Multimolecular complex of Par-4 and E2F1 binding to Smac promoter contributes to glutamate-induced apoptosis in human-bone mesenchymal stem cells

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ABSTRACT

Neural cells undergo glutamate-induced apoptosis in ischaemic brain tissue, in which prostate apoptosis response-4 gene (Par-4) is involved. Human-bone mesenchymal stem cells can be utilized as an effective therapy for ischemic brain injury. In this study, we found that glutamate could induce apoptosis in human-bone mesenchymal stem cells, accompanied by increased expression of Par-4 gene and Smac release from mitochondria. Repressing Par-4 expression attenuated the glutamate-induced apoptosis. Both Par-4 protein and E2F1 protein could bind to E2F1-binding BS3 site on Smac promoter and participated in the formation of a proteins-DNA complex. Moreover, in the complex, E2F1, not Par-4, was found to be directly bound to the Smac promoter, suggesting that Par-4 exerted indirectly its transcriptional control on the Smac gene though interacting with E2F1. Expression of full-length Par-4 in human-bone mesenchymal cells resulted in increased activity of the Smac promoter. In addition, the indirect transcriptional regulation of Par-4 on Smac depended on its COOH terminus-mediated interaction between Par-4 and E2F1. We conclude that the formation of proteins-DNA complex, containing Par-4 protein, E2F1 protein and the Smac promoter, contributes to the pro-apoptotic effect on glutamate-treated human-bone mesenchymal stem cells.

INTRODUCTION

As a major contributor to death and disability, ischaemic brain injury does not only affect adults (1,2), it is also an important cause of mortality and severe neurodevelopmental morbidity in children, such as cerebral palsy, mental retardation, epilepsy and learning disability (3). Human-bone mesenchymal stem cells (hBMSCs) are self-renewing multipotent cells, which can be utilized as an effective therapy for ischemic brain injury. However, although considerable studies have been devoted to investigating the therapeutic efficacy of hBMSCs transplantation in ischemic brain, rather less attention has been paid to the biochemical regulation of the stem cell itself in a pro-apoptotic microenvironment of ischaemic brain tissue. Thus, it is of paramount importance to investigate the events and factors that predispose hBMSCs to undergo apoptosis in ischaemia tissue.

Glutamate, an excitatory amino acid, binds to postsynaptically located glutamate receptors that regulate calcium channels in ischaemic brain tissue. The resulting calcium influx activates proteases, lipases and endonucleases, which in turn destroy the cellular skeleton, finally leading to cells necrosis or switch on apoptosis (4). The implanted stem cells will suffer from the impairment of excitotoxic glutamate as well in such a microenvironment. Hence, for alleviating suffering and improving survival of implanted cells in ischaemic brain tissue, it is important to determine how hBMSCs respond to glutamate.

The prostate apoptosis response factor-4 (Par-4) gene was originally identified by differential screening for up-regulated genes in apoptotic prostate cell (5). Subsequently, Par-4 was found to possess potent apoptotic activity in various cellular systems. In the past few years, several studies have investigated the role of Par-4 in cell apoptosis and found that Par-4 had been attributed a crucial function to its COOH terminus domain (6,8). One of the most noticeable structural features of Par-4 COOH terminus was the leucine zipper repeats, a putative death domain, which was found in several other proteins involved in apoptosis, suggesting the importance of this region in Par-4 function (7,9). However, despite the advances made in understanding the role of Par-4 in apoptosis, little was known about whether Par-4 is involved in the response of glutamate-treated hBMSCs.

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Snac (the second mitochondria-derived activator of caspase), also known as DIABLO (direct IAP-binding protein with low pI), is a pro-apoptotic protein and is normally compartmentalized and stored in mitochondria upon receiving apoptotic stimuli; Snac is released into cytosol, where it binds to IAPs, and active caspases either by eradicating the binding capability of IAPs to caspases or enhancing the proteosome-mediated degradation of IAPs (10,11). In addition, E2F1, a member of E2F transcription factor family, can bind to the Smac promoter and transcriptionally upregulate Smac expression, which results into the mitochondria-mediated apoptosis (12). However, it remains unclear whether Snac plays a role in glutamate-induced hBMSCs apoptosis.

In this study, we demonstrate for the first time that excitotoxic glutamate can induce hBMSCs apoptosis, accompanied by increased expression of Par-4 and Snac release from mitochondria. Our results indicate that the glutamate-induced pro-apoptotic effect on hBMSCs is partially due to the formation of multimolecular complex of Par-4, E2F1 and Snac promoter. Our results show that Par-4 can transcriptionally modulate Smac expression through the indirect interaction between Par-4 and Smac promoter. The association of Par-4 with E2F1 which depended on the COOH terminus of Par-4, enhances the capability of E2F1 binding to Smac promoter. In conclusion, our findings provided the detailed molecular mechanisms responsible for glutamate-induced apoptosis in hBMSCs.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Par-4 rabbit polyclonal antibody, E2F1 rabbit polyclonal antibody and FITC conjugated anti-rabbit IgG secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The majority of reagents used for this study, including glutamate and DAPI, were obtained from Sigma–Aldrich (St Louis, MO, USA). Anti-CD105 (endoglin), -CD73, -CD106 (VCAM-1), -CD44, -CD90, -CD29, -CD45, -CD34, -CD38, -CD80, -CD86 antibodies, were purchased from BD Biosciences (La Jolla, CA, USA). pGEMT-easy vector, Gel Shift Assay kit, pGL3 Luciferase Reporter Vector were obtained from Promega (Madison, WI, USA). Advantage 2 DNA polymerase and pcDNA3.1/myc-his vector were obtained from BD Biosciences (La Jolla, CA, USA). Restriction endonucleases were purchased from New England BioLab (Beverly, MA, USA). Endo-free Plasmid Maxi kit was obtained from Qiagen (Hilden, Germany). Lipofectamine 2000 reagent was obtained from Invitrogen (Carlsbad, CA, USA). Nuclear Protein Extraction kit was purchased from Active Motif (Carlsbad, CA, USA).

