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ORIGINAL ARTICLE

Umbilical cord blood mesenchymal stem cells protect amyloid-β42 neurotoxicity *via* paracrine

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Abstract

AIM: To understand the neuroprotective mechanism of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) against amyloid-β42 (Aβ42) exposed rat primary neurons.

METHODS: To evaluate the neuroprotective effect of hUCB-MSCs, the cells were co-cultured with Aβ42-exposed rat primary neuronal cells in a Transwell apparatus. To assess the involvement of soluble factors released from hUCB-MSCs in neuroprotection, an antibody-based array using co-cultured media was conducted. The neuroprotective roles of the identified hUCB-MSC proteins was assessed by treating recombinant proteins or specific small interfering RNAs (siRNAs) for each candidate protein in a co-culture system.

RESULTS: The hUCB-MSCs secreted elevated levels of decorin and progranulin when co-cultured with rat primary neuronal cells exposed to Aβ42. Treatment with recombinant decorin and progranulin protected from Aβ42-neurotoxicity *in vitro*. In addition, siRNA-mediated knock-down of decorin and progranulin production in hUCB-MSCs reduced the anti-apoptotic effects of hUCB-MSC in the co-culture system.

CONCLUSION: Decorin and progranulin may be involved in anti-apoptotic activity of hUCB-MSCs exposed to Aβ42.

Key words: Human umbilical cord blood-derived mesenchymal stem cells; Decorin; Progranulin; Aβ42; Anti-apoptosis

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INTRODUCTION

Alzheimer's disease (AD) is currently an incurable neurodegenerative disease. The proposed main causes of AD are amyloids, tau theory, mitochondria dysfunction

and chronic inflammation[1,2]. These causes gradually and progressively induce neuronal loss in the brain, which progressively diminishes mental activity. Mesenchymal stem cells (MSCs) have shown promise in the treatment of AD *in vitro* and *in vivo*[3-5]. MSCs can be collected from various human sources including bone marrow, umbilical cord blood, adipose tissue and Wharton's jelly[6,7]. Although MSCs have the capacity for differentiation of into bone, cartilage and adipose, and so are compelling candidates for regenerative therapies, the paracrine action of MSCs has been spotlighted as the major role of stem cells in disease models[8-13].

Our group has studied the paracrine effect of human umbilical cord blood-derived MSCs (hUCB-MSCs) in the treatment of AD *in vitro* and *in vivo*. We found that hUCB-MSC secretes a variety of proteins including galectin-3 for neuron survival[14] and soluble intercellular adhesion molecule-1 (sICAM-1) for the induction of Aβ degrading enzyme and neprilysin on microglia when co-cultured with rat primary neuronal cells during exposure to amyloid-β42 (Aβ42)[15]. These actions reduce Aβ and improve memory deficit in an AD-transgenic mouse model[3].

In this study, we identify two additional soluble proteins, progranulin and decorin, which are released from hUCB-MSC under AD microenviroment. The data provide another example of the paracrine action of hUCB-MSCs for AD therapy.

MATERIALS AND METHODS

Cell culture

This study was approved by the Institutional Review Board of MEDIPOST. Umbilical cord blood was collected from umbilical veins after neonatal delivery with informed consent of the pregnant mothers. hUCB-MSCs were isolated and expanded as described previously[15]. Pregnant Sprague-Dawley rats were purchased from Orientbio (Kyeonggi, South Korea). Brain tissue was dissected from embryonic-day-14 rat cortex and hippocampus, and cells were mechanically dissociated in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution as we have described before[14,15]. Rat primary neuronal cultures were treated with Aβ42 (Sigma-Aldrich, St. Louis, MO, United States) for 12 h or 24 h. hUCB-MSCs (8×10^4 cells/cm^2) were co-cultured in the upper chamber (pore size: 1 μm) of a Transwell apparatus (Falcon) with Aβ42-exposed rat primary neuronal cells or BV2 cells.

