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Different expression patterns of growth factors in rat fetuses with spina bifida aperta after *in utero* mesenchymal stromal cell transplantation

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Abstract

Background aims. In a previous study, we successfully devised a prenatal surgical approach and transplanted mesenchymal stromal cells (MSCs) to fetal rat spinal column to treat retinoic acid–induced neural tube defects in rat. Our results show that MSCs survived, migrated and differentiated into neural lineage cells. We intended to study various growth factor expressions in rat fetal spinal cords with spina bifida aperta after *in utero* MSC transplantation and the effect of *in vivo* growth factor introduction for prenatal spina bifida treatment. *Methods.* Pregnant rats were treated with retinoic acid on embryonic day 10 and then received fetal surgery for MSC transplantation and/or lentiviral epidermal growth factor (EGF) injection on embryonic day 16; various growth factor expression in spinal cords from embryonic day 20 fetuses were analyzed by means of quantitative reverse transcriptase–polymerase chain reaction. Terminal deoxynucleotidyl transferase dUTP nick end labeling analysis was performed to observe spinal tissue apoptosis. *Results.* Growth factor expression was dysregulated in spinal cords with spina bifida. After MSC transplantation, we observed significantly increased expression of EGF, fibroblast growth factor (FGF)-8, FGF-2 and FGF-20 in the MSC transplantation group compared with blank injection; Furthermore, EGF expression positively correlated with surviving MSC amounts. Expression of other growth factors was not significantly different. *In vivo* EGF introduction reduced spinal tissue apoptosis. *Conclusions.* Our results suggest that intrinsic EGF and FGF-2, FGF-8 and FGF-20 might affect the *in vivo* fate of transplanted MSCs in a fetal rat spina bifida model. *In vivo* EGF introduction together with MSC transplantation might serve as a new strategy for prenatal spina bifida treatment.

Key Words: *growth factors, in utero MSC transplantation, mesenchymal stromal cells, neural tube defects, prenatal treatment*

Introduction

Neural tube defects (NTDs) are complex congenital malformations resulting from incomplete neurulation during the fourth week of gestation. Spina bifida and anencephaly are the most common and severe forms of NTDs, affecting approximately one in 2000 live births worldwide (1). Despite early diagnosis of the defects, in most of the cases with prenatal alpha-fetoprotein (AFP) screening and ultrasonography, treatment options for the affected fetuses are still limited and not satisfactory. Fetal surgical repair of NTDs in fetuses has been shown to reduce the incidence of shunt-dependent hydrocephalus as well as hindbrain herniation in some patients. However, prenatal repair of the defects did not improve neurological outcome of patients. Individuals with lumbosacral spina bifida continue to have motor and sensory dysfunction of the lower limbs and dysregulated anal and urethral sphincters after birth (2).

We have shown that sensory and motor neuron deficiency is a primary anomaly coexisting with spinal malformation, which suggests that poor functional outcomes of surgical repair of the unclosed neural tube could be caused by deficiency of sensory and motor neurons (3,4). Thus, in a previous study, we successfully established a new therapy for the potential treatment of spina bifida aperta with *in utero* mesenchymal stromal cell (MSC) transplantation, and our data indicate that MSCs survived, migrated and differentiated into neurons in the spinal cord and reduced spinal neuron apoptosis (5). Our results suggest that prenatal MSC transplantation can be used to treat spinal neuron deficiency in NTDs and might serve as a potential treatment option for other congenital anomalies; however, the mechanism of *in vivo* survival and migration of transplanted MSCs were not yet investigated.