**Cell culture and hBMSCs phenotype analysis**

Culture and characterization of hBMSCs were performed as described previously (13). Briefly, bone marrow cells were obtained from 5 ml aspirates from the iliac crest of normal donors after informed consents were given. Cells were plated at a density of $5 \times 10^6$ per 25 cm$^2$ flask in 5 ml Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum, incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. After 72 h, non-adherent cells were discarded and adherent cells were washed gently with DMEM medium and cultured for ~21 days. The culture medium was replaced with fresh complete medium twice a week. Upon reaching near confluence, cells were detached with a solution of 0.25% trypsin and 100 μMol/l EDTA for 2–3 min at 37°C and plated at 1000 cells/cm$^2$ with medium replacement twice a week. Cells used in our experiments were the fifth or sixth passage of cultivation. hBMSCs were immunophenotyped by fluorescence-activated cell sorting (FACS). Cells were detached with trypsin-EDTA, washed in PBS, and immediately stained with the following labelled antibodies: CD105-FITC, CD73-FITC, CD44-FITC, CD90-FITC, CD147-FITC, HLA Class I-FITC, CD29-FITC, CD34-PE, CD45-PE, CD14-PE, CD11b-PE and HLA-DR-PE and then analysed using a flow cytometry.

**Fluorescence-activated cell sorter analysis**

hBMSCs were harvested in the presence or absence of 1—100 μM glutamate for 6, 12 or 24 h. Cells were collected by trypsinization and washed in PBS. After incubation with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI, 50 μg/ml) at room temperature for 15 min in the dark, cells were analysed by flow cytometry. The percentage of apoptosis in the cells were calculated by data from FACS analysis and the result is presented in the bar graph relative to the apoptotic cell rate in hBMSCs without treatment of glutamate.

**DNA fragmentation assay**

DNA fragmentation was assessed using a soluble DNA preparation as previously described (14). hBMSCs were harvested in the presence or absence of 100 μM glutamate for 24 h. Then cells were lysed in 10 mM Tris–HCl (pH 7.5) containing 10 mM EDTA and 0.2% Triton X-100. The lysate was centrifuged at 12 000g for 10 min. The supernatant was treated with proteinase K (0.3 mg/ml) and RNase A (0.3 mg/ml) and then extracted in the presence of 300 mM NaAc. The DNA was precipitated with isopropanol and dissolved in 10 mM Tris–HCl (pH 8.0) containing 100 μM EDTA. The DNA was electrophoresed in 1.5% agarose gel in Tris borate-EDTA buffer. The DNA bands were then imaged by ethidium bromide staining and photographed.

**Subcellular fractionation**

Cells were washed once in PBS and harvested in isonic buffer (250 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.4) supplemented with 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotonin. After a brief sonication, samples were transferred to 1.5-ml tubes and centrifuged at 900g for 10 min at 4°C to eliminate nuclei and unbroken cells. Supernatant was then centrifuged at 9500g for 15 min at 4°C to obtain the heavy membrane pellet enriched for mitochondria.
and the resulting supernatant was stored as the cytosolic fraction.

Western blotting analysis

Cells were collected by trypsinization and washed in PBS. Then cell pellets were lysed in 1% Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), to which 2 mM phenylmethylsulfonyl fluoride, 3.3 μg/ml leupeptin and 5 μg/ml pepstatin were added fresh before use. Fifty micrograms of proteins was loaded on precast SDS/Tris/glycine gels, and after electrophoresis, proteins were transferred to nitrocellulose membranes, which were blocked in 5% non-fat dry milk and blotted with the appropriate primary antibody. Membranes were then incubated with the appropriate secondary antibody linked to horseradish peroxidase and developed using the ECL kit according to the manufacturer’s protocols. Smac levels were represented as ratios between the levels in mitochondria versus cytosol.

Small interfering RNA-based (siRNA) experiments

A small interfering RNA-based (siRNA) strategy was employed to silence endogenous human Par-4 in hBMSCs. Par-4 and scrambled siRNAs were generated using the procedure of siSTRIKE U6 Hairpin Cloning Systems (Promega, Madison, WI). The four siRNAs had the following sense strand sequences:

Par-4-siRNA-1:
5′-ACCGTCAAGGCGTGGTGAATAATATTTCCAAG GAAATATTTCAACAAGCGTGTACCTTTTTC-3′.

Par-4-siRNA-2:
5′-ACCGGAAACGAGAAGATGCAATTATACCG AGATATAATCGATTTCTGTTTCTTTTTTTC-3′.

Par-4-siRNA-3:
5′-ACCGTGAGACTGATGCAAAGATATATCCAG AGATTTATCTTGACTGACGTTTCTTTTTTC-3′.

Par-4-siRNA-4:
5′-ACCGAACAGTTTCAGGCGATATATTTCCAAG AGATATACGTGCCTGAAAATCTGTCTTTTTTC-3′.

The target sequences for the Par-4 gene are underlined.

RT–PCR analysis

Cells were harvested, and total RNA extraction was performed. RNA purity (A260/A280 >1.6) was checked by a spectrophotometer (GeneQuant, Type II; Pharmacia, Uppsala, Sweden), and RNA integrity was confirmed by visualization of 28 and 18 s bands (2:1) on 1% agarose gel.

Real time quantitative PCR

Total RNA extraction was performed using TriZol reagent followed by chloroform-isopropanol extraction and ethanol precipitation. Subsequently, duplicate samples of 1 μl of each cDNA were used as a template. Real time quantitative PCR was performed using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Wellesley, MA, USA). We used primers and a probe generated by the program Primer Premier. Par-4 primers were F (forward) 5-GCCCGAGATGCTTAGATGAG-3 and Par-4 R (reverse) 5-GCAGATA GGAACGTGCGAAGAAGAGG-3 was added to the PCR mixture to a final concentration of 200 nM. GAPDH primers were F (forward) 5-GAAGGTGA GGTCCGAGTC-3 and GAPDH R (reverse) 5-GAAGATGGTGATGGGATTC-3 and TaqMan probes were FAM-labeled 5′-ACCAAGAGAAGGCAAGAAGG-3 was added to the PCR mixture to a final concentration of 200 nM. GAPDH primers were F (forward) 5-GAAGGTGAA GGTCCGAGTC-3 and GAPDH R (reverse) 5-GAAGATGGTGATGGGATTC-3 and TaqMan probe fluorescence labeled 5′F-CAAGCTTCCCGTTCG for 60 s. GAPDH was co-amplified with Par-4. Average threshold cycle (Ct) values from the triplicate PCR reactions for Par-4 were normalized against the average Ct values for GAPDH from the same cDNA sample. The fold change of Par-4 transcript levels between Par-4-siRNA-1,2,3,4 and the control equals 2ΔΔCt, where ΔCt = CtPar-4–CtGAPDH, and ΔΔCt = ΔCtcontrol−ΔCtPar-4-siRNA-1,2,3,4. Real time quantitative PCR for Smac was similarly performed.

Construction of expression plasmids and transient transfection

To construct and clone the eukaryotic expression vector of human Par-4, full-length cDNA of Par-4 was amplified by RT–PCR from human neuroblastoma cell line SK-N-SH (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, Peoples’ Republic of China).

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After RT–PCR amplification, Par-4 cDNA was inserted to plasmid pGEM-T and transformed into Escherichia coli JM109. Positive clones were amplified, screened and identified by sequencing. Then Par-4 cDNA was subcloned into pcDNA3.1 vector between the restriction enzyme sites HindIII and XhoI to generate the pcDNA3.1-Par-4 construct. For construction of pcDNA3.1-myc-Par-4, which coded a myc-tagged Par-4, RT–PCR amplification was performed with another pair of primers, from which the stop codon of Par-4 was removed. pcDNA3.1-myc-ΔPar-4 was similarly constructed, which encoded a myc fusion protein containing a Par-4 deletion mutant, the latter lacking 70 amino acids at the COOH terminus of Par-4. Transfections were performed using the Lipofectamine 2000 procedure following the manufacturer's protocol. Green fluorescent protein phMGFP vector was cotransfected to determine transfection efficiency with flow cytometry assay.

**Northern blotting analysis**

Samples of total RNA (10 µg) were separated by electrophoresis through denaturing 1.2% agarose gels containing 1% formaldehyde and transferred onto nylon or nitrocellulose membranes using standard molecular biological techniques. Probes were derived from PCR products amplified with gene-specific primers for human Smac cDNA and labeled with [γ-32P]dCTP using the Prime-It random priming kit (Stratagene, La Jolla, CA). Filters were reprobed with the cDNA for GAPDH to correct for the amount of RNA loaded onto the filters. Pre-hybridization and hybridization were performed at 62°C for 1 h and overnight, respectively. The blot was subjected to low and high stringency washes using the buffers provided in the NorthernMaxTM kit for Northern blots (Ambion Inc., Inc., Austin, TX). After hybridization, the membranes were washed and exposed at -70°C to X-ray film using an intensifying screen.

**Immunoprecipitations**

Cells were lysed in icecold immunoprecipitation buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, and a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN, USA). Lysis with immunoprecipitation buffer was complemented with sonication (5 pulses) on ice. The lysate was cleared of cell debris by centrifugation at 16 000g for 30 min. The supernatant was saved for immunoprecipitation using anti-Par-4 or anti-E2F1 conjugated to Sepharose beads overnight at 4°C with gentle agitation. The beads were washed 3 times in lysis buffer, resuspended in 2 x Laemmlı buffer and boiled for 5 min to release bound proteins. Proteins were analysed by SDS–PAGE and immunoblotting after transfer to polyvinylidene difluoride membranes.

**Isolation of nuclear proteins binding to biotinylated probes**

Nuclear proteins binding to biotinylated probes were isolated as described previously (28). Briefly, oligonucleotides representing E2F1-binding site on Smac promoter sequence were synthesized, and 5 µg of the sense strand was 3'-labelled using the terminal deoxynucleotidyltransferase and biotin-14-dATP. The biotin-labelled sense strand was annealed to its complementary antisense strand and purified over a Sephadex column. The concentration of purified oligonucleotide was measured by absorbance at 260 nm, and equal amounts of annealed oligonucleotide were incubated with streptavidin-coated magnetic beads (Promega) for 30 min at room temperature. Coupling of the oligonucleotides to the beads was measured by absorbance. For the binding reaction, 50 µg of nuclear extract alone or preincubated with anti-Par-4 antibody (R334) was used. The beads were captured by a magnet, washed three times with a high salt buffer, and resuspended in Laemmlı buffer. The samples were heated at 95°C for 5 min to elute all the proteins, loaded onto a 12% SDS–PAGE, electrophoresed, and transferred to a nitrocellulose membrane. Par-4 and E2F1 were detected using the Par-4 antibody (R334) or E2F1 antibody by immunoblotting.

**Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)**

The double-stranded oligonucleotides used for EMSA of E2F1-binding sites in Smac promoter are shown as follows: E2F1-binding BS3 site (underlined), 5'-GCCGGAGATT-3'; mutated E2F1-binding BS3 site (underlined and in boldface), 5'-GCCGGCTTGAAGTT-3'; E2F1-binding BS2 site (underlined), 5'-GGCCAGCGGATGC-3'. The oligonucleotides were 5'-end-labeled with [γ-32P]dATP. Nuclear proteins were extracted using a Nuclear Protein Extraction Kit. Electrophoretic mobility shift assay was performed by using a Gel Shift Assay Kit following the manufacturer's instructions. The nuclear extracts containing 30 µg of total proteins were preincubated with gel shift binding buffer for 10 min, followed by the addition of 1 µl of a [γ-32P]-labelled, double-stranded oligonucleotide probe of E2F1 and incubation for 1 h. Formed nuclear protein–DNA complexes were dissolved in 4% nondenaturing polyacrylamide gels, and electrophoresis was performed under 90 V for 2 h. Gels were dried and exposed to Kodak Biomax films at -70°C for 48 h. To assess the specificity of the reaction, competition assays were performed with 100-fold excess of unlabeled consensus oligonucleotide pairs of E2F1. The unlabelled probes were added to binding reaction mixture 10 min before the addition of the labelled probes. For the blocking reaction, 500 ng each of anti-Par-4 antibody (R334) and anti-E2F1 antibody were used.

**Chromatin immunoprecipitation**

hBMSCs cultured in the presence or absence of 100 µM of glutamate for 24 h were subjected to chromatin immunoprecipitation. Chromatin immunoprecipitation was done as described earlier (28). Cells were plated in a 100-mm dish. About 70% confluent dishes were treated with formaldehyde (1%) for 10 min at 37°C to cross-link proteins to DNA. The cells were washed twice with PBS, pH 7.4, containing protease/inhibitor mixture (Roche Applied Science) and then lysed with lysis buffer containing 1% SDS. Sonication of cross-linked chromatin was performed.
at 30% of maximum power with two rounds of 10 s pulses so that chromatin fragments thus obtained ranged from 500 to 2000 bp in size. Soluble chromatin was subjected to overnight immunoprecipitation with either anti-E2F1 antibody or anti-Par-4 antibody. A portion of the chromatin solution was kept to check the amount of input DNA in different samples before immunoprecipitation. Following immunoprecipitation and elution, the eluent was heated to 65°C for 6 h to reverse the cross-link. Phenol/chloroform extraction was performed, and the supernatant was ethanol-precipitated. DNA thus obtained was resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0 and subjected to PCR. Forward and reverse primers selected for Smac promoter are as follows: sense primer, 5'-TTC CTTCAAGCCTGGCAGAAC-3'; antisense primer, 5'-ACGCCCCCACTCAGGGACGCAGGCCG-3'. PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

Luciferase assays

pGL3-Smac-P-Luc reporter was constructed as described previously (28). Briefly, the Smac promoter fragment (~600 to ~100 bp), which contained E2F1-binding BS3 site, was cloned into the Xho I/Hind III site of pGL3-Basic plasmid with the primer pairs indicated below. Sense, 5'-CCGCTCGAGCCTGGTGCCTGCCTGAGCAGGACGGCCG-3'; Antisense, 5'-CGAAGCTTACGATCTGGGAATTGGGCAGCCG-3'. hBMSCs were transiently cotransfected with appropriate pGL3-Smac-P-Luc reporter plasmids (200 ng) and either pcDNA3.1-myc-Par-4 or pcDNA3.1-myc-ΔPar-4 (200 ng) using Lipofectamine 2000. In each transfection, cells were also cotransfected with Renilla luciferase reporter plasmid pRL-CMV, which served as an internal control for transfection efficiency. Cell extracts were assayed for firefly and Renilla luciferase activity. Fold-activation values were measured relative to the levels of luciferase activity in cells transfected with empty vectors and normalized by Renilla luciferase activities.

RESULTS

Par-4 played an important role in glutamate-induced hBMSCs apoptosis

We investigated whether glutamate could induce apoptosis in hBMSCs. The cells were exposed continuously to 1–100 μM glutamate with different time lengths. Upon FACS analysis, we found that glutamate induced a dose-dependent apoptosis in hBMSCs, which started at 1 μM and peaked at 100 μM (Figure 1A), and glutamate at 100 μM induced a time-dependent cells apoptosis starting at 6 h and culminating at 24 h (Figure 1A and Supplementary Figure S1), which was confirmed by the following DNA laddering assay. As shown in Figure 1B, after exposure to 100 μM glutamate for 24 h, nucleosomal DNA fragmentation, an end stage apoptotic event, was observed in hBMSCs. By contrast, no DNA laddering was found in the cells without treatment of glutamate. These data showed that glutamate could induce apoptosis in hBMSCs.

To examine whether Par-4 was involved in the regulation of glutamate-induced apoptosis in hBMSCs, Western blotting was performed to detect Par-4 expression in the cells treated with or without glutamate. Because the pro-apoptotic function of Par-4 was attributed to its nuclear translocation (13), Western blotting was performed with nuclear extracts. As shown in Figure 1C, glutamate induced a dose-dependent increase of Par-4 expression in hBMSCs as compared with control.