Enzyme-linked immunosorbant assay and antibody array

Enzyme-linked immunosorbant assay (ELISA) was performed according to the manufacturer's instructions. A progranulin ELISA kit (R and D Systems, Minneapolis, MN, United States) and a kit for decorin (R and D Systems) were used in analyzing each conditioned medium. The capture antibody was diluted in phosphate-buffered saline and added to a 96-well plate for pre-coating. After overnight incubation, each plate was washed and blocked by the reagent. After aspiration, 100 μL of medium was incubated in wells coated with capture antibody for 2 h. After incubation of streptavidin-horseradish peroxidase, substrate solution was added. For the antibody array, the collected medium was dialyzed at 4 ℃ for 3 h then labeled with biotin reagent according to the recommended protocol. Biotin-labeled proteins in the medium were reacted with a glass chip assembly and further incubated with streptavidin-conjugated fluorescent dye. The glass chip was scanned and analyzed using Analysis Tool Software (Ray-Bio, Norcross, GA, United States).

Small interfering RNAs treatment

Small interfering RNAs (siRNAs) for decorin and progranulin were purchased from Bioneer (Daejon, South Korea) and non-targeted control siRNA was from Thermo Scientific Dharmacon (Chicago, IL, United States). siRNA was treated with Lipofecamine-2000 (Invitrogen, Carlsbad, CA, United States) for 6 h without fetal bovine serum and antibiotics. siRNA treated cells were washed and cultured overnight in Dulbecco's modified Eagle's medium without antibiotics.

Immunostaining

Fixed cells were stained with antibodies for microtubule associated protein 2 (MAP2; Millipore, Billerica, MA, United States) and fluorescein isothiocynate (FITC; Cy3) secondary antibodies (Jackson Immunoresearch Laboratories, Bar Harbor, ME, United States). Stained cells were photographed using a fluorescence microscope (Nikon, Tokyo, Japan).

RESULTS

Co-culture of rat primary neuronal cells enhances secretion of decorin and progranulin by hUCB-MSCs

Since we already reported that co-culture of hUCB-MSCs protects from Aβ-mediated neurotoxicity *in vitro*[14], we sought to confirm these results in a co-cultured system using Live/Dead staining (Figure 1). hUCB-MSCs were co-cultured with Aβ42-exposed rat primary neuronal cells for 24 h in a Transwell chamber. Green and red fluorescence indicated lived and dead rat primary neuronal cells, respectively. The co-culture of hUCB-MSC protects Aβ42-mediated neurotoxicity in rat primary neuronal cells (Figure 1A and B). To identify soluble factors from hUCB-MSC during co-culture, we analyzed secreted proteins in the medium using an antibody-based array (unpublished data). Among the identified proteins, we focused on progranulin and decorin because their protein levels were highly elevated when hUCB-MSCs were co-cultured with rat primary neuronal cells in the presence of Aβ42 (Figure 1C). To confirm the accuracy of the antibody-based array, we performed ELISA to quantify the progranulin and decorin presence in the medium. Progranulin and decorin were also elevated during co-culture compare to hUCB-MSCs cultured alone (Figure 1D). However, Aβ42 exposure slightly inhibited

