Cellular and Genetic Characterization of Human Adult Bone Marrow-Derived Neural Stem-Like Cells: A Potential Antiglioma Cellular Vector

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ABSTRACT

We describe the in vitro isolation and expansion of cells capable of forming neurosphere-like aggregates from human adult bone marrow. Cells within these passaged spheroids can differentiate into astrocytes, specific neuronal subtypes, and oligodendrocytes and have gene expression profiles similar to human fetal brain-derived neural stem cells. Genetically modified neural-competent bone marrow-derived cells efficiently migrate toward distant sites of brain injury and tumor in vivo, where they differentiate and express therapeutic transgenes when transplanted into the brains of mice. These studies suggest that adult bone marrow may serve as a large reservoir for autologous neural stem-like cells for future therapeutic strategies.

INTRODUCTION

Malignant gliomas represent an important cause of cancer-related mortality for which standard treatments are suboptimal (1). Although gliomas do not generally metastasize, their propensity to deeply infiltrate adjacent cerebral cortex precludes definitive surgical resection or other local therapies. Intrinsic resistance of glioma cells to radiation and chemotherapy limits traditional cancer therapies, whereas newer approaches such as gene therapy are confounded by the inefficiencies of viral vectors to transduce the deeply infiltrating glioma cells. An autologous cellular vector that could migrate through brain parenchyma and deliver a therapeutic transgene to the site of infiltrating tumor would theoretically represent an optimal gene delivery strategy for brain tumors. Neural stem cell (NSC) lines have been demonstrated to be effective for delivering transgenes to brain tumors based on their unique migratory properties within the central nervous system [CNS (2–4)]; however, the ability to safely obtain sufficient numbers of autologous NSCs represents a significant and potentially prohibitive technical challenge. The demonstration that freshly isolated bone marrow cells injected into the brains of animals can express neuronal and glial markers in mice suggests that tissue-specific stem cells possess broad developmental potential and raises the possibility of a readily available source of autologous NSC-like cells (5-15). Here we demonstrate the in vitro isolation, expansion, and therapeutic potential of a neural-competent population of cells from adult human bone marrow.

MATERIALS AND METHODS

Cell Culture. RBCs from human adult (donors, 20–40 years old) bone marrow cells purchased from AliCells (Berkeley, CA) were depleted by density gradient, and the remaining cells were plated in uncoted tissue culture plastic dishes. After 10 days of culture in 15% ES-qualified serum (Life Technologies, Inc.) with bFGF/FGF8/sonic hedgehog. Floating aggregates were separated and cultured for 10 days. Brain-derived neural stem cells (BDNSCs) were obtained from Clonetics (Walkersville, MD). Conditioned media from BDNSC culture were filtered through 0.22-μm Stericup (Millipore, Bedford, MA) and used in the culture of BDNSCs and marrow-derived neural-competent cells [MDNCCs (25% conditioned media +75% fresh media)]. To induce differentiation in vitro, cell aggregates were plated on fibronectin-coated plates in B27 media containing 10% fetal bovine serum. In vivo conditioning consisted of the injection of cell aggregate-disassociated, nestin-positive cells into the lateral ventricle of neonatal severe combined immunodeficient mouse. Transplanted human cells were examined by confocal microscopy (Zeiss LSM 510; Zeiss). For ex vivo characterization, mice transplanted with human cells were killed; brains were dissociated by digestion with trypsin, collagenase, and papain; and then the harvested cells were cultured in the presence of bFGF and differentiated by the removal of bFGF (16).

Immunohistochemistry. Immunohistochemistry was carried out using standard protocols. The following primary antibodies were used at the following dilutions: (a) TuJ1 rabbit polyclonal antibody, 1:2000; (b) nestin rabbit polyclonal antibody, 1:2000; (c) F4/80 rat monoclonal antibody, 1:50; (d) CD45 mouse monoclonal antibody, 1:100; (e) Th3 monoclonal antibody, 1:100; (f) CD11b mouse monoclonal antibody, 1:100; (g) CD255 mouse monoclonal antibody, 1:100; (h) GFAP mouse monoclonal antibody, 1:500; (i) CD31 mouse monoclonal antibody, 1:100; (j) β-3-tubulin mouse monoclonal antibody, 1:100; (k) nestin rabbit monoclonal antibody, 1:1000; (l) nestin mouse monoclonal antibody, 1:100; (m) BDNSC monoclonal antibody, 1:1000; and (n) TuJ1 mouse monoclonal antibody, 1:1000. Secondary antibodies were used at the following concentrations: (a) α·CD91 antibody, 1:2000; (b) α·CD31 antibody, 1:100; (c) α·nestin antibody, 1:200; (d) α·GFAP antibody, 1:2000; (e) α·nestin antibody, 1:100; (f) α·BDNSC antibody, 1:100; (g) α·TuJ1 antibody, 1:1000; (h) α·MAP2 antibody, 1:1000; (i) α·nestin antibody, 1:1000; (j) α·BDNSC antibody, 1:100; (k) α·GFAP antibody, 1:2000; (l) α·nestin antibody, 1:2000; (m) α·BDNSC antibody, 1:200.