To further confirm the role of Par-4 in glutamate-induced apoptosis, we studied the effect of reducing Par-4 expression on apoptosis of hBMSCs after treatment with glutamate. hBMSCs was transfected with either Par-4 specific siRNA, scrambled siRNA, or no siRNA (Mock). The mRNA expression levels of Par-4 in transfected cells were assessed by real time quantitative PCR analysis. As shown in Figure 1D and Supplementary Figure S2, Par-4-siRNA-1, one of the tested siRNA, caused an effective reduction of Par-4 mRNA. In all, 93% of Par-4 mRNA was suppressed by Par-4-siRNA-1. Then, the transfected hBMSCs were treated with or without equal concentration of glutamate (100 μM) for 24 h. FACS analysis showed that the suppression of Par-4 expression significantly attenuated the glutamate–induced apoptosis, which started at 1 μM (Figure 1A), and glutamate induced a time-dependent cells apoptosis start- ing at 6 h and culminating at 24 h (Figure 1A and Supplementary Figure S1). To characterize the involvement of E2F1 in the regulation of Par-4, we blocked the expression of E2F1 with specific siRNA, scrambled siRNA, or no siRNA (Mock). The mRNA expression on apoptosis of hBMSCs after treatment with glutamate was suppressed by Par-4-siRNA-1. By contrast, transient overexpression of Par-4 increased the apoptosis cells in glutamate-treated hBMSCs (Figure 1F). Together, these data indicated that Par-4 played an important role in glutamate-induced hBMSCs apoptosis.

Ectopic expression of Par-4 enhanced the expression of Smac, which could be abrogated by blocking endogenous E2F1

To determine whether Smac is involved in glutamate-induced hBMSCs apoptosis, Western blotting was performed to detect the release of Smac from mitochondria. Figure 2A showed that Smac protein releasing from mitochondria exhibited an apparent increase upon the treatment with glutamate in a dose-dependent manner.

We next examined the relationship between Par-4 and Smac. hBMSCs were transfected with plasmid pcDNA3.1-Par-4, which contained complete Par-4 CDS. The same amount of empty pcDNA3 vector was also introduced into cells as a control. Smac protein expression was found to be upmodulated in the Par-4 overexpressing hBMSCs (Figure 2B and Supplementary Figure S4). Northern blotting showed that Smac transcript in hBMSCs was enhanced as well (Figure 2C), suggesting a previously undefined transcriptional mechanism that Par-4 could mediate upmodulation of Smac protein.

To characterize the involvement of E2F1 in the regulation of Smac in hBMSCs, we blocked the expression of endogenous E2F1 protein using siRNA. hBMSCs were cotransfected with pcDNA3-Par-4 and plasmid pSiRNA-E2F1-1, the latter specifically targeting E2F1 for gene silencing. Western blotting analysis demonstrated Smac expression was restrained following the inhibition of
E2F1 expression (Figure 2D and Supplementary Figure S5). Real time quantitative RT-PCR showed that pSiRNA-E2F1-1 could repress pcDNA3-Par-4-induced increase of Smac mRNA transcription (Figure 2E). These data indicated that E2F1 participated in Par-4-induced Smac transcription and expression.

**Par-4 and E2F1 formed a protein complex in glutamate-treated hBMSCs**

Based on the strong correlation among Par-4, E2F1 and Smac from our above study, we further characterized the interaction among these molecules. As shown...
Figure 2. Ectopic expression of Par-4 enhanced Smac protein and transcript. Blocking endogenous E2F1 abrogated the enhancement of Par-4-induced Smac expression. (A) Glutamate induced a release of Smac from mitochondria. hBMSCs were treated with glutamate at the dose from 1 to 100 μM. Subcellular localization of Smac was determined by western blotting after 24 h, and protein levels were quantified by computer-assisted densitometry. Tubulin levels were analysed to confirm equal protein loading in cytosol. Hsp60 was used to monitor the absence of mitochondrial contamination in the cytosolic fraction and to verify equal protein loading in mitochondrial fraction. Intensities of bands from western blotting analysis were quantified. Results are indicated as the ratio between Smac levels in mitochondria versus Smac levels in cytosol and are mean ± S.E. of three independent experiments performed in duplicate. *P < 0.05 versus the cells without treatment of glutamate. (B) Overexpression of Par-4 enhanced Smac protein in hBMSCs. hBMSCs were transfected with either pcDNA3.1-Par-4 or empty pcDNA3.1 vector. Subcellular localization of Smac was determined by Western blotting after 48 h, and protein levels were quantified by computer-assisted densitometry. Tubulin levels were analysed to confirm equal protein loading in cytosol. Hsp60 was used to monitor the absence of mitochondrial contamination in the cytosolic fraction and to verify equal protein loading in mitochondrial fraction. Results are indicated as the ratio between Smac levels in mitochondria versus Smac levels in cytosol and are the mean ± SE of three independent experiments performed in duplicate. *P < 0.05 versus the cells transfected with empty vector. (C) Overexpression of Par-4 enhanced Smac transcript in hBMSCs. Northern blotting was used to determine Smac mRNA in hBMSCs. hBMSCs were transfected with either pcDNA3.1-Par-4 or empty pcDNA3.1 vector. After 24 h of transfection, total cellular RNAs were separated and hybridized to 32P-labelled DNA probes. GAPDH probe was used to normalize for differences in RNA loading. Intensities of bands from northern blotting analysis were quantitated and normalized with GAPDH. Arbitrary unit = (ASmac/AGAPDH) × 100%. Values are mean ± S.E of three independent experiments performed in duplicate. *P < 0.05 versus the cells transfected with empty vector. (D) Blocking endogenous E2F1 by siRNA abrogated the enhancement of Par-4-induced Smac protein in hBMSCs. hBMSCs were co-transfected with pcDNA3.1-Par-4 and psiSTRIKE-E2F1-siRNA-1 vector. As controls, these plasmids or empty vectors were respectively transfected into hBMSCs. Subcellular localization of Smac/DIABLO was determined by western blotting after 48 h, and protein levels were quantified by computer-assisted densitometry. Tubulin protein was analysed to confirm equal protein loading in cytosol. Hsp60 was used to monitor the absence of mitochondrial contamination in the cytosolic fraction and to verify equal protein loading in mitochondrial fraction. Intensities of bands from western blotting analysis were quantitated. Results are indicated as the ratio between Smac levels in mitochondria versus Smac levels in cytosol and are the mean ± S.E. of three independent experiments performed in duplicate. *P < 0.05 versus untransfected cells (the first bar at left). #P < 0.05 versus hBMSCs transfected only with pcDNA3.1-Par-4. (E) Blocking endogenous E2F1 by siRNA abrogated enhancement of Par-4-induced Smac transcript in hBMSCs. hBMSCs were co-transfected with pcDNA3.1-Par-4 and psiSTRIKE-E2F1-siRNA-1 vector. As a control, empty vector was respectively transfected into hBMSCs. After 24 h of transfection, relative amount of Par-4 mRNA was measured by real-time quantitative RT–PCR and normalized against GAPDH. Bars depict the percentage of Par-4 mRNA versus that of untransfected cells. Values are mean ± S.E of three independent experiments. *P < 0.05 versus untransfected cells (control, the first bar at left). #P < 0.05 versus transfected cells only with pcDNA3.1-Par-4.
Par-4 was performed using anti-Par-4 antibody. Indeed, we found that Par-4 and E2F1 existed in nuclear extracts from glutamate-treated cells (Figure 4A, lane 1; Figure 4B, lane 1). The Par-4-specific antibody (R334) has been shown previously (28) to disrupt the interaction between Par-4 and other molecules. We could not almost detect Par-4 in the nuclear extracts preincubated with the R334 antibody and then incubated with the biotin-labeled E2F1-binding site on Smac promoter (Figure 4A, lane 2). Similarly, E2F1 also decreased markedly upon the pre-incubation of the neutralizing antibody against E2F1 (Figure 4B, lane 2). Together, this set of experiments indicated that Par-4 and E2F1 bound either indirectly or directly to Smac promoter in glutamate-treated hBMSCs.

To further elucidate the molecular mechanisms by which Par-4 and E2F1 interact with the Smac promoter, EMSA experiments in vitro were performed with the nuclear extracts from hBMSCs treated with glutamate. Previously defined E2F1-binding site BS3 (−200 to −193 bp relative to ATG) on Smac promoter were used as probes (12). Two complexes were formed with the BS3 oligonucleotide (Figure 4C, lane 2). Competition with increasing amounts of cold competitor demonstrated that both complexes were specific (Figure 4C, lane 3). To determine the presence of Par-4 and E2F1 in the complexes formed, nuclear extracts from the glutamate-treated hBMSCs were incubated with the BS3 probe along with antibodies directed against Par-4 or E2F1. The anti-Par-4 antibody is directed against the COOH terminus of Par-4, which directly bound to Smac promoter, and has been demonstrated previously as a blocking antibody (28). The anti-Par-4 antibody caused disappearance of the slower migrating complex, indicating the presence of Par-4 (Figure 4C, lane 4). The neutralizing antibody against E2F1 caused disappearance of both the faster and the slower migrating complex, thus indicating the presence of E2F1 in both the complexes (Figure 4C, lane 5). These results supported that it was E2F1, not Par-4, in Par-4-E2F1 proteins complex that directly bound to Smac promoter, and that Par-4 might indirectly exert its transcriptional control on Smac gene though interacting with E2F1. The BS3 E2F1-binding site was also used as a probe to perform EMSA with nuclear extracts from the hBMSCs without treatment of glutamate. However, no DNA–protein complex was found (Figure 4C, lane 7). Therefore, the presence of glutamate was critical for the formation of these DNA–protein complexes.

**Par-4-E2F1 complex bound to the BS3 Site**

(200 to −193 bp relative to ATG) within Smac promoter in glutamate-treated hBMSCs in vivo

To evaluate directly the potential significance of physical interactions among Par-4, E2F1 and Smac promoter, we investigated whether Par-4 and E2F1 associate on the chromatin of endogenous Smac promoter using the chromatin immunoprecipitation assay. We immunoprecipitated chromatin from the glutamate-treated hBMSCs, using specific antibodies against Par-4 and E2F1 or no beads, with the help of a magnet. The proteins binding to the promoter were eluted by boiling in Laemmli’s buffer and subjected to SDS–PAGE, and Western blotting for Par-4 was performed using anti-Par-4 antibody. Indeed, we found that Par-4 and E2F1 existed in nuclear extracts from glutamate-treated cells (Figure 4A, lane 1; Figure 4B, lane 1). The Par-4-specific antibody (R334) has been shown previously (28) to disrupt the interaction between Par-4 and other molecules. We could not almost detect Par-4 in the nuclear extracts preincubated with the R334 antibody and then incubated with the biotin-labeled E2F1-binding site on Smac promoter (Figure 4A, lane 2). Similarly, E2F1 also decreased markedly upon the pre-incubation of the neutralizing antibody against E2F1 (Figure 4B, lane 2). Together, this set of experiments indicated that Par-4 and E2F1 bound either indirectly or directly to Smac promoter in glutamate-treated hBMSCs.

To further elucidate the molecular mechanisms by which Par-4 and E2F1 interact with the Smac promoter, EMSA experiments in vitro were performed with the nuclear extracts from hBMSCs treated with glutamate. Previously defined E2F1-binding site BS3 (−200 to −193 bp relative to ATG) on Smac promoter were used as probes (12). Two complexes were formed with the BS3 oligonucleotide (Figure 4C, lane 2). Competition with increasing amounts of cold competitor demonstrated that both complexes were specific (Figure 4C, lane 3). To determine the presence of Par-4 and E2F1 in the complexes formed, nuclear extracts from the glutamate-treated hBMSCs were incubated with the BS3 probe along with antibodies directed against Par-4 or E2F1. The anti-Par-4 antibody is directed against the COOH terminus of Par-4 and has been demonstrated previously as a blocking antibody (28). The anti-Par-4 antibody caused disappearance of the slower migrating complex, indicating the presence of Par-4 (Figure 4C, lane 4). The neutralizing antibody against E2F1 caused disappearance of both the faster and the slower migrating complex, thus indicating the presence of E2F1 in both the complexes (Figure 4C, lane 5). These results supported that it was E2F1, not Par-4, in Par-4-E2F1 proteins complex that directly bound to Smac promoter, and that Par-4 might indirectly exert its transcriptional control on Smac gene though interacting with E2F1. The BS3 E2F1-binding site was also used as a probe to perform EMSA with nuclear extracts from the hBMSCs without treatment of glutamate. However, no DNA–protein complex was found (Figure 4C, lane 7). Therefore, the presence of glutamate was critical for the formation of these DNA–protein complexes.
antibody at all as a control. Genomic DNA fragments bound to Par-4 or E2F1 were analysed by PCR using primers upstream of the BS3 (−200 to −193 bp relative to ATG) site within Smac promoter. Analysis of genomic DNA immunoprecipitated with anti-Par-4 antibody revealed Smac promoter in glutamate-treated hBMSCs (Figure 5, lane 4). Also, immunoprecipitates from anti-E2F1 antibody revealed the presence of Smac promoter (Figure 5, lane 5). As expected, when minus the antibodies in the immunoprecipitation procedure, no genomic DNA was pulled out (Figure 5, lane 6). Chromatin immunoprecipitated with anti-Par-4 antibody or anti-E2F1 antibody was subjected to PCR using primers representing BS2 (−542 to −535 bp relative to ATG) site, another previously reported E2F1-binding site. No DNA could be amplified from the chromatins, (data not shown), demonstrating that Par-4 and E2F1 specifically bind to the BS3 (−200 to −193 bp relative to ATG) site on Smac promoter. PCR analysis of the total input DNA was also performed. Products of chromatin immunoprecipitation and PCR amplification were analysed by 2% agarose gel electrophoresis and beta-actin was performed as a negative control. Results are representative of three independent experiments.

Figure 4. Par-4 and E2F1 bound to Smac promoter in hBMSCs with exposure of glutamate. (A and B) hBMSCs were treated with or without 100 μM glutamate for 24 h. Nuclear proteins were isolated. Before the binding reaction, 50 μg of nuclear extract was preincubated with either anti-Par-4 antibody (R334) or anti-E2F1 antibody. Subsequently, these nuclear extracts were co-incubated with the magnetic beads containing biotinylated oligonucleotide sequence of SB3 sites within Smac promoter for binding reaction. The beads were captured by a magnet, washed three times with a high salt buffer, and resuspended in Laemmli buffer. The samples were heated at 95°C for 5 min to elute all the proteins, loaded onto a 12% SDS–PAGE gel, and transferred to a nitrocellulose membrane. The samples were probed with goat anti-rabbit IgG conjugated to horseradish peroxidase and visualised by chemiluminescence. Immunoblotting was performed as described under ‘Materials and Methods’ section. Immunoprecipitated DNA was amplified using primers representing the BS3 site (−200 to −193 bp relative to ATG) on Smac promoter. PCR analysis of the total input DNA was also performed. Products of chromatin immunoprecipitation and PCR amplification were analysed by 2% agarose gel electrophoresis and beta-actin was performed as a negative control. Results are representative of three independent experiments.

Figure 5. Demonstration of *in vivo* binding of Par-4 and E2F1 to BS3 site (−200 to −193 bp relative to ATG) on the Smac/DIABLO promoter by chromatin immunoprecipitation. hBMSCs were treated with or without 100 μM glutamate for 24 h. Chromatin lysates were immunoprecipitated (IP) with either antibody against Par-4 or antibody against E2F1. The samples were processed as described under ‘Materials and Methods’ section. Immunoprecipitated DNA was amplified using primers representing the BS3 site (−200 to −193 bp relative to ATG) on Smac promoter. PCR analysis of the total input DNA was also performed. Products of chromatin immunoprecipitation and PCR amplification were analysed by 2% agarose gel electrophoresis and beta-actin was performed as a negative control. Results are representative of three independent experiments.

The indirect transcriptional regulation of Par-4 on Smac was dependant on its COOH terminus-mediated interaction between Par-4 and E2F1 in hBMSCs

To identify structural determinants of Par-4 responsible for its regulatory function on Smac transcription, we focused on investigating the role of the COOH terminus of Par-4, since a stretch of 70 amino acids at this region showed homology to a domain referred to as the death domain, which was also found in other pro-apoptotic proteins such as Fas, FADD and TNFR-1 (6,20). We engineered and constructed plasmids pcDNA3.1-myc-Par-4 and pcDN3.1-myc-DPar-4, encoding a myc fusion protein
cotransfection with increasing amounts of full-length Par-4 resulted in an increased activity of Smac promoter in a dose-dependent manner, whereas mutant Par-4 or empty vector failed to give rise to noticeable activation of Smac promoter. These data indicated the COOH terminus of Par-4 was essential for Par-4-mediated activation of Smac promoter.