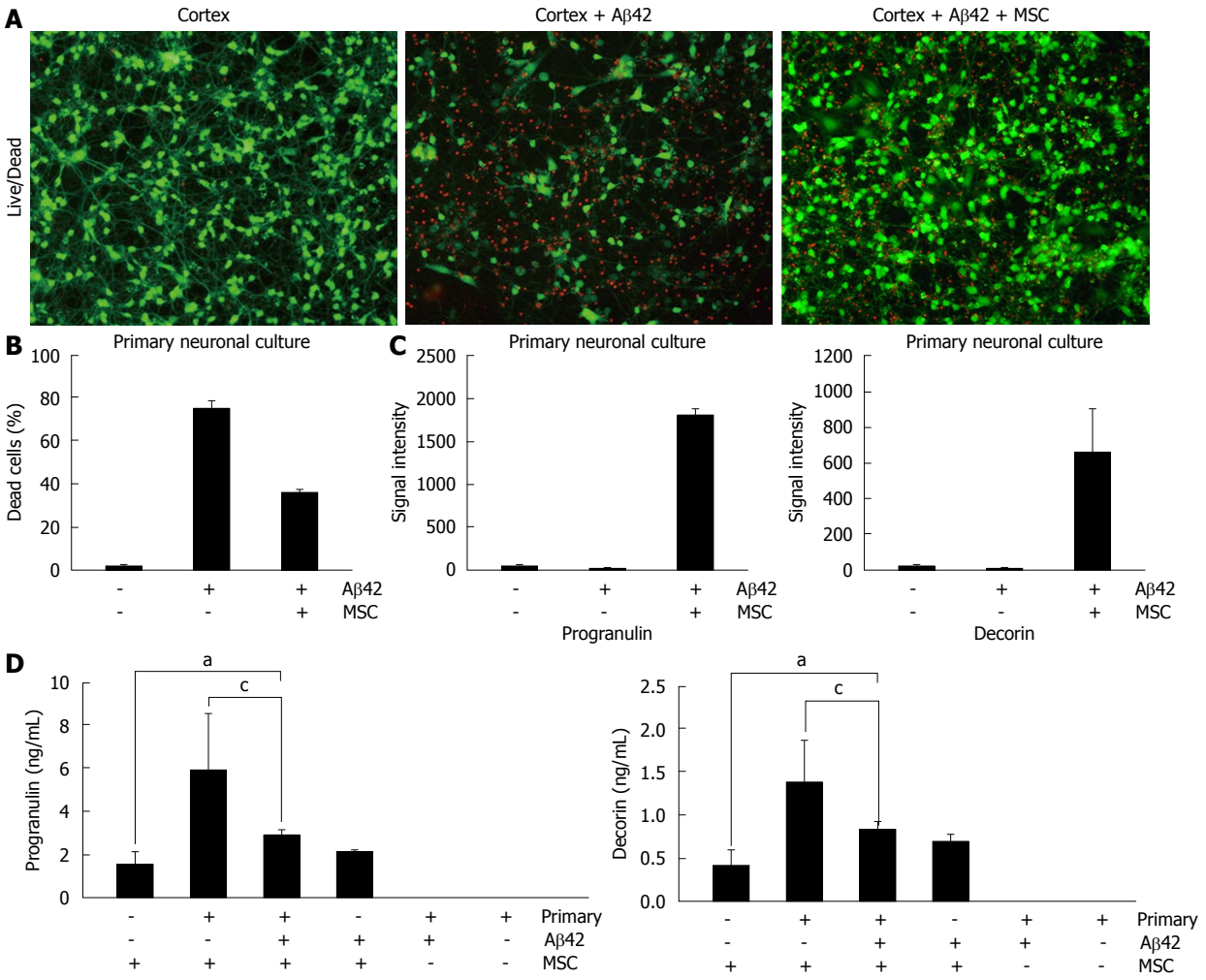

Figure 1 Decorin and progranulin are highly secreted from human umbilical cord blood-derived mesenchymal stem cells. A: Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) were co-cultured with amyloid-β42-exposed rat primary neuronal cells for 24 h in a Transwell chamber. Then, rat primary neuronal cells were stained by Live/Dead staining. Green color indicates surviving neuronal cells. B: Percentage of dead cells was calculated ($P < 0.05$, $n = 4$ per group); C: To identify paracrine factors, co-cultured media was analyzed by antibody-based array (RayBio). Spot intensity of progranulin and decorin in co-cultured media was much higher compared to rat primary neuronal cells in the absence of hUCB-MSC ($P < 0.05$, $n = 3$ per group); D: Each medium used in the Transwell was analyzed by enzyme-linked immunosorbant assay for progranulin and decorin ($^{a}P < 0.05$, $^{c}P < 0.05$, $n = 3$ per group).

secretion of decorin and progranulin ($P < 0.05$). Each ELISA was human specific because the medium used for rat primary neuronal cells did not react with decorin and progranulin of hUCB-MSCs. These data suggest that secretion of decorin and progranulin were induced in hUCB-MSCs by the co-culture of rat primary neuronal cells in the presence or absence of Aβ42.

