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Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells

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Abstract

Neural tissue has limited capacity for intrinsic repair after injury, and the identification of alternate sources of neuronal stem cells has broad clinical potential. Preliminary studies have demonstrated that adipose-derived adult stromal (ADAS) cells are capable of differentiating into mesenchymal and non-mesenchymal cells in vitro, including cells with select characteristics of neuronal/glial tissue. In this study, we extended these observations to test the hypothesis that murine (mu) ADAS cells can be induced to exhibit characteristics of neuronal and glial tissue by exposure to a cocktail of induction agents. We characterized the differentiation of muADAS cells in vitro using immunohistochemistry and immunoblotting, and examined whether these cells respond to the glutamate agonist *N*-methyl-D-aspartate (NMDA). We found that induced muADAS cells express proteins indicative of neuronal/glial cells, including nestin, GFAP, S-100, NeuN, MAP2, tau, and β-III tubulin. Induced muADAS cells express γ-aminobutyric acid (GABA), the NR-1 and NR-2 subunits of the glutamate receptor, GAP-43, synapsin I, and voltage-gated calcium channels. Finally, induced muADAS cells demonstrate decreased viability in response to NMDA. These findings suggest that muADAS cells can be induced to exhibit several phenotypic, morphologic, and excitotoxic characteristics consistent with developing neuronal and glial tissue.
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Keywords: Adipose-derived adult stromal cell; Mesenchymal stem cell; Neural differentiation

Introduction

Cells capable of neuronal differentiation have broad potential for cellular therapies. Neural tissue has a limited capacity for repair after injury, and adult neurogenesis is limited to select regions of the brain (Gage, 2000; Magavi et al., 2000; Rakic, 2002; Temple and Alvarez-Buylla, 1999). Commonly proposed cell sources for neuronal cellular therapies include embryonic stem cells (ESCs), and neural stem cells (NSCs) from embryonic or adult brain tissue (Bain et al., 1995; Brustle et al., 1999; Freed et al., 2001; Lindvall et al., 1990; McKay, 1997). However, the use of ESCs and NSCs is limited by various ethical and logistical constraints, and adult peripheral tissues may be an alternative source of stem and progenitor cells. For example, recent studies have shown that adult bone marrow contains a population of mesenchymal stem cells capable of differentiating into several lineages, including neuronal and glial tissues (Brazelton et al., 2000; Jiang et al., 2002; Kopen et al., 1999; Mezey et al., 2000; Sanchez-Ramos, 2000; Woodbury et al., 2000).

Adipose tissue has been identified as an alternative source of stromal cells capable of differentiating into mesodermal lineages. Previous studies have demonstrated the osteogenic, chondrogenic, adipogenic, myogenic, cardiomyogenic, and hematopoietic potential of stromal cells isolated from adipose tissue (Charriere et al., 2003; Erickson et al., 2002; Halvorsen et al., 2001; Rangappa et al., 2003; Sen et al., 2001; Winter et al., 2003; Zuk et al., 2001). Recently, we and others have shown in preliminary studies that adipose-derived adult stromal (ADAS) cells can express select properties of neuronal and glial cells (Ashjian et al., 2003; Safford et al., 2002; Zuk et al., 2002).

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In the current study, we have extended these observations to test the hypothesis that adipose tissue contains a population of cells that are capable of exhibiting phenotypic, morphologic, and excitotoxic characteristics of neuronal and glial tissues.

Materials and methods

Cell harvest and culture

For isolation of murine ADAS (muADAS) cells, we used Balb/C mice (Charles River Laboratories, Wilmington, MA). Animals were housed under standard conditions, and all animal procedures were approved by the Duke University Institutional Animal Care and Use Committee. After sacrifice of the mice, we harvested subcutaneous adipose tissue, and isolated ADAS cells using a modification of published methods (Safford et al., 2002). Briefly, we mechanically dissociated the adipose tissue, performed a digestion with collagenase type I (Sigma, St. Louis, MO), and centrifuged the suspension to separate the floating adipocytes from the stromal vascular fraction. We plated the cells in the stromal vascular fraction in T75 flasks (Corning, Acton, MA) at a density of 8000–10,000 cells/cm^2. We cultured the cells with Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (GibcoBRL, Rockville, MD) (control media). After 24 h, we removed the non-adherent cells and expanded the adherent ADAS cells by serial passage. All experiments were performed on whole populations of ADAS cells after two to five passages.

Neuronal induction

To induce neuronal differentiation, we exposed the muADAS cells to a cocktail of induction agents as previously published (Safford et al., 2002). Briefly, muADAS cells from passages 2–5 were grown to 80% confluence in control media. To initiate neuronal differentiation, the cells were washed with PBS and neuronal induction media (NIM) was added. NIM consisted of alpha-MEM without serum, with added butylated hydroxyanisole (200 μM), KCL (5 mM), valproic acid (2 mM), forskolin (10 μM), hydrocortisone (1 μM), and insulin (5 μg/ml). All experiments on ADAS cells were performed within 5 h to 5 days following exposure to neuronal induction media.

Our neuronal induction protocol is a modification of previously published protocols (Woodbury et al., 2000; Zuk et al., 2002). Specifically, we modified these protocols to use ethanol (0.5% final concentration) as the solvent for BHA, since we found that dimethylsulfoxide (DMSO, 2% final concentration) resulted in high rates of cell death. Also, we modified these protocols to avoid the exposure of ADAS cells to a pre-induction media of 20% FCS and 1 mM β-mercaptoethanol, since the cell culture media for ADAS cells includes 10% FCS, and our neuronal induction media contain the antioxidant BHA.

