***In Vitro* Differentiation of Canine Celiac Adipose Tissue-Derived Stromal Cells into Neuronal Cells**

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(Received 18 July 2007/Accepted 10 December 2007)

ABSTRACT. To investigate *in vitro* differentiation of canine adipose tissue-derived stromal cells (ATSCs) into neuronal cells, ATSCs from celiac adipose tissue in clinically healthy beagle dogs were treated with 100 μ M dibutyl cyclic adenosine monophosphate (dbcAMP) and 125 μ M isobutylmethylxanthine (IBMX). ATSCs were morphologically changed into differentiated ATSCs from spindle-shaped cells to neuron-like cells with numerous processes after the treatment. Expression of neuron-specific enolase (NSE) as an early neuron specific marker protein was detected in both ATSCs and differentiated ATSCs, however diachronic increase of NSE expression was observed in differentiated ATSCs after the treatment with dbcAMP/IBMX. In addition, neurofilament-68 (NF-68) as an early to mature neuron specific marker protein was weakly expressed in differentiated ATSCs. Neuron specific glutamate and glucose transporter (EAAC1 and GLUT-3, respectively) mRNAs were strongly expressed in differentiated ATSCs compared with those in ATSCs, although glia specific glutamate transporter mRNA (GLT-1) was also detected in differentiated ATSCs. ATSCs can differentiate into early to mature neuronal cells and are candidate cells for autologous nerve regeneration therapy, although additional research is needed to examine functional characteristics of differentiated ATSCs.

KEY WORDS: adipose tissue-derived stromal cell, canine, dbcAMP/IBMX, *in vitro* differentiation, neuronal cell.

J. Vet. Med. Sci. 70(4): 353–357, 2008

Regeneration therapy transplanting progenitor cells into necrotized and/or degenerated tissues has been applied to various diseases, especially nervous diseases, since the neuronal cell has poor ability of self-renewal. Various stem cells included embryonic stem cells (ES cells) have been examined to obtain transplantable progenitors of neural cells for nerve regeneration therapy [5, 17, 23]. However, there are ethical and legal problems, immune rejection, and forming organoid tumors despite of their powerful potentials. Adult stem cells, neural stem cells (NSCs), are also obtained from forebrain subventricular zone, however great damages and problems are usually developed like as ES cells [19, 20].

Among various adult stem cells, adipose tissue-derived stromal cells (ATSCs) are multipotential adult mesenchymal stem cells and differentiate into mesenchymal lineage cells such as adipocytes, osteocytes, chondrocytes, and myocytes [22, 24]. Recently, Ashjian *et al.* [1] reported that ATSCs was able to differentiate into nonmesodermal cells included ectodermal neural cells. However, there are few reports on *in vitro* differentiation of ATSCs into neuronal cells in the dog. In this study, *in vitro* differentiation of canine celiac ATSCs into neuronal cells by chemical treatment with dibutyl cyclic adenosine monophosphate

(dbcAMP) and isobutylmethyl- xanthine (IBMX) was investigated for obtaining transplantable autologous progenitors of neuronal cells.

MATERIALS AND METHODS

In this study, animal experiments were carried out in accordance with the Guidelines for Use of Laboratory Animals, Postgraduate School of Agricultural and Life Sciences, the University of Tokyo, Japan.

Reagents: Anti-human neuron-specific enolase (NSE) antibody was obtained from Dako (A/S, Denmark). Anti-pig neurofilament 68 kDa (NF-68) and anti-bovine microtubule associated protein (MAP-2) antibody, Dulbecco's modified Eagle's Medium (DMEM), dbcAMP, Triton-X 100, and Tween-20 were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Fetal bovine serum (FBS) was obtained from GIBCO laboratories (Grand Island, NY, U.S.A.). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Goat serum was obtained from Bethyl Laboratory (Montgomery, TX, U.S.A.). Isobutylmethylxanthine (IBMX) and all other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Adipose tissue-derived stromal Cells (ATSCs): ATSCs were prepared according to the method of Wu *et al.* [22]. Briefly, celiac adipose tissues were collected aseptically from 1- to 2-years-old beagle dogs under general anesthesia