Treatment of recombinant decorin and progranuiln increases neuron viabilty

To test whether decorin and progranulin participate in the neuroprotection against Aβ42-neurotoxicity, recombinant decorin and progranulin were treated in Aβ42-exposed rat primary neurons at three doses (10 ng/mL, 20 ng/mL and 50 ng/mL). After treatment of recombinant decorin or progranulin for 36 h, rat primary neuronal cells were analyzed by Live/Dead staining. Almost the same neuroprotective effect of decorin and progranulin was apparent at each dose (data not shown). Representative percentage of dead cells using 10 ng of decorin or progranulin in Aβ42-exposed rat primary neuronal cells is shown in Figure 2B. Since neuron and glia cells were mixed in the rat primary neuronal cells, we tried to stain MAP2-positive neurons in recombinant decorin- and progranuin-treated cells exposed to Aβ42 (Figure 2C). Treatment of each protein reduced Aβ-mediated neurotoxicity because MAP2-positive cells were very apparent in Aβ-exposed neurons with decorin or progranulin, compared to controls. These data suggest that secreted decorin and progranulin from hUCB-MSCs have an anti-apoptotic effect against Aβ42-neurotoxicity *in vitro*.

Knock-down of decorin and progranulin secretion reduces the neuroprotective effect of hUCB-MSC

To assess whether the anti-apoptotic effect of hUCB-MSC against Aβ42 was mediated by the action of decorin and progranulin, each siRNA for decorin and