Viability assay

To determine the viability of muADAS cells after exposure to neuronal induction media, we used a dye exclusion assay. At daily time points from 1 to 5 days after induction, we added Hoechst dye (Intergen, Purchase, NY, 200 μg/ml)

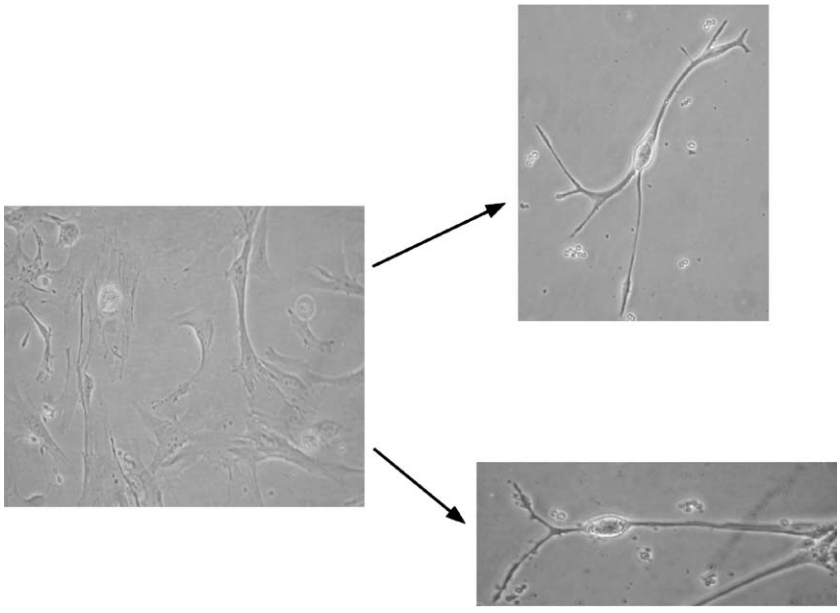

Fig. 1. Morphologic changes following neuronal induction of muADAS cells. (Left) MuADAS cells grown under control conditions grow as a monolayer of large, flat cells. (Right) MuADAS cells after 24 h culture in neuronal induction media display cytoplasmic retraction and a spherical cell body appearance (200 × magnification).

to the cells in culture. The viability of cells grown in control media was determined for comparison to neuronally induced cells. Viability was determined by counting live cells using phase microscopy in three non-overlapping fields. Differences in cell viability between control and induced ADAS cells were compared using Student's *t* test.

Immunocytochemistry

To determine cell phenotype, muADAS cells were grown under either control conditions or exposed to neuronal induction media on chambered slides (LabTek, Napersville, IL). At various time points after neuronal induction (5 h to 5 days), cells were fixed with 4% paraformaldehyde. After fixation, cells were incubated with murine specific monoclonal antibodies directed against the following markers of neuronal and glial cells: nestin, GFAP, S-100, NeuN, MAP2, β-tubulin III, tau, NMDAR-1, γ-aminobutyric acid (GABA), 5HTP, TH, DDC, GAP-43, synapsin I, and pan α-1 voltage-gated calcium channel (all obtained from Chemicon, Inc., Temecula, CA), and NMDAR-2 (Santa Cruz Biotechnology, Santa Cruz, CA). We used an ABC amplification kit (Vector Laboratories, Burlingame, CA and Santa Cruz Biotechnology) with all antibodies. To identify the co-expression of NeuN and GFAP, or MAP2 and tau by muADAS cells, cells were incubated with each antibody sequentially. We used an avidin/biotin blocking kit with Texas Red and fluorescein avidins (Vector Laboratories and Santa Cruz Biotechnology) to label the antibodies.

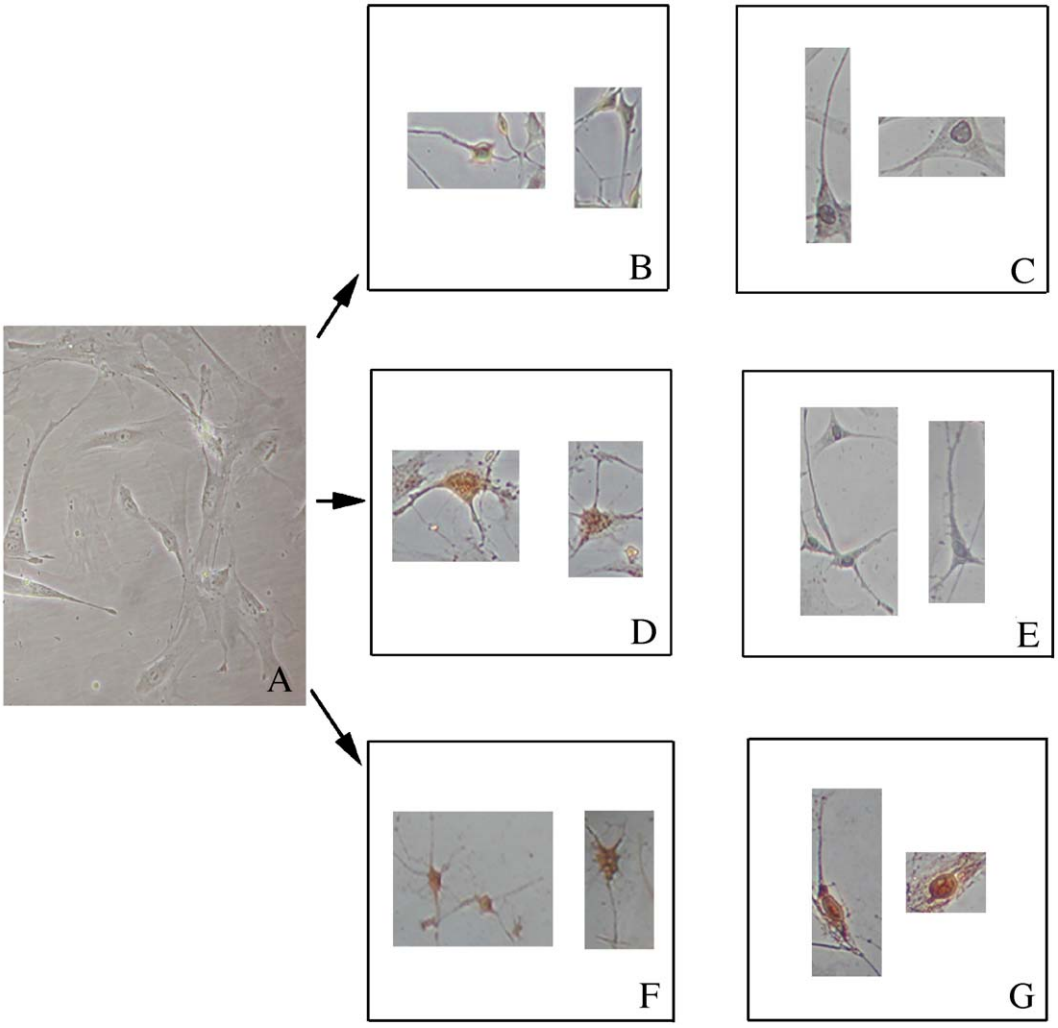

Fig. 2. Immunocytochemistry of muADAS cells for neuronal and glial markers. (A) MuADAS cells grown under control conditions in DMEM/10% FCS. MuADAS cells grown under control conditions expressed low levels of nestin, and undetectable levels of the other following markers, representative image shown. (B) Nestin expression in muADAS cells following neuronal induction, with staining concentrated in the nucleus. (C) NeuN expression in muADAS cells following neuronal induction, with staining primarily nuclear. (D) GFAP expression in muADAS cells following neuronal induction. Staining is evident in both the cytoplasm and nuclei of the majority of cells. (E) β-III tubulin staining after neuronal induction. Staining is evident in both the cytoplasmic processes and the nuclei of the cells. (F) S-100 expression following neuronal induction, with expression seen in both the cytoplasm and nuclei. (G) MAP2 staining after neuronal induction in both the neurite-like processes as well as the cytoplasm and nuclei. Diamino benzidine (DAB) served as a brown chromagen for nestin, GFAP, S-100, and MAP2; nickel solution was added to the DAB substrate as a blue/gray chromagen for NeuN and β-III tubulin detection.

Nestin is an intermediate filament protein expressed by neural stem or progenitor cells (Lendahl et al., 1990). The glial fibrillary acidic protein (GFAP) is a structural element of fibrillary astrocytes. S100 is a calcium binding protein found in astrocytes throughout the brain (Kandel et al., 1991). Neuronal nuclear antigen (NeuN) is a marker of intermediate and mature neuronal nuclei (Mullen et al., 1992). Microtubule-associated protein 2 (MAP2) is a cytoskeletal protein required for neuronal development and maintenance (Kandel et al., 1991). Tau is a microtubule-associated protein found in axonal processes (Kandel et al., 1991; Kosik and Finch, 1987). β-III tubulin is a neuronal cytoskeletal dimer (Kandel et al., 1991). Dopa decarboxylase (DDC) is involved in the synthesis of dopamine and serotonin (Sumi-Ichinose et al., 1992). Derived from tryptophan, 5-hydroxytryptophan (5-HTP) is converted by DDC to serotonin (Kandel et al., 1991). Tyrosine hydroxylase identifies dopaminergic neurons (Feany and Bender, 2000). NR-1 and NR-2 are *N*-methyl-D-aspartate (NMDA) receptor subunits 1 and 2 for glutamate (Patton et al., 1998). GABA is the main inhibitory neurotransmitter in the mammalian CNS (Chebib and Johnston, 1999). Growth-associated protein-43 (GAP-43) is expressed in growing axons, active growth cones, and in the axons of mature neurons (Kandel et al., 1991). Synapsin I is a synaptic vesicle protein (Kandel et al., 1991). Since most neurons have voltage-gated calcium channels, we used a pan α-1 voltage-gated calcium channel marker for neuronal tissue (Kandel et al., 1991).

We incubated ADAS cells with 5–10 μg/ml primary biotinylated antibodies according to kit instructions, and viewed images at 100×–400× magnification. At time points from 1 day to 5 days after neuronal induction, we examined and photographed morphology using bright field and phase-contrast microscopy. Two independent investigators counted the percentage of positive cells per field in random non-overlapping visual fields, using cultures from a minimum of three different experiments.

NMDA-induced excitotoxicity assay

To measure the response of muADAS cells to the glutamate antagonist NMDA, we quantified the excitotoxic effect of NMDA on cell viability. Triplicate cultures of muADAS cells were grown in either control media or neuronal induction media for 24 h. We exposed muADAS cells to 500–1000 μM NMDA for 30 min. As a control, we exposed one group of cells to both 1000 μM NMDA as well as the NMDA antagonist, MK-801 (10 μM), for 30 min. We examined cell viability 24 h after NMDA exposure by incubating cells with Hoechst dye and counting live cells by phase microscopy in three non-overlapping fields, with values expressed as means ± SD.

Western blot

To confirm protein expression following neuronal induction, we harvested muADAS cells grown under control conditions or after 1–5 days of neuronal induction for Western blot analysis by lysing cells in 1× Laemmli (1–4% SDS, 10% glycerol, 100 mM dithiothreitol, 50 mM Tris, pH 6.8). All samples were sonicated and boiled for 5 min. We separated 25 μg protein extracts from whole cell lysates of undifferentiated and neuronal-induced cells on 4–20% gradient or 7.5% non-gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred them to a nitrocellulose membrane using electrophoresis. Blots were

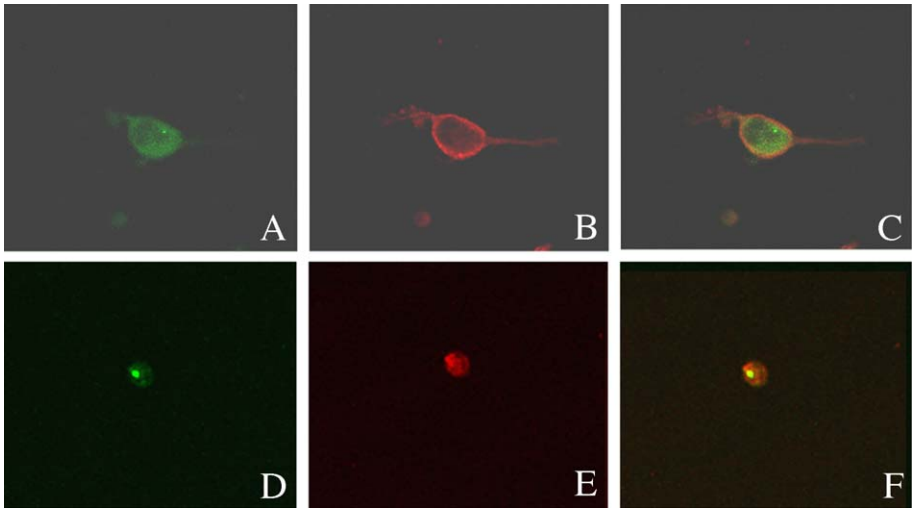

Fig. 3. Fluorescent images of muADAS cells after exposure to neuronal induction media for 24 h. (A–C) Cells were co-stained with monoclonal antibodies against MAP2 (FITC-labeled) and tau (Texas Red-labeled). (A) MAP2 expression; (B) tau expression; (C) merged image confirming co-expression of MAP2 and tau in the same cell. Note that MAP2 and tau expression segregate along the cell body and neurite-like processes, respectively, in a similar fashion to developing neurons. (D–F) Cells were co-stained with monoclonal antibodies against NeuN (FITC-labeled) and GFAP (Texas Red-labeled). (D) NeuN expression in select muADAS cell; (E) GFAP expression in same cell; (F) merged image confirming co-expression of NeuN and GFAP in the same cell, suggestive that select muADAS cells express markers of both neuronal and glial pathways.

stained with amido black (0.5% w/v in 5% acetic acid) and destained with water to confirm transfer and equal loading. We blocked blots in Tris-buffered saline (0.8% NaCl, 2.7 mM KCl, 25 mM Tris, pH 7.4) with 0.05% Tween 20 (TBS-T) and 1% milk/1% bovine serum albumin for 1 h at room temperature. We incubated blots in fresh blocking solution and probed overnight at 4°C with a 1:1000 dilution of primary antibodies against murine nestin, NeuN, GFAP, MAP2, β-tubulin, S-100, synapsin I, and GAP-43 (Chemicon). The blots were washed 3 × 5 min in TBS-T and then incubated with a 1:4000 dilution of peroxidase-conjugated secondary antibody (Chemicon) in blocking solution for 1 h at room temperature. We developed the blots using enhanced chemiluminescence (Pierce, Inc., Rockford, IL). Murine brain protein extract served as a positive control, and actin served as an internal protein control.

Results

In vitro viability of muADAS cells

Undifferentiated muADAS cells maintained 90–95% viability through 5 days of culture. In contrast, after exposure to neuronal induction media, muADAS cells show decreased viability of 70% at day 1, followed by a 10–25% decrease each additional day that the cells remained in neuronal induction media. By day 5, only 35% of the muADAS cells are still viable ($P < 0.01$), and all die within 14 days of culture. Additional experiments will clarify whether surviving induced ADAS cells retain proliferative potential, and whether the decrease in viability is due to apoptosis, cell necrosis, or a combination of these factors.

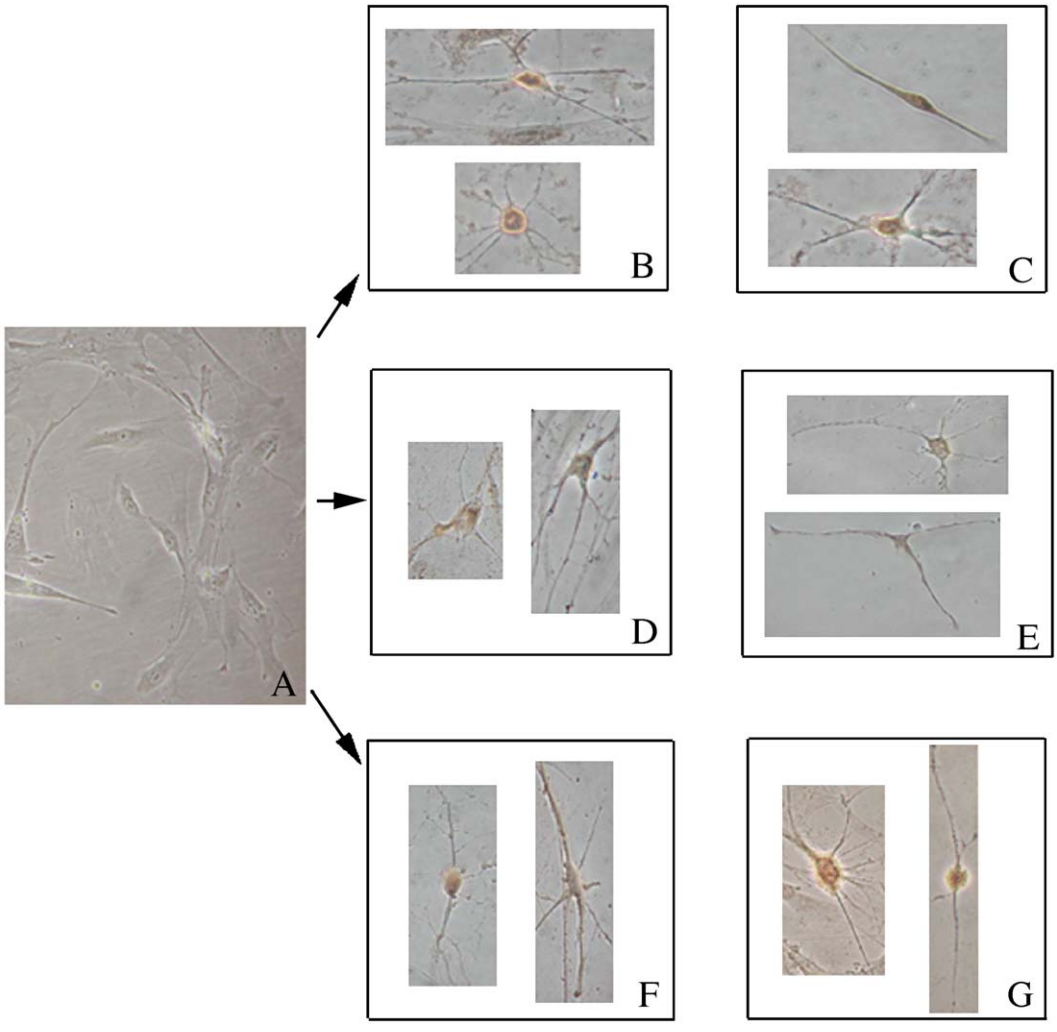

Fig. 4. Immunocytochemistry of muADAS cells stained with antibodies against GAP-43, synapsin I, GABA, NMDA receptor subunits 1 (NR-1) and 2 (NR-2), and pan α-1 subunit of voltage-gated calcium channels. (A) MuADAS cells grown under control conditions expressed low levels of pan α-1 calcium channel marker and synapsin I, and undetectable levels of the other following markers, representative image shown. (B) GAP-43 expression after neuronal induction. (C) Synapsin I staining in muADAS cells after neuronal induction. (D) NR-1 expression seen in muADAS cells following neuronal induction. (E) NR-2 expression seen in muADAS cells following neuronal induction. (F) GABA staining seen in muADAS cells after neuronal induction. (G) Pan α-1 calcium channel marker staining seen in muADAS cells following neuronal induction. Diamino benzidine (DAB) served as a brown chromagen for all markers.

Table 1
Summary of immunocytochemical profile in neuronally induced and control muADAS cells

	Induced ADAS cells	Control ADAS cells
Nestin	+	[a]
GFAP	+	–
S100	+	–
NeuN	+	–
MAP2	+	–
Tau	+	–
β-III tubulin	+	–
GABA	+	–
NMDAR 1	+	–
NMDAR 2	+	–
α-1 Calcium channel	+	[a]
GAP-43	+	–
Synapsin I	+	+
Dopa decarboxylase	–	–
Tyrosine hydroxylase	–	–
5-Hydroxytryptophan	–	–

Immunocytochemical profile of muADAS cells either after neuronal induction or under control conditions (see text). Results summarize the expression of various neuronal and glial cell markers as demonstrated both by immunohistochemistry as well as by Western blot analysis.
[a] Control muADAS cells express low levels of nestin, pan α-1 voltage-gated calcium channel marker, and synapsin I by immunohistochemistry, although only synapsin I was detected by Western analysis.

Histologic changes following neuronal induction

Following transfer of muADAS cells to neuronal induction media, most cells within the culture displayed rapid changes in cellular morphology (Fig. 1). Many cells displayed retraction of the cytoplasm toward the nucleus, forming compact cell bodies with cytoplasmic extensions. The cell bodies became increasingly spherical with multiple cell processes.

Immunocytochemistry following neuronal induction

To characterize neuronal and glial differentiation, we stained control and neuronally induced muADAS cells with a panel of markers against neuronal and glial cells at various stages of differentiation. Before exposure to neuronal induction media, muADAS cells express very low levels of nestin, synapsin I, and the pan α-1 voltage-gated calcium channel marker. Control muADAS cells do not express any of the other neuronal or glial markers examined.

Following exposure to NIM, muADAS cells exhibit immunocytochemical changes consistent with both immature and mature neuronal and glial cells. Following 24 h of exposure to NIM, select muADAS cells stain brightly for nestin (14.4 ± 3.3% of cells/culture) as well as the astrocyte markers GFAP (87.6 ± 5.6%) and S-100 (83.5 ± 4.4%). Induced MuADAS cells also express the neuronal markers β-tubulin III (74.6 ± 6.7%), MAP2 (71.2 ± 5.6%), and NeuN (80.7 ± 13.7%) (Fig. 2). Double staining induced muADAS cells with NeuN and GFAP revealed cells that express both markers, as well as cells that express either the neuronal or astrocyte marker (Fig. 3). Examination of muADAS cells at day 2 and day 5 following exposure to NIM demonstrated similar staining, although cell viability was decreased at each time point. We double-labeled muADAS cells with MAP2 and tau to examine the morpholog-

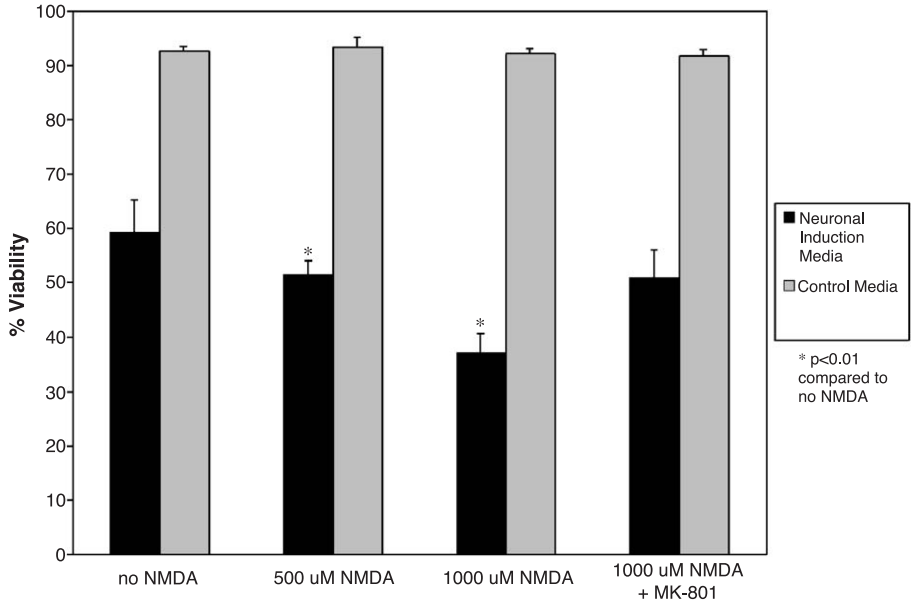

Fig. 5. Excitotoxic effect of NMDA on viability of muADAS cells. Viability was measured by Hoechst dye exclusion. MuADAS cells in control media do not respond to NMDA. Neuronally induced muADAS cells exposed to either 500 or 1000 μM NMDA for 30 min show a significant decrease in viability compared to both cells grown in control media as well as to induced cells not exposed to NMDA. ($P < 0.01$, compared by Student's t test). Exposure of cells to the NMDA antagonist MK-801 blocked the excitotoxic effect of NMDA on induced ADAS cells.

ical polarity of the cells after neuronal induction. A subset of induced cells expresses both tau and MAP2 (Fig. 3).

To see if ADAS cells can be induced to exhibit phenotypes of mature neuronal tissues, we stained induced muADAS cells with a panel of markers against neurotransmitter, neurotransmitter precursor, and neural protein markers. Before exposure to NIM, muADAS cells do not express GABA or NMDAR 1 and 2. Following 24 h of exposure to NIM, we detected GABA and NMDAR 1 and 2 expression in select muADAS cells. The number of cells expressing NMDAR-1 was 5.3 ± 0.9% of the total number of induced cells; 4.6 ± 1.1% of induced cells express NMDAR-2. Examination of muADAS cells at day 5 following exposure to NIM demonstrated similar staining patterns. Following 24 h of exposure to NIM, 38 ± 4.1% of induced cells express GAP-43; 21 ± 2.5% of induced cells express synapsin I. We detected the pan α-1 voltage-gated calcium channel marker on 23 ± 3.8% of neuronally induced cells (Fig. 4). In contrast to GABA, NMDAR, and calcium channel staining, muADAS cells do not stain for the proteins TH, DDC, or 5HTP either under control conditions or after exposure to neuronal induction media. Immunocytochemical results for neuronally induced and control muADAS cells are summarized in Table 1.

NMDA-induced excitotoxicity assay

Before exposure to NIM, muADAS cells did not respond to NMDA as measured by a loss of cell viability in response to NMDA (Fig. 5). In contrast, muADAS cells exposed to NIM for 24 h before exposure to NMDA show a significant decrease in viability in response to both 500 and 1000 μM NMDA ($P < 0.01$), compared to cells grown in control media or exposed to NMDA and MK-801.

Western blot analysis

We found that muADAS cells after exposure to neuronal induction media express select neuronal or glial proteins as demonstrated by Western blot analysis. Before exposure to NIM, muADAS cells do not express nestin, GFAP, NeuN, MAP2, or β-tubulin III. In contrast, after exposure to NIM, muADAS cells demonstrated the presence of GFAP, NeuN, nestin, MAP2, and β-tubulin III (Fig. 6). As reported earlier, the NeuN band detected in muADAS cells after neuronal induction was at a molecular weight of 150 kDa, while the three bands detected in the control mouse brain were present at weights of 46, 48, and 66 kDa (Safford et al., 2002).

Discussion

Our findings suggest that murine ADAS cells can be induced to differentiate into cells with select characteristics of neuronal and glial tissues. Using a series of immunohistochemistry and immunoblotting experiments, we have found that muADAS cells can be induced to undergo morphologic and phenotypic changes consistent with developing neuronal and glial cells. Murine ADAS cells appear to exhibit excitotoxic characteristics in response to NMDA. These cells express several phenotypic properties of neuronal tissue, including the expression of GABA, the NMDAR-1 and

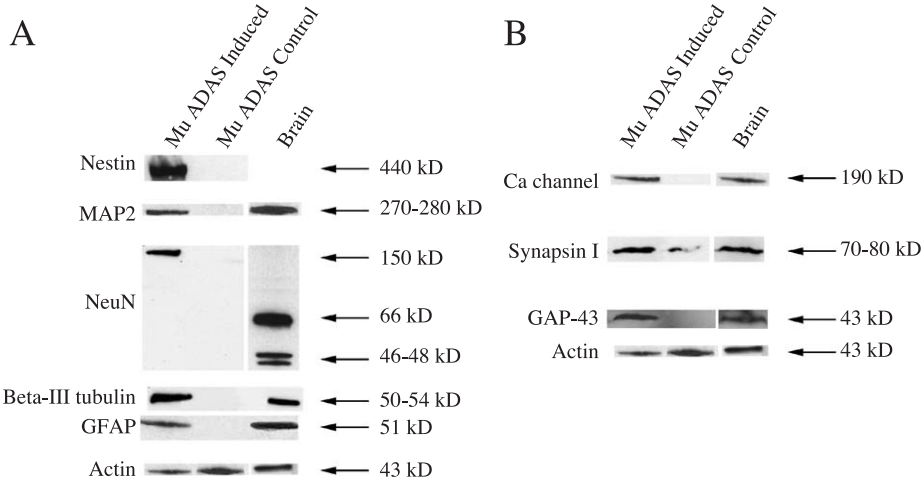

Fig. 6. Western blots of whole cell lysates of muADAS cells grown under either control conditions or following neuronal induction for 24 h. Cell blots were probed with antibodies to nestin, MAP2, NeuN, β-tubulin, GFAP, pan α-1 subunit of voltage-gated calcium channels, GAP-43, and synapsin I, followed by HRP-conjugated secondary antibodies, and developed using enhanced chemiluminescence. Murine brain served as a positive control, and actin served as an internal control. (A) No immunoreactivity from the antibodies tested is detected in muADAS cells grown under control conditions. Following neuronal induction, nestin, MAP2, NeuN, β-tubulin, and GFAP immunoreactivity is seen in the muADAS cells. The NeuN band for muADAS cells is present at a molecular mass of approximately 150 kDa. The three bands for NeuN staining in control brain tissue have molecular weights of approximately 46, 48, and 66 kDa. (B) MuADAS cells grown under control conditions display immunoreactivity for synapsin I. Following neuronal induction, pan α-1 calcium channel marker, GAP-43, and synapsin 1 immunoreactivity is seen in the muADAS cells. All experiments performed in triplicate, representative immunoblots shown.

NMDAR-2 subunits of the glutamate receptor, GAP-43, synapsin I, and α-1 voltage-gated calcium channels.

The expression of markers seen in ADAS cells is similar to the pattern of expression seen in developmental neurogenesis and glialgenesis. Undifferentiated muADAS cells express low levels of the neuronal progenitor marker nestin, similar to several other stem and progenitor cell populations (Gottlieb and Huettner, 1999; Romero-Ramos et al., 2002; Song et al., 2002; Woodbury et al., 2000). After neuronal induction, muADAS cells express β-tubulin III, MAP2, and tau. β-tubulin III and tau are expressed in neurons at an early stage of development, and certain isoforms of MAP2 are expressed early during the neuronal differentiation of neural precursor cells (Moody et al., 1996; Sah et al., 1997). After neuronal induction, select muADAS cells demonstrate MAP2 and tau segregation along the cell body and neurite-like processes, respectively, in a similar fashion to developing neurons. Segregation of MAP2 and tau staining is characteristic of the polarization of axonal and dendritic processes during neuronal differentiation (Kosik and Finch, 1987). Following neuronal induction, select muADAS cells express the astrocyte markers GFAP and S-100; both of these markers are detectable during fetal glial development (Eng et al., 2000; Malloch et al., 1987). Finally, other muADAS cells co-express both NeuN and GFAP, which suggests that at least in this short-term culture, some muADAS cells may retain the potential for neuronal as well as glial development. The regulation of lineage commitment, including possible environmental cues for differentiation, remains to be determined.

To determine further the neuronal phenotypic characteristics of ADAS cells after exposure to neuronal induction media, we stained muADAS cells with a broad panel of markers against neurotransmitter, neurotransmitter precursor, and neural protein markers. We found that select induced ADAS cells express markers of both GABA and glutamate pathways. Recent data propose that early astrocytes or other glial cells, in addition to neurons, may secrete both GABA and glutamate in a form of early nonsynaptic transmitter signaling, suggestive that ADAS cells may be induced along similar pathways (Demarque et al., 2002; Owens and Kriegstein, 2002; Verhage et al., 2000).

In terms of phenotypic markers of functional neuronal tissues, we found that select induced ADAS cells express both subunits of the glutamate NMDA receptor, which suggests that these cells may have the potential to respond to neuronal agonists. Furthermore, neuronally induced ADAS cells demonstrate an excitotoxic response to NMDA with a loss of cell viability, suggesting that cells within this population may have formed functional NMDA receptors. Recent studies have found that NMDA receptor activation by glutamate may induce early gene transcription in developing neurons, and determine the rate of neuronal proliferation in the brain (Bading et al., 1995; Cameron et al., 1998). Undifferentiated muADAS cells express low levels of the synaptic vesicle protein marker synapsin I. After neuronal induction, muADAS cells express synapsin I and the axonal marker GAP-43. Synapsins play a role in neuronal development as well as the regulation of neurotransmitter release (Ferreira and Rapoport, 2002). Growth-associated protein 43 is expressed in almost all neurons during developmental axonal outgrowth (Skene, 1989). Although these data show a broad spectrum of phenotypic changes seen in induced ADAS cells, we must emphasize that short-term exposure of ADAS cells to neuronal induction media does not demonstrate that these cells can be induced to functional neurons, which can integrate in vivo with electrophysiologic potentials and synaptic properties of neuronal tissues (Reh, 2002).

Currently there are few effective treatments for many diseases of the central nervous system, including degenerative disorders, trauma, and ischemic injury. Options for neuronal cell transplantation include ESCs and fetal neuronal stem cells. For example, fetal dopaminergic neurons have been transplanted into adult Parkinson's disease patients, resulting in promising clinical success (Freed et al., 2001). However, the use of embryonic and fetal stem cell therapy is still accompanied by many logistic and ethical issues that limit their use. Adult neuronal stem cell sources are limited, as neurogenesis is known to occur in limited regions of the adult brain (Gage, 2000; Temple and Alvarez-Buylla, 1999). Though recent reports speculate that neuronal stem cells may reside throughout the adult CNS, adult NSCs remain an inaccessible population for clinical cell therapies (Aboody et al., 2000; Gage, 2000).

Marrow-derived mesenchymal cells have been identified as a potential source of adult cells for neuronal therapies. The ability of bone marrow cells to develop into cells with characteristics of neuronal cells has been demonstrated in vitro (Jiang et al., 2002, 2003; Mezey et al., 2000; Sanchez-Ramos, 2000; Woodbury et al., 2000; Zhao et al., 2002) and in vivo (Eglitis and Mezey, 1997). Varying degrees of functional restoration have been reported after the transplantation of marrow stromal cells into rats injured by stroke or trauma (Chen et al., 2001; Chopp et al., 2000; Li et al., 2000; Mahmood et al., 2001).

Adipose tissue represents an alternative source of multipotent mesenchymal cells capable of differentiating along mesodermal and ectodermal pathways (Ashjian et al., 2003; Erickson et al., 2002; Gronthos et al., 2001; Halvorsen et al., 2001; Safford et al., 2002; Sen et al., 2001; Zuk et al., 2001, 2002). We have termed these cells ADAS cells, and these cells can be obtained in high numbers from the waste materials generated from liposuction procedures. After expansion in culture, ADAS cells display a distinct phenotype based on cell surface protein expression and cytokine expression (Gronthos et al., 2001). In particular, hematopoietic and epithelial markers are not present by two to three passages of culture (Gronthos et al., 2001; Safford et al., 2002). This phenotype is similar to that described for both bone marrow and skeletal muscle-derived stem cells (Young et al., 1999). The current study complements the findings of

neuronal differentiation of marrow-derived mesenchymal stem cells, and lends support to the hypothesis that stromal cells within adipose tissue are capable of differentiating along neuronal and glial pathways.

The ability of cells to alter their developmental fate remains difficult to explain, as normal tissue differentiation requires sequential restriction in developmental potential. Our findings of transdifferentiation may be explained by alternative mechanisms, including modification of normal developmental pathways, contaminating cell populations, or persistence of a primitive stem cell (Liu and Rao, 2003). For example, the ability of brain-derived NSCs to form mesodermal tissue may involve induction of neural crest differentiation (Galli et al., 2000; Weissman et al., 2001). We must emphasize that our findings do not demonstrate long-term differentiation in these short-term cultures, as the replacement of serum to cultures causes the cells to revert to a undifferentiated phenotype. Distinguishing among these alternative mechanisms and transdifferentiation using techniques such as clonogenic and genomic analysis of differentiation pathways will better define which cells within the ADAS population are capable of neuronal and glial differentiation, and the mechanism underlying this differentiation.

In summary, we have demonstrated that murine adipose tissue contains a population of stromal cells that can differentiate into cells with several phenotypic and functional characteristics of neuronal and glial tissues. These findings support the further development of adipose tissue as an alternative tissue source for cellular therapies, and for the comparison of these cells with other neuronal stem cell sources. These studies establish a foundation for further phenotypic characterization, functional studies, and disease modeling for neuronal therapies using ADAS cells.

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