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or euthanasia. The tissue (approximately 5 g) was minced into small pieces and digested in DMEM containing 2 mg/ml collagenase, 4 mg/ml bovine serum albumin (BSA), and 20 mM N-2-Hydroxyethyl- piperazine-N'-2-ethanesulfonic acid (HEPES) in a shaking water bath at 37°C for 1 hr. The digested solution was filtered through a nylon mesh with 100- μ m pores, treated with hemolysis buffer (154 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) for 10 min, and centrifuged. The cell pellet obtained was washed 3 times with DMEM containing 10% FBS, resuspended in the same medium, and cultured at a cell density of 0.5×10^4 in 90 mm culture plate at 37°C under 5% CO_2 atmosphere. The cells passaged 3 times were used as ATSCs in this study. The medium was replaced every 5 days.

In vitro differentiation of ATSCs: For differentiation of ATSCs, chemical treatment was performed on the confluent ATSCs in accordance with a protocol reported by Deng *et al.* [4]. Briefly, the cells were cultured in differentiating medium (DMEM containing 100 μ M dbcAMP and 125 μ M IBMX) for 10 days.

Immunoblot and immunocytochemical analysis: ATSCs and differentiated ATSCs were washed 3 times with PBS, added 0.5 ml detergent solution (135 mM NaCl-Tris, 0.1% Triton-X 100, 1.6 mM phenylmethanesulfonyl fluoride, pH 7.4), mixed, and centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatants were collected and measured protein concentration by a protein assay kit (Bio-Rad, Laboratory, Hercules, CA, U.S.A.) using bovine serum albumin as a standard. Then, 20 μ g of each cellular protein was applied to 8% polyacrylamide gel and separated by electrophoresis. The separated protein was transferred onto nitrocellulose membrane using Trans-Blot Cell (Bio-Rad). The membrane was blocked with 5% skim milk in Tris-buffered saline containing Tween-20 (TBS-T; 50 mM Tris, 153 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 hr, and washed 3 times with TBS-T. Then, the membrane was incubated with anti-NSE (1:1,000), anti-NF 68 (1:500), or anti-MAP-2 (1:500) antibody as first antibody for 2 hr, and washed 3 times with TBS-T. The membrane was incubated with HRP-conjugated anti-mouse IgG (1:1,000) as second antibody for 1 hr, washed 3 times with TBS-T, and visualized by chemiluminescence on ECL films (Amersham).

Expression of NSE in differentiated ATSCs was also examined by immunocytochemical analysis. Differentiated ATSCs cultured on PDL/laminin coated coverslip (ϕ 12mm) were fixed in Phosphate buffered saline (PBS) containing 2% paraformaldehyde for 30 min at 4°C and incubated in

PBS containing 0.1% Triton-X 100 for 30 min at 4°C, followed by washing 3 times with PBS. Then, the cells were incubated with anti-NSE antibody (1:500) as first antibody in PBS containing goat serum (1:500) for 2 hr at room temperature and washed 3 times with PBS. The reaction was visualized by incubating with fluorescein thiocyanate (FITC) conjugated anti-mouse IgG for 1 hr at room temperature. The cells were washed 3 times with PBS, dried, mounted by Prolong antifade kit (Invitrogen, Carlsbad, CA, U.S.A.), and observed by fluorescence microscopy (ORCA-1394; Hamamatsu Photonics, Hamamatsu, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR): Expression of neuron and glia specific glutamate transporter (EAAC1 and EAAT4, and GLT-1, respectively) mRNA, and neuron specific glucose transporter (GLUT-3) mRNA in ATSCs and differentiated ATSCs were examined by RT-PCR. Briefly, total RNA was extracted from both cells using Rneasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacture's extraction protocol. Total RNA was treated with DNase I, and then purified by a clean up procedure. Single-stranded cDNA was synthesized from 1 μ g RNA using SuperScript II 1st strand system (Invitrogen, Gaithersburg, MD, U.S.A.). Primer pairs against EAAC1, EAAT4, GLT-1, and GLUT-3 were made from each human sequence (Table 1). Templates of cDNA were amplified with Ampli Taq Gold (Roche, Branchburg, NJ, U.S.A.). Briefly, a protocol of initial denaturation at 95°C for 9 min and 35 cycles amplification with 30 sec denaturation at 95°C, 30 sec annealing at 58°C, and 90 sec extension at 72°C was carried out. Amplification products were visualized by agarose gel electrophoresis.