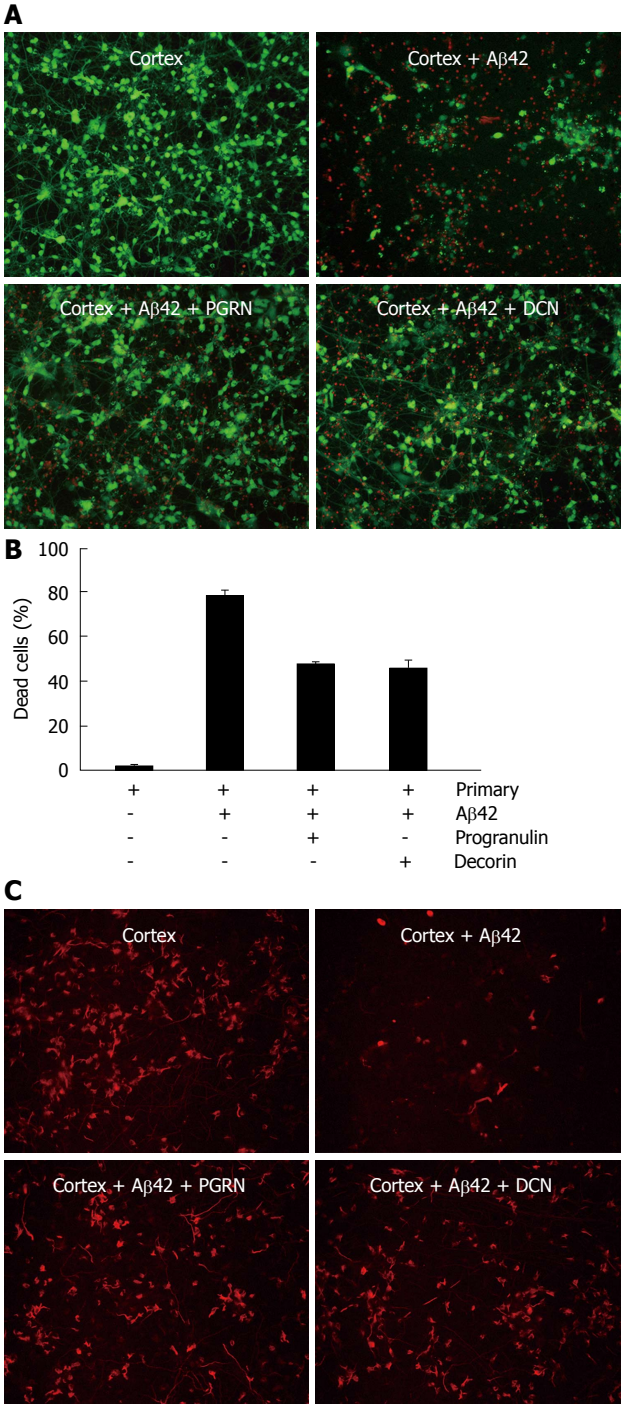

Figure 2 Recombinant progranulin and decroin protect against amyloid-β42-neurotoxicity *in vitro*. A: Instead of human umbilical cord blood-derived mesenchymal stem cells, 10 ng of progranulin and decroin were used to treat amyloid-β42 (Aβ42)-exposed rat primary neuronal cells, After 36 h, each cell was assayed by Live/Dead staining. The green and red colorindicate live and dead cells, respectively; B: Percentage of dead cells in decroin- and progranulin-treated cells exposed to Aβ42; C: To show survived neurons in rat primary neuronal cells, cells were stained by anti-microtubule associated protein 2 antibody. PGRN: Progranulin; DCN: Decorin.

progranulin were used for a 6-h pretreatment of hUCB-MSCs cocultured with Aβ42-exposed rat primary neurons for further 24 h. Pretreatment using either siRNA inhibited the secretion of decorin and progranulin (Figure 3A). To analyze the surviving neurons in rat primary neuronal culture by siRNA treated hUCB-MSCs, MAP2 was visualized by staining with anti-MAP2 antibody (Figure 3B). siRNA treated hUCB-MSCs were not protective against Aβ42 neurotoxicity. The percentage of surviving neurons is depicted in Figure 3C. The data suggested that decroin and progranulin released from hUCB-MSC mediate an anti-apoptotic effect of hUCB-MSCs against Aβ42 neurotoxicity *in vitro*.

DISCUSSION

MSC display paracrine action in pathological conditions[16]. Especially, we observed that hUCB-MSCs also secreted a variety of proteins by incubation with body fluid collected from patients (unpublished data). When we analyzed co-cultured media by various biochemical approaches such as antibody array, we identified several proteins including galectin-3 and sICAM-1. We have previously reported on the function of each identified protein in an AD model[14,15]. Here, we report on the role of decorin and progranulin in hUCB-MSC-action in an AD model. hUCB-MSCs seem to act simultaneously in an AD microenviroment because Aβ reduction and increased neuron survival were observed after application of the hUCB-MSCs.

During co-culture of hUCB-MSCs with rat primary neurons, secretion of decorin and progranulin were increased in the presence or absence of Aβ42 (Figure 1). These elevations were confirmed by two-different methods: antibody-based array and ELISA. Since hUCB-MSCs protected from Aβ42 neurotoxicity *in vitro*, we tested whether decorin and progranulin participated in the neuroprotection of hUCB-MSCs in an AD model. Human recombinant decorin and progranulin were used to treat Aβ42-exposed rat primary neurons for 24–36 h and then neuronal survival was tested *in vitro*. The two proteins protected against Aβ42 neurotoxicity *in vitro*. These data support the suggestion that decorin and progranulin are soluble factors which are released from hUCB-MSC to protect Aβ42 neurotoxicity *in vitro*.

The role of decorin in cell survival has been reported in various disease models. Decorin expressed in endothelial cells prevents apoptosis of the cells in a collagen lattice[17]. In a myocardial infarction model, decorin treatment significantly mitigated fibrosis compared to control[18]. In addition, decorin stimulated cell proliferation and reduced apoptosis in the infarct area. Especilly, decorin promotes robust axon growth on inhibitory CSPGs and myelin *via* a direct effect on neurons[19]. Decorin pretreatment of meningial fibroblasts *in vitro* resulted in a three-fold increase in neurite outgrowth from co-cultured adult sensory neurons[20]. The secretion of decorin has been reported[21, 22]. Expression of decorin in adipose progenitor cells[23] supports the idea that hUCB-MSCs secrete decorin.