Although, as delineated previously (15), no evidence was provided that Par-4 could directly bind to DNA molecule, our above studies has demonstrated that Par-4 and E2F1 could form protein complex and bind to Smac promoter. Hence, we could set up a hypothesis that the COOH terminus of Par-4 might contribute to proteins complex formation of Par-4 and E2F1, and then via recruitment of E2F1 to Smac promoter, Par-4 induced indirectly Smac transcription. In order to further corroborate this hypothesis, we performed the co-immunoprecipitation assays for measuring the interaction between Par-4 and E2F1. As shown in Figure 6B (lane 2), from nuclear extracts of the cells transfected with pcDNA3.1-myc-Par-4 and following treated with glutamate, myc-Par-4 was immunoprecipitated using anti-myc antibody, and associated E2F1 were detected by immunoblotting. However, Par-4 deletion mutant, which lacked the COOH terminus of wild-type Par-4 protein, failed to associate with E2F1 (Figure 6B, lane 5). The findings validated the essentiality of the COOH terminus of Par-4 for Par-4-E2F1 complex formation. Taken together, we concluded that the COOH terminus of Par-4, by which Par-4 associated with E2F1 and bound to Smac promoter, was functionally required for Par-4-mediated activation of Smac promoter in hBMSCs.

**DISCUSSION**

In this study, we report for the first time that glutamate can induce apoptosis in hBMSCs by a mechanism involving pro-apoptotic gene Par-4. Glutamate-induced neurotoxicity is an important contributor in acute neuronal damages and in chronic neurodegenerative diseases (16,17). Therefore, implanted stem cells will be sure to suffer from glutamate-mediated injury. We observed that glutamate induced a dose- and time-dependent apoptosis in hBMSCs, which was consistent with previous studies carried out in neural cells (4,16–18). As a pro-apoptotic factor, Par-4 is initially identified as a product of gene upregulated in prostate tumour cells undergoing apoptosis (5). Recently, growing evidence show that increased expression of Par-4 also regulates neuronal apoptosis, especially when exposed to metabolic insults such as glutamate and intracellular calcium elevation (15,19). In the current study, we demonstrated that the suppression of Par-4 expression greatly attenuated apoptosis in glutamate-treated hBMSCs, whereas Par-4 overexpression increased the percentage of apoptotic cells, implying that Par-4 played a critical role in glutamate-induced hBMSCs apoptosis.

Previously, several signal pathways, including WT1/Bcl-2, Fas/Fas ligand, PKC/p38 mitogen-activated protein kinase (MAPK), Ras/NF-kB, DAP-like or ZIP kinase...
Par-4 transcriptional control on Smac ectopic expression in hBMSCs resulted in increased Par-4 mediated apoptosis (6,9,20,21). Our studies showed that blocking endogenous E2F1 decreased Par-4-induced Smac transcription, indicating that both Par-4 and E2F1 were required for Smac transcription regulation. Numerous studies showed that ectopic expression of E2F1 induced apoptosis by different mechanisms. Apoptosis-related E2F1 target genes, such as p73, Apaf1, noxa, bim and akt, are involved in both p53-dependent and p53-independent apoptotic regulation (22–27). A recent study demonstrated that enhanced accumulation of nuclear E2F1 upregulated Smac expression and subsequently accelerated mitochondria-mediated apoptosis, implying that Smac was an E2F1 target gene (12). In agreement with the study, our data showed that the increased expression of Smac gene in glutamate-treated hBMSCs were E2F1-dependent, at least partially.

We further confirmed that both Par-4 and E2F1 proteins participated in the formation of a complex that bound to the BS3 site (~200 to ~193 bp relative to ATG) on Smac promoter, which was previously identified as a site where E2F1 bound to and increased transcription of Smac gene in human H1299 non-small lung carcinoma cells (12). Our results indicated that this site was also employed to drive the transcription of Smac in hBMSCs. Similar mechanism of Par-4 mediated indirect transcriptional regulation was delineated by Sangeeta and colleagues as well. They demonstrated that Par-4 associated with WT1 and bound to the bcl-2 promoter to transcriptionally regulate Bcl-2 expression in an androgen-independent prostate cancer cell line (28). In particular, our results suggested that it was E2F1, not Par-4, that directly bound to Smac promoter in the DNA–proteins complex, which implied that Par-4, indirectly, exert its transcriptional control on Smac gene though interacting with E2F1.

Consistent with previous studies (6,20), our study validated the functional significance of the COOH terminus of Par-4 protein in hBMSCs apoptosis. The COOH terminus of Par-4 protein containing a leucine zipper domain has been shown to be essential in Par-4 binding to PKC, WT1, ZIPK/Daxx and THAP, and their interactions promoted cell apoptosis (6,20,29). Here we showed that the mutant of Par-4, which lacked 70 amino acids at the COOH terminus, failed to co-immunoprecipitate with E2F1 protein, implying that this region was also required for the interaction between Par-4 and E2F1. With luciferase activity analysis, we found that the increased expression of Par-4 resulted in an increased activity of Smac promoter in a dose-dependent manner, whereas Par-4 mutant failed to activate Smac promoter. Therefore, we further corroborated that the indirect transcriptional regulation of Par-4 on Smac was depended on its COOH terminus-mediated interaction between Par-4 and E2F1.

Recently, it was reported that the COOH terminus of or possibly the full-length Par-4 protein was not required for apoptosis in primary fibroblasts (30). This discrepancy may be due to the different cell types, since in the same experiment settings, depletion of Par-4 in HeLa cells conferred a significant resistant to various apoptotic agents (30).

In summary, we show that the proteins–DNA complex, containing Par-4 protein, E2F1 protein and Smac promoter, contributes to glutamate-induced apoptosis in hBMSCs. The Par-4 mediated indirect transcriptional regulation on Smac gene depended on its COOH terminus-mediated interaction between Par-4 and E2F1. Our studies provide a further understanding of molecular mechanism in glutamate induced hBMSCs apoptosis, which benefit stem-cell therapy in ischemic brain damage.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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