturation. JNK1 was shown to be activated by oxidative stress and, subsequently, upregulated the Mst3 in trophoblasts (Wu et al., 2008). Although Mst3 was demonstrated to trigger the trophoblast apoptosis by oxidative stress, its role in the hypoxia-induced apoptosis of human trophoblast has not been fully explored.

In this study, Mst3 was demonstrated to play an important role in the signaling pathway of hypoxia-induced trophoblast apoptosis. Similar results were also observed in human explant cultures under hypoxic stress. Furthermore, this study shows that hypoxia may activate Mst3 in human trophoblasts by elevating oxidative stress. Both NOS and NOX were shown to play a role in generating ROS in human trophoblasts under hypoxic stress.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), α-MEM, penicillin G, streptomycin, trypsin and lipofectamine were obtained from GIBCO Life Technologies. N^G-nitro-L-arginine (L-NNA) and DL-α-lipoic acid (LA) were bought from Cayman. G418-sulfate was obtained from Invitrogen. SP600125 was obtained from Calbiochem. Hypoxyprobe™-1 Kit for the detection of tissue hypoxia was purchased from Chemicon. Other reagents were regent grade.

2.2. Histology and immunohistochemistry

Fresh placental tissues for explant culture were obtained from normal pregnant women undergoing elective Cesarean sections (ECS) at term without labor pain (36-week; $n = 6$) with informed consent. The placental specimens were cut into a size of 1 cm × 1 cm × 0.5 cm and incubated in α-MEM medium (containing 10% FBS and 100 units/mL penicillin and 100 (g/mL streptomycin) under the hypoxia (1% O_2, 5% CO_2 and 94% N_2) or the normal condition (20% O_2, 5% CO_2 and 75% N_2) at 37 °C for 24 h. The histological and immunohistochemistry analyses of the explants were performed as described previously (Wu et al., 2008). The placental specimens were examined by a pathologist to confirm the histological preservation of the microanatomical structure and the absence of any abnormality in placenta.

The fixed human placental specimens were incubated with anti-Mst3 (1:100 dilution, Santa Cruz), anti-nitrotyrosine (1:200 dilution, ABcam Cat#ab52309) or anti-active caspase 3 (1:100 dilution, Santa Cruz) antibody for 2 h. Placental specimens were washed once with PBST (0.13 M NaCl, 0.1 M NaH_2PO_4 and 0.05% Tween-20) and then incubated with peroxidase-conjugated antibody against rabbit or mouse IgG (1:300 dilution, Santa Cluz). The activity of antibody-conjugated peroxidase was determined with diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC). Slides were then dried and sealed for microscopic analysis (Leica, DMLS). Images were obtained using a digital camera (Olympus, DP70) and processed by the free PLUS software supplied with the camera.

2.3. Cell culture and apoptosis assay

Human placental trophoblast, SV40 transformed cell line 3A-sub-E (3A) from American Type Culture Collection (ATCC # CRL-1584) was maintained in the α-MEM medium. Cells were incubated under hypoxia or 20% O_2 in a humidified 37 °C incubation chamber. The stable clones 3A(pNEO), 3A(siMst3)-1 and -2 (Wu et al., 2008) were maintained in the same cultural medium containing 0.3 mg/mL G418-sulfate (Invitrogen).The TUNEL (TdT-mediated, dUTP-incorporated nick-end labeling) staining of human placental specimens were performed by using Frag EL™ DNA Fragmentation Detection kit (Calbiochem). The specimens were covered with 60 μL TUNEL mix (TdT, HRP-12-dUTP, and cobalt chloride) at 37 °C for 1.5 h. A negative control was performed by omitting TdT. Human placental specimen treated with DNase I was used as a positive control. After incubation, specimens were rinsed three times with 1× PBS prior to incubating with 5 mg/mL DAB under room temperature for 10 min. The specimens were washed twice with 1× PBS and once with double-deionized H_2O before mounting for the microscopic analysis.The TUNEL assay of 3A cells was performed by using DeadEnd™ Fluorometric TUNEL System kit (Promega). Briefly, 3A cells (2×10^5 cells) were harvested and fixed with 0.1% formaldehyde for 10 min. After fixation, cells were washed three times with 1× PBS and then suspended in 5 mL 70% ice-cold ethanol. After incubating at −20 °C for at least 4 h, cells were then rinsed three times with 1× PBS and equilibrated with 100 μL of equilibration buffer (10 mM EDTA, 10 mM Tris–HCl, pH7.5, 10 mM DTT) at room temperature for 10 min. After equilibration, cells were incubated with 60 μL TUNEL mix (TdT, FITC-12-dUTP, and cobalt chloride) at 37 °C for 1 h. Reaction was terminated by adding 1 mL of 20 mM EDTA. Cells were then rinsed and suspended in 1× PBS for the analysis under a fluorescence-activated cell sorter Cytometer (CyFlow® SL, Partec). The negative control was prepared by omitting TdT in the TUNEL mix. Cell treated with DNase I was used as positive control.

For Annexin V binding assay, cells were collected from 100 mm dishes and washed twice with cold 1× PBS. Subsequently, cells were resuspended in 500 μL of 1× binding buffer (0.01 M Hepes/NaOH buffer, pH 7.4 containing 0.14 M NaCl, 2.5 mM $CaCl_2$) and stained with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (50 μg/mL). After incubated at room temperature for 5 min in the dark, number of cells bound Annexin V-FITC was analyzed on flow cytometer (CyFlow® SL, Partec).

2.4. Immunoblot analysis

Cells lysate was prepared by incubating 3A cells (about 2×10^6) with 50 μL RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0,150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Na-deoxycholate, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) at room temperature for 15 min. The particulates were removed from cell lysates by centrifugation at 4 °C and a speed of 16,100 × g for 5 min. The explant cultures of fresh placental villous were transfected with

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