Recently, mutations in the progranulin (*PGRN*) gene were found to cause familial and apparently sporadic

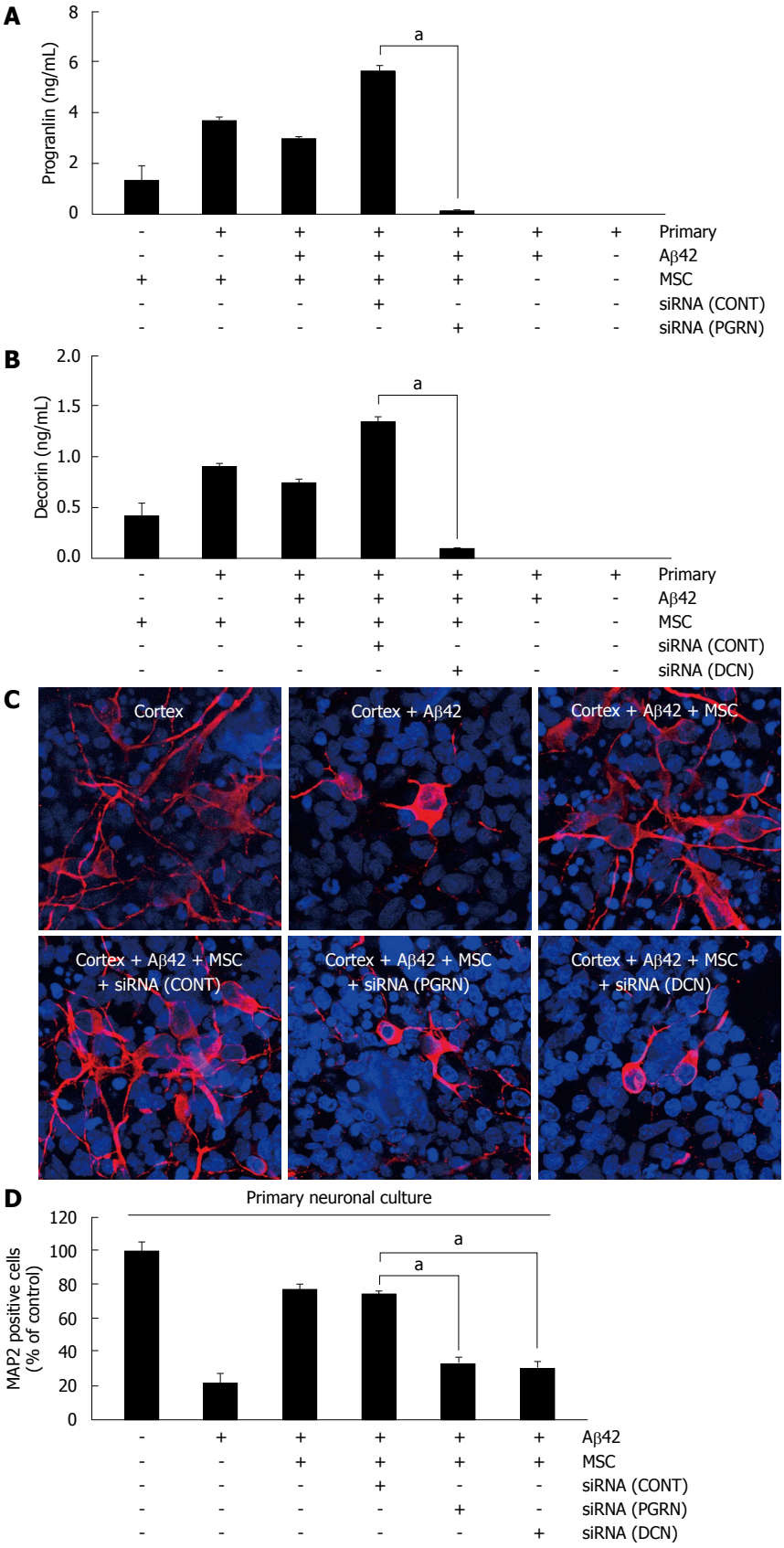

Figure 3 Knock-down of decorin and progranulin by siRNAs reduces the anti-apoptotic effect on human umbilical cord blood-derived mesenchymal stem cells in a Transwell chamber. A, B: Each siRNA was pretreated in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSC) for 6 h. These cells were co-cultured with rat primary neuronal cells for 24 h. After co-culturing, the medium was analyzed by enzyme-linked immunosorbant assay for progranulin (A) ($^aP < 0.05$, $n = 3$ per group) and decorin (B) ($^aP < 0.05$, $n = 3$ per group); C: Rat primary neurons in each condition were stained by anti-microtubule associated protein 2 (MAP2) antibody. Red color indicates MAP2-positive neurons and nuclei were visualized by DAPI (blue); D: The percentage of MAP2-postive neuron in each conditions were analyzed ($^aP < 0.05$, $n = 3$ per group). CONT: Control; PGRN: Progranulin; DCN: Decorin.