Various growth factors have been reported to be involved in stem cell survival, migration, differentiation

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and tissue regeneration (6–9). Vascular endothelial growth factor (VEGF) is involved in many central nervous system disorders, is a key regulator of the paracrine effects of MSCs and was reported to significantly enhance the dopaminergic differentiation of human umbilical cord MSCs *in vivo* in a rat model of Parkinson disease (10,11). Transforming growth factor (TGF)-β, the prototypical member of the TGF-β family, regulates a broad range of cellular responses, including cell proliferation, differentiation, adhesion, migration and apoptosis. It was reported to regulate differentiation, expansion and migration of MSCs *in vitro* (12,13). TGF-α is an important mediator of wound healing and the injury response; it increased paracrine factors secretion of MSCs *in vitro* (14). Insulin-like growth factor (IGF-1) mediates several regenerative processes, including modulation of inflammatory responses, apoptosis and proliferation, and was shown to stimulate differentiation and migration of cultured MSCs (15). Epidermal growth factor (EGF) is a general growth factor that exerts various actions including cell migration and proliferation on a wide variety of cells. MSCs showed significantly increased proliferation and migration in response to EGF, and EGF significantly protected MSCs from apoptosis (16,17). Platelet-derived growth factor A (PDGF-A) affects cell proliferation, survival, migration and differentiation by way of paracrine or autocrine interaction, and the migratory activity of MSCs was greatly increased with PDGF-A treatment *in vitro* (18). Furthermore, fibroblast growth factors (FGFs) have been implicated in numerous cellular processes, including proliferation, migration, differentiation and survival. FGF family members also have broad effects on nervous system development, neural disease and neural repair (19), and various FGF family members (FGF-2, FGF-8, FGF-20 and so on) were used for neural induction of MSCs *in vitro*. On the basis of the above observation, to explore which growth factor affects the fate of MSCs after *in utero* MSC transplantation in our retinoic acid–induced NTD rat model, we evaluated VEGF, EGF, IGF, TGF-α, TGF-β, PDGF and FGF family members FGF-2, FGF-4, FGF-7, FGF-8, FGF-10 and FGF-20 expression in an NTD rat model.

Methods

Experiment animals

Outbred Wistar rats, 10–12 weeks of age (250–300 g) and 4 weeks of age (approximately 100 g), were purchased from the animal center of China Medical University. The appearance of vaginal plugs in female rats the morning after mating was timed as the embryonic day 0 (E0). Spina bifida aperta was induced with the use of intragastric retinoic acid administration (single dose, 140 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) on E10 as previously described (5,20). All animal experiments were performed with approval obtained from the ethics committee of China Medical University.

Isolation, culture expansion and transfection of bone marrow–derived MSCs

MSC cultures were performed as previously reported (5). Rat MSCs were cultured in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 IU/mL penicillin–100 μg/mL streptomycin (Life Technologies) on 25-cm^2 tissue culture flasks (BD Biosciences, Franklin Lakes, NJ, USA). Twenty-four hours before transplantation, MSCs were transfected with enhanced green fluorescent protein (GFP) expression adeno-5 vector (SinoGenoMax Co, Ltd, Beijing, China) (100 pfu per cell), for the visualization of MSCs after transplantation into the rat fetus. Before transplantation, cells were trypsinized, centrifuged and resuspended in small aliquots of fresh medium.

Lentiviral EGF preparation, purification and in vivo microinjection

Oligonucleotides encoding for rat EGF (ATGAACAGCGACAGCGAGTGCCC CCTGAGCCACGACGGATACTGTCTGCACGACGGCGTGTGCATGTACATCGAGGCCCTGGATAAGTACGCCTGCAACTGCGTCGTGGGCTACATCGGCGAGAGATGCCAGTACAGGGACCTGAAGTGGTGGGAGCTGAGA) were synthesized and fused with DsRed monomer and were then cloned into pLenti6.3_MCS vector under the control of cytomegalovirus promoter (customized services provided by Life Technologies). Purified plasmid was transfected into 293T cells together with ViraPower Lentiviral Packaging Mix (K497500; Life Technologies); conditioned medium were collected 48 h after transfection, centrifuged at 50,000 rpm for 2 h and the lentiviral EGF was then resuspended with opti-MEM (minimum essential media). The titer of lentiviral EGF used for *in vivo* injection was 1.45*108 TU/mL. For each injection, approximately 0.2 μL of lentiviral EGF or control lentivirus was injected into the defective region of spinal cord with the use of a micropipette.