RESULTS

Morphology: ATSCs were morphologically changed from flat and spindle-shaped cells into small rounded neuron-like cells with several branching extensions and refractile characteristics by 3 days after the treatment with dbcAMP/IBMX (Fig. 1). Approximately 60% of ATSCs were changed into differentiated ATSCs at 10 days.

Expression of neuron marker protein: Strong expression of NSE as an early neuron marker protein was detected in differentiated ATSCs compared with that in ATSCs by immunoblot analysis. NF-68 as an early to mature neuron marker protein was weakly expressed in differentiated ATSCs. On the other hand, expression of MAP-2 as a mature neuron marker protein was detected neither in

Table 1. Primers used in RT-PCR

EAAC1	sense:1001→1023 antisense:1411←1433	5'-GATCTCTTCCAGTTCAGCCACAC-3' 5'-GAAACATCCATCTGCTCCAGCTC-3'
EAAT4	sense:261→283 antisense:622←644	5'-ACCAGCTTACCTACCGCCAGATC-3' 5'-CCAACCTCGTGCTATACTGCGTC-3'
GLT-1	sense:103→125 antisense:488←510	5'-ACGACAGCCATCTCAGCTCAGAG-3' 5'-GTCCAGCTGCTTCTTGAGTTTG-3'
GLUT-3	sense:491→513 antisense:1018←1040	5'-CAACCTCTTGGCTGTCGCTGGTG-3' 5'-CTGGGAGAGTTGGAGCATGATGG-3'

Fig. 1. Morphological changes of ATSCs by the treatment with dbcAMP/IBMX. Remarkable morphological changes of ATSCs were observed by 3 days after the treatment from flat and spindle-shaped cells into small rounded cells with several branching extensions and refractile characteristics.

ATSCs nor differentiated ATSCs (Fig. 2-a). On immunocytochemical findings, approximately 60% of differentiated ATSCs strongly expressed NSE (Fig. 2-b), while 3% of ATSCs weakly expressed it. In addition, NSE expression was gradually increased in differentiated ATSCs until 10

days during the treatment with dbcAMP/IBMX by immunoblot analysis (Fig. 3).

Expression of neuron-specific glutamate and glucose transporter mRNA: Expression of neuron and glia specific glutamate transporter (EAAC1 and EAAT4, and GLT-1, respectively) mRNA, and neuron specific glucose transporter (GLUT-3) mRNA in ATSCs and differentiated ATSCs were examined by RT-PCR. Neuron specific glutamate and glucose transporter (EAAC1 and GLUT-3, respectively) mRNAs were strongly expressed in differentiated ATSCs compared with those in ATSCs, whereas glia specific glutamate transporter mRNA (GLT-1) was also detected in differentiated ATSCs. No remarkable expression of EAAT4 mRNA as neuron specific glutamate transporter, however, was detected in differentiated ATSCs or ATSCs (Fig. 4).

DISCUSSION

Transplantable progenitors of neural cells are required for autologous nervous regeneration therapy, since neural cells show a limited capacity for intrinsic repair after injury. Various adult stem cells have been examined to obtain progeni-

Fig. 2. Expression of neuron marker proteins. Strong expression of NSE as an early neuron marker protein was detected in differentiated ATSCs compared with that in ATSC by immunoblot analysis (a) and also detected in those cell body and processes by immunocytochemical analysis (b). NF-68 as an early to mature neuron marker protein was weakly expressed in differentiated ATSCs ((a), arrow head).

Fig. 3. Changes in NSE expression in differentiated ATSCs during the treatment with dbcAMP/IBMX. Dichronic expression of NSE was observed in differentiated ATSCs for 10 days after the treatment with dbcAMP/IBMX.

Fig. 4. Expression of neuron specific glutamate and glucose transporter mRNAs in ATSCs and differentiated ATSCs. Strongly expressions of EAAC1, GLT-1, and GLUT-3 mRNAs were observed in differentiated ATSCs after the treatment with dbcAMP/IBMX compared with those in ATSCs.

tor cells from alternative sources of ES cells and NSCs, both of which have ethical and legal problems, and also induce immune rejection [18]. Among adult stem cells, ATSCs prepared from the adipose tissue are multipotential stem cells to differentiate into neural cells [1, 24] as well as bone marrow-derived stroma cells (BMSCs) [9, 12, 13, 21]. In our knowledge, this is the first report on *in vitro* differentiation of ATSCs into neuronal cells in the dog.

In this study, approximately 60% of ATSCs were morphologically changed into differentiated ATSCs from flat and spindle-shaped cells into small rounded neuron-like cells with various processes by the treatment with dbcAMP/IBMX *in vitro*. Deng *et al.* [4] reported that about 25% of human BMSCs were differentiated into neural cells with neuron-like cell morphology by the same treatment. ATSCs may have a higher potentiality than BMSCs for differentiating into neural cells. Strong expression of NSE as an early neuron marker protein was observed in differentiated ATSCs compared with that in ATSCs by western blotting and immunocytochemical analysis. Differentiated human BMSCs also show NSE expression by the same treatment *in vitro* [4, 15]. The treatment with dbcAMP and IBMX, both of which elevate intracellular cAMP level, induces terminal neuroendocrine differentiation in prostate tumor cells and C6 glioma cells [2, 3, 8, 16]. Therefore, an increase of intracellular cAMP is a trigger of differentiation into early neural

cells for ATSCs like as reported for BMSCs [4].

On the other hand, NSE expression in differentiated ATSCs increased diachronically for 10 days during the treatment in this study. ATSCs can differentiate into early neuronal cells by the treatment with dbcAMP/IBMX *in vitro*, since NSE is a specific neuron marker protein, which is expressed in the early stage of development and is increased by morphological and functional differentiation of neuronal cells [11]. In addition, expression of NF-68 as an early to mature neuron marker protein [10] was detected in differentiated ATSCs, although no expression of MAP-2 as a mature neuron marker protein [6] was detected in differentiated ATSCs. No expression of mature neuron marker proteins such as MAP-2 and NF-70 are also observed in differentiated human BMSCs by the same treatment with dbcAMP/IBMX [4]. ATSCs may have a higher potentiality to differentiate into neuronal cells than BMSCs.

Differentiation of NSCs into neurons is generally accepted to need specific gene activation and expression of specific gene products [5, 17]. Adult rat and human BMSCs are capable to differentiate into neurons by the treatment with dimethylsulfoxide (DMSO)/butylated hydroxyanisole, associated with the expression of NSE and neuron specific intermediate filament (NF-M) [21]. Zuk *et al.* [24] also demonstrated that human ATSCs (processed lipoaspirate cells; PLA cells) differentiated into neurogenic cells, show-

ing neuron-like cell morphology and expressing several proteins consistent with neuronal phenotype. In this study, neuron specific glutamate and glucose transporter mRNAs (EAAC1 and GLUT-3, respectively) were strongly expressed in differentiated ATSCs compared with those in ATSCs, although glia specific glutamate transporter mRNA (GLT-1) was also detected in differentiated ATSCs. Several reports on *in vitro* differentiation of NSCs have shown neuronal cell induction with some accompanying induction of glial cells [14, 21]. ATSCs can differentiate into early to mature neuronal cells with some glial cells by the chemical treatment with dbcAMP/IBMX *in vitro*. It is suggested that another induction condition or prolonged induction time are probably required for ATSCs to differentiate into mature neuronal cells as reported by Ashjian *et al.* [1].

In conclusion, ATSCs can differentiate into early to mature neuronal cells and are candidate cells for autologous nerve regeneration therapy, although additional research is needed to examine functional characteristics of differentiated ATSCs.

REFERENCES

- Ashjian, P. H., Elbarbary, A. S., Edmonds, B., DeUgarte, D., Zhu, M., Zuk, P. A., Lorenz, H. P., Benhaim, P. and Hedrick, M. H. 2003. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. *Plast. Reconstr. Surg.* **111**: 1922–1931.
- Bang, Y., Pirnia, F., Fang, W., Kang, W. K., Sartor, O., Whitesell, L., Ha, M. J., Tsokos, M., Sheahan, M. D., Nguyen, P., Niklinski, W. T., Myers, C. E. and Trepel, J. B. 1994. Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 5330–5334.
- Cox, M. E., Deeble, P. D., Lakhani, S. and Parsons, S. J. 1999. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: Implication for prostate cancer progression. *Cancer Res.* **59**: 3821–3830.
- Deng, W., Obrocka, M., Fischer, I. and Prockop, D. J. 2001. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. *Biochem. Biophys. Res. Commun.* **282**: 148–152.
- Dezawa, M., Kanno, H., Hoshino, M., Cho, H., Matsumoto, N., Itokazu, Y., Tajima, N., Yamada, H., Sawada, H., Ishikawa, H., Mimura, T., Kitada, M., Suzuki, Y. and Ide, C. 2004. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J. Clin. Invest.* **113**: 1701–1710.
- Fukumitsu, H., Ohashi, A., Nitta, A., Nomoto, H. and Furukawa, S. 1997. BDNF and NT-3 modulate expression and threonine phosphorylation of microtubule-associated protein 2 analogues, and alter their distribution in the developing rat cerebral cortex. *Neurosci. Lett.* **238**: 107–110.
- Gage, F. H. 2000. Mammalian neural stem cells. *Science* **287**: 1433–1438.
- Ghosh, P. and Singh, U. N. 1997. Intracellular communication in rapidly proliferating and differentiated C6 glioma cells in culture. *Cell Biol. Int.* **21**: 551–557.
- Jiang, Y., Henderson, D., Blackstad, M., Chen, A., Miller, R. and Verfaillie, C. M. 2003. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**: 11854–11860.
- Lee, M. K., Xu, Z., Wong, P. C. and Cleveland, D. W. 1993. Neurofilaments are obligate heteropolymers *in vivo*. *J. Cell Biol.* **122**: 1337–1350.
- Marangos, P. J., Schmechel, D.E., Parma, A. M. and Goodwin, F. K. 1980. Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. *Brain Res.* **190**: 185–193.
- Petersen, B. E., Bowen, W. C., Patrene, K.D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. and Goff, J. P. 1999. Bone marrow as a potential source of hepatic oval cells. *Science* **284**: 1168–1170.
- Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.
- Sanchez-Ramos, J. R. 2002. Neural cells derived from adult bone marrow and umbilical cord blood. *J. Neurosci. Res.* **69**: 880–893.
- Sanchez-Ramos, J., Song, S., Pelaez, C., Hazzi, C., Stedeford, T., Willing, A., Freeman, T. B., Saporta, S., Janssen, W., Patel, N., Cooper, D. R. and Sanberg, P. R. 2000. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp. Neurol.* **164**: 247–256.
- Sharma, S. K. and Raj, A. B. J. 1987. Transient increase in intracellular concentration of adenosine 3':5'-cyclic monophosphate results in morphological and biochemical differentiation of C6 glioma cells in culture. *J. Neurosci. Res.* **17**: 135–141.
- Su, X., Kameoka, S., Lentz, S. and Majumder, S. 2004. Activation of REST/NRSF target genes in neural stem cells is sufficient to cause neuronal differentiation. *Mol. Cell. Biol.* **24**: 8018–8025.
- Swijnenburg, R. J., Tanaka, M., Vogel, H., Baker, J., Kofidis, T., Gunawan, F., Lebl, D. R., Caffarelli, A. D., de Bruin, J. L., Fedoseyeva, E. V. and Robbins, R. C. 2005. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. *Circulation* **112**: I166–I172.
- Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H. and Weissman, I. L. 2000. Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **97**: 14720–14725.
- Weiss, S., Reynolds, B. A., Vescovi, A. L., Morshead, C., Craig, C. G. and van der Kooy, D. 1996. Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci.* **19**: 387–393.
- Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* **61**: 364–370.
- Wu, P., Sato, K., Yukawa, S., Hikasa, Y. and Kagota, K. 2001. Differentiation of Stromal-Vascular Cells Isolated from Canine Adipose Tissues in Primary Culture. *J. Vet. Med. Sci.* **63**: 17–23.
- Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O. and Thompson, J. A. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* **19**: 1129–1133.
- Zuk, P. A., Zhu, M., Ashjian, P., DeUgarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Benhaim, P. and Hedrick, M. H. 2002. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**: 4279–4295.