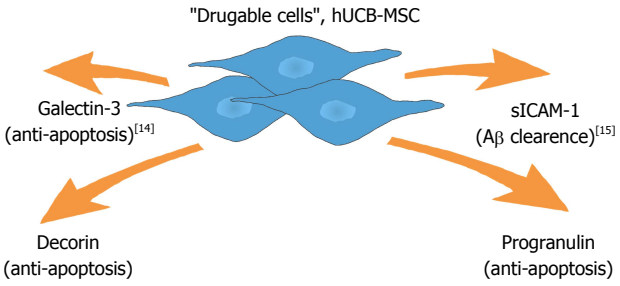

Figure 4 Paracrine action of human umbilical cord blood-derived mesenchymal stem cells in amyloid-β42 neurotoxicity *in vitro*. We previously reported that human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) secrete soluble intercellular adhesion molecule-1 (sICAM-1) and galectin-3 for Aβ clearance and neuron survival, respectively. We also showed anti-apoptotic role of decorin and progranulin in amyloid-β42-neurotoxicity *in vitro*. Recently, MSCs have become regarded as drugable cells because they secrete various beneficial proteins for healing of damaged sites.

frontotemporal lobe dementia[24]. Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. These data indicated that mutations in PGRN as a cause of neurodegenerative disease and indicate the importance of PGRN function for neuronal survival[25]. Interestingly, it has been demonstrated that progranulin neurotrophic factor enhances neuronal survival and axonal growth[26]. PGRN stimulates ribosomal S6 kinase (p90RSK) and phosphatidylinositol-3 kinase/Akt cell survival pathways, and rescues cortical neurons from cell death induced by glutamate or oxidative stress. Although progranulin is a well-known secreted protein[25], there is no report regarding secretion of progranulin in MSCs. Since we confirmed mRNA expression of progranulin in hUCB-MSCs (unpublished data) and since ELISA with rat-derived progranulin was negative (Figures 1 and 3), progranulin is expected to be secreted from hUCB-MSCs. Collectively, these reports support the view that decorin and progranulin are survival factor for cells in neurodegenerative diseases.

In previous reports, we have been shown that hUCB-MSCs exhibited neuroprotection in an AD model *via* paracrine action. Especially, sICAM-1 was released from hUCB-MSCs and stimulated microglia to produce the A degrading enzyme neprilysin[15]. Presently, we implicate progranulin and decorin as additional paracrine factors that exert an anti-apoptotic effect against Aβ42-neurotoxicity (Figure 4). Since hUCB-MSCs seem to act as part of a cocktail of several drugs, we expect the emergence of paradigm-shifting approaches such as stem cell therapeutics for AD in the near future.

COMMENTS

Background

Human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) has been regarded as a fascinating candidate of stem cell therapy in alzheimer's disease (AD). Recently, we reported that transplantation of hUCB-MSC reduced amyloid plaques *via* release of soluble intercellular adhesion molecule-1 *in vitro* and *in vivo*. The paracrine action has been spotlighted as a main mechanism of action for hUCB-MSC.

Research frontiers

In this study, the authors identified paracrine factors of hUCB-MSC for Aβ42 neurotoxicity *in vitro*. This data will be additional example of paracrine action of hUCB-MSC in AD microenvironment.

Innovations and breakthroughs

Stem cells are important source for not only regeneration but also paracrine action. The data provided real protein factors to protect Aβ42 neurotoxicity in vitro.

Applications

The study results suggest that hUCB-MSC is a potential therapeutic material that could be used in treatment for AD.

Peer review

This is a good descriptive study in which authors analyze the therapeutic effect of hUCB-MSC on AD Induced by toxix amyloid beta proteins. The results are interesting and suggest that hUCB-MSC is a potential therapeutic source of stem cells that could be used in treatment of AD.

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