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8877
scanned, and image analysis was performed as described online.1 Briefly, fluorescence-labeled cDNA was synthesized from 1 μg of sample or reference mRNA by random hexamers and oligo(dT)-primed reverse transcription in the presence of either Cy3 or Cy5 fluor-deUTP (Amersham Pharmacia Biotech, Piscataway, NJ). All samples were compared with the same reference RNA to allow for normalization of each clone’s expression relative to the reference for each sample. The reference consisted of RNA extracted from adult whole bone marrow depleted of RBCs. Image analyses were performed using DeArray software.9 The two fluorescent images (red and green channel) obtained constitute the raw data from which differential gene expression ratio values were calculated. The ratio normalization was performed based on all data points. All data from all experiments were then entered into a relational database using the Filemaker Pro software.

**Microarray Analysis.** All analyses were done on median centered log (base 2) expression ratios. Hierarchical clustering was performed based on Pearson correlation and average linkage. We used multidimensional scaling (MDS) to graphically assess the similarity between groups. MDS was performed using Euclidean distance (root sum of squared differences) as the distance metric.

Commonly overexpressed/underexpressed genes between the differentiated and undifferentiated BDNSC groups were selected based on all samples either being overexpressed (over 1.8) or underexpressed (0.56): 133 genes were identified. We compared the median correlation between BDNSC and MDNCC groups with the median correlation between BDNSCs and other groups as a measure of the closeness of BDNSCs and MDNCCs. A permutation test (17) was used to assess the statistical significance of the difference between these two median correlations. The permutation P was obtained using a null distribution estimated by scrambling individual labels for the control, differentiated MDNCC, and undifferentiated MDNCC groups, and recomputing the difference in the two median correlations (10,000 permutations were generated).

Genes that were differentially expressed between differentiated and undifferentiated BDNSCs were selected based on (a) all of the differentiated (undifferentiated) BDNSCs being overexpressed (over 1.8), and none of the undifferentiated (differentiated) BDNSCs being overexpressed (under 1.8), and (b) all of the differentiated (undifferentiated) BDNSCs being underexpressed (under 0.56), and none of the undifferentiated (differentiated) BDNSCs being underexpressed (over 0.56). There were 78 genes that met this criterion. We assessed the closeness of the expression patterns between MDNCC and BDNSC groups by averaging the median correlation between undifferentiated MDNCC and BDNSC samples and the median correlation between differentiated MDNCC and BDNSC samples. A permutation test was used to test whether MDNCC and BDNSC expression patterns of the same differentiation status were more highly correlated than MDNCC and BDNC patterns of a different differentiation status. The permutation P was obtained using a null distribution estimated by scrambling labels for the differentiated and undifferentiated MDNCC samples and recomputing the average correlation measure described above.

**In Vitro Models.** The freezing insult was induced by stabbing the brain of anesthetized animals with a Hamilton syringe chilled in liquid nitrogen under stereotactic guidance (2 mm lateral and 1 mm anterior to bregma). CM-DiI (Molecular Probes)-labeled MDNCCs (2 × 10³), suspended in 5 μl of HBSS, were then stereotactically implanted into a distant site within the ipsilateral or contralateral hemisphere (2 mm lateral and 2.5 mm posterior to bregma; depth of 2.5 mm from dural surface; 5 mice/group). On the specified days, animals were sacrificed, and brains were processed for immunohistochemical staining and microscopy.

Experimental gliomas were produced by injecting U87-MG human glioma cells (5 × 10⁴), suspended in 5 μl of HBSS, into the forebrain (2 mm lateral and 1 mm anterior to bregma; depth of 2.5 mm from dura) of female adult nude mice over a 3.5–minute period. Three days later, animals received a second stereotactic injection (2 mm lateral and 2.5 mm posterior to bregma; depth of 2.5 mm from dura) of genetically transduced MDNCCs.

**In Vitro Migration Assays.** In vitro migration assays were performed using a 96-well microchemotaxis chamber with polypyrrolidone-free polycarbonate filters with a pore size of 8 μm (Neuroprobe, Gaithersburg, MD). Various concentrations of the cytokines (R&D Systems), U87-MG cells (2.5 × 10⁴), or supernatant from homogenized tissues were placed in B27 media (serum free, 0.1% BSA), and 29 μl of the solution were placed in the lower chamber. After placing the filter over the lower chamber, 2.5 × 10⁴ MDNCCs, BDNSCs, or human bone marrow cells were resuspended and pipetted onto the surface of the filter. The apparatus was then incubated for 5 h at 37°C in a humidified chamber with 5% CO₂ to allow cell migration. After the incubation period, the filter was removed, and the upper side of the filter was washed and scraped with a rubber policeman. The filters were fixed with methanol and stained with Giemsa. We quantified migration by counting cells in four random high-power fields (×400) in each well. Experiments were conducted in quadruplicate.

**RESULTS**

**Isolation of Neural Competent Cells in Vitro from Human Bone Marrow.** We proceeded under the assumption that the bone marrow contains a population of pluripotent stem cells that could be driven toward a cell type with NSC-like properties, given that neural fate is one of the principle default differentiation pathways of uncommitted multipotent cells in vitro (15, 18, 19). To select for such an undifferentiated population of cells in vitro, we chose to culture adult bone marrow under conditions that are conducive to the expansion of pluripotent embryonic stem cells [Fig. 1A (18–20)]. Small clusters of cells began to appear after 10 days in embryonic stem cell culture conditions, at which point we switched the cells to serum-free growth conditions similar to those used for culturing NSCs [Fig. 1B (20)]. The media contained basic FGF2, sonic hedgehog, and FGF8, cytokines known to be important both in proliferation and fate determination of NSCs in early development (21, 22). The majority of these clumped cells began to grow as nestin-positive spheroids with morphologies identical to typical BDNSC-derived neurospheres within 10 days in NSC culture conditions [Fig. 1B, b–e (23)]. These floating spheroids were first seen as 10–20-cell aggregates but then quickly grew to clusters consisting of hundreds of floating cells, facilitating their separation from cells that remained attached to the plates. Approximately 2000 spheroids were typically generated from 10⁵ human adult bone marrow cells after 20 days in culture. Our cultures generally yielded between 5 × 10⁴ and 5 × 10⁵ cells after 1 month in culture starting from a population of 10 million whole bone marrow cells. Cells from dissociated marrow-derived neurosphere-like aggregates continued to proliferate, reformed spheroids, and could be passaged for more than 2 months.

We next sought to determine whether these bone marrow-derived cellular aggregates could be induced to differentiate into cells of neural lineage, given their morphological similarity to NSC-derived neurospheres. After the addition of serum and the removal of growth factors, spheroid-derived cells attached to the polyornithine/fibronectin-coated plates and spread out (Fig. 1B). Some nestin-positive cells also became positive for A2B5, a marker for glial progenitor cells, during the first few days in the differentiation culture conditions (Fig. 1B, g). Immunohistochemical staining of cells after 7 days in differentiation conditions demonstrated that 10–15% of cells were positive for TuJ1 (neuron-specific β III tubulin), whereas 50–80% of cells were positive for GFAP, which are markers for neuronal and glial differentiation, respectively (Fig. 1B, h–i). GFAP cells were also positive for $\varepsilon$100β staining, another marker of astrocytic differentiation (Fig. 1B, i). All cells were negative for HuMac (marker for human macrophages) and CD45 (marker for differentiated hematopoietic cells), respectively (Fig. 1B, f and h). Despite the abundance of cells with astrocytic and neuronal morphology and immunophenotypes, we saw only a few O4-positive cells with morphology consistent with oligodendrocyte differentiation (data not shown).

We performed single cell limiting dilution assays to evaluate the
clonogenicity of individual cells derived from our cellular aggregates. Growth of individual cells derived from dissociated spheroids met with limited success; however, clumps of a minimum of 10 cells in a 96-well microtiter plate were sufficient to generate cellular aggregates characteristic of neurospheres in 23/11006.3% of the wells (89 of 384 wells). To evaluate the growth and differentiation potential of single cells, we performed mixing experiments whereby single cells expressing GFP from a dissociated GFP-transduced spheroid were mixed with 9 cells from non-GFP-expressing spheroids. We observed GFP-positive spheroids forming in 2.1/11006.0.85% of the microtiter wells (6 of 288 wells). Both GFP-expressing astrocytes and neurons were formed after exposure of these GFP-positive spheroids to differentiation conditions. We therefore estimate that approximately 2% of the cells within our bone marrow-derived spheroids are clonogenic and capable of differentiating toward both glial and neuronal lineage.

Further evidence for the stem cell-like nature of the cells within the bone marrow-derived neurosphere-like aggregates was their expression of telomerase, as demonstrated in various other stem cell populations (16, 24). By contrast, the differentiated progeny of these spheroids were negative for telomerase expression (Fig. 1C). Based on these in vitro data, we named these cells MDNCCs. All MDNCCs and their differentiated progeny demonstrated a normal 2N human karyotype (data not shown).

In Vivo Differentiation of MDNCCs. We transduced MDNCCs with GFP and injected them (50,000 GFP-expressing cells) into the ventricles of neonatal (P1 to P3) immunodeficient severe combined immunodeficient mice (25, 26) to evaluate the effects of in vivo conditioning on the differentiation of MDNCCs. We subsequently harvested the brains and performed immunohistochemistry on brain-derived single cell suspensions (Fig. 2A) or on whole brain mounts (Fig. 2B) at various time points after injection (5 days, 10 days, 1 month, and 2 months). Examination of the single cell suspensions demonstrated the presence of GFP-expressing TuJ1/neurofilament M-, GFAP-, and O4-positive cells with morphology consistent with neuronal, astrocytic, and oligodendroglial differentiation, respectively (Fig. 2A). We next evaluated the whole brain sections of the 1-month-old MDNCC intracerebrally injected mice by exclusively using confocal microscopy to unambiguously identify labeled cells in situ. Approximately 32.9% of the GFP-positive cells coexpressed GFAP (342 of 1038 cells counted), whereas 6.5% of the GFP-expressing...
cells coexpressed the neuronal marker NeuN (49 of 757 cells). Immunostained whole brain mounts revealed GFP-expressing cells with immunophenotypes and morphologies consistent with glial and neuronal subtypes including GFAP-positive astrocytes, CNPase-positive oligodendrocytes, and neurons positive for TuJ1, NeuN, GABA, and tyrosine hydroxylase expression (Fig. 2B). In addition, we found numerous GFP-positive cells located within the ventricular lining zone. We did not detect GFP-positive glial and neuron-like cells when we injected either 50,000 or 500,000 fresh bone marrow cells (data not shown).

Global Gene Expression Profiling of MDNCCs. To first confirm the neural lineage of the spheroids containing MDNCCs and examine the similarity between BDNSC-derived neurospheres and MDNCC-derived spheroids during the process of differentiation, we characterized the global gene expression profiles of these two populations of cells using cDNA microarrays. Total RNA was isolated from different populations of cells, and the gene expression pattern of 13,826 genes was analyzed (27, 28). MDS analysis and hierarchical clustering were performed to compare gene expression profiles of undifferentiated and differentiated BDNSCs with MDNCCs as well as with other bone marrow-derived and other unrelated cell populations [Fig. 3 (29, 30)]. For the first analyses, we sought to identify a group of genes with expression profiles representative of the neural lineage to confirm the neural characteristics of MDNCCs. We reasoned that the genes in the undifferentiated and differentiated BDNSC groups demonstrating the most extreme concordant gene regulation compared with a control
nonneural control cell would define such a neural-selective gene set. We identified 133 such genes and compared them across all groups. We found a high degree of similarity in gene expression patterns between the BDNSC and the MDNCC groups as compared with the other cell populations [Fig. 3B (median correlation between BDNSCs and MDNCCs = 0.872; median correlation between BDNSCs and other groups = 0.628, \( P < 0.001 \)].

The pattern of gene expression necessary to differentiate a stem cell to a specific terminally differentiated cell must involve a unique and regulated sequence of events. Thus, for our second analyses, we asked whether the gene expression patterns seen during the differentiation of BDNSC-derived neurospheres were similar to those of MDNCC-derived spheroids. We identified 78 genes that were highly up- or down-regulated during the process of BDNSC differentiation, based on April 15, 2017. © 2003 American Association for Cancer Research.
on the assumption that a subgroup of these genes may be coregulated in cells with NSC-like potential. When we performed hierarchical clustering analysis using 78 weighted genes and their respective clone IDs that demonstrated significant change during BDNSC differentiation, we found that the undifferentiated MDNCC spheroids were closely correlated with undifferentiated BDNSC neurospheres, and the differentiated MDNCCs showed strong correlation with the differentiated BDNSC populations (Fig. 3C). Clustering analyses graphically demonstrate the relatedness of these groups compared with each other and all other control cells (Fig. 3C). As expected, analyses of the expression data using MDS revealed little similarity between undifferentiated (red) and differentiated (yellow) BDNSCs. By contrast, the MDS demonstrated a strong similarity between the undifferentiated BDNSCs (red) and undifferentiated MDNCCs (pink) and between the differentiated BDNSC spheroids (yellow) and the differentiated MDNCCs (light yellow). The average of these two median correlations is 0.836, which is substantially higher than a similar correlation measure relating MDNCC spheroids and BDNSC neurospheres of different differentiation status (average correlation of 0.579), suggesting a high degree of similarity between these two cell populations relative to their differentiation status (P < 0.01 by a permutation test). In both analyses, we obtained similar results when we selected genes based on t-values and average log expression values with the largest magnitude, although the weighted genes were different (data not shown). As strong as these correlations are, however, some genes did have different expression patterns between the MDNCC and BDNSC groups. It remains to be determined whether these differences simply reflect differences in the conditions used for in vitro acquisition and propagation of cells or contamination of a minor cell population (i.e., hematopoietic and/or stromal progenitors) or rather represent intrinsic differences between NSC-like populations. Taken together, these microarray data suggest that MDNCC-derived spheroids...
roids have genetic profiles and neural differentiation potential very similar to those of BDNSC-derived neurospheres.

In Vivo Migration of MDNCCs in Freeze Injury Model. We were interested in determining whether MDNCCs have migratory properties similar to NSCs, given the known ability of such cells to migrate within the brain in response to physiological and pathological stimuli [i.e., the rostral migratory stream and trauma, respectively (31, 32)]. We therefore investigated the migratory properties of the MDNCCs using an in vitro migration assay by using lysates from normal and freeze-injured brain tissue. Similar to BDNSCs, MDNCCs migrated toward freeze-injured brain tissue lysates to a far greater extent than to normal brain lysates (7-fold increase; Fig. 4A). By contrast, our starting population of whole bone marrow cells demonstrated significantly less migration (2-fold increase; *P* = 0.001, *t* test). The effect of time from injury on the migration of cells within each group (fresh bone marrow, BDNSCs, and MDNCCs) was analyzed using a two-way ANOVA. The effect of time on migration varied significantly between the different groups when all groups were considered [F = 17.41; degree(s) of freedom (df) = 8,45; *P* < 0.001]. However, there was no significant variation in the effect of time on cell migration when only the BDNSC (F = 1.62; df = 4,30; *P* = 0.2) and MDNCC (F = 2.23; df = 1.34; *P* = 0.14) groups were analyzed, suggesting no strong differences in temporal migration patterns between BDNSCs and MDNCCs.

To evaluate the response of MDNCCs to brain injury in vivo, we stereotactically injected CM-DiI (fluorescent red)-labeled MDNCCs at a distance from the site of freeze-injury. Seven days after injury, fluorescence-labeled cells from both the ipsilateral and contralateral MDNCC injection sites were found within the area of the injured brain (Fig. 4B). In all of the animals we examined (n = 5 MDNCCs and 5 controls), the migration patterns of the MDNCCs were largely through white matter tracts, including the corpus callosum and external capsule. MDNCCs migrated in a chain-like formation, characteristic of the pattern of NSC migration seen in the rostral migratory stream [Fig. 4C (31)]. Human ribonucleoprotein-specific antibody staining colocalized with the DiI-labeled cells, confirming that the labeled cells were of human origin. The transplanted MDNCCs were found adjacent to reactive astrocytes, which were identified by intense GFAP and nestin immunopositivity (33). Immunostaining of GFP-expressing MDNCCs at the trauma site also revealed dual nestin- and GFAP-positive cells, demonstrating the ability of the MDNCCs to differentiate along a glial lineage in response to brain injury (gliosis).
This pattern of glial differentiation and cellular migration was mirrored identically by the BDNSCs, in contrast to the starting population of freshly isolated bone marrow cells that demonstrated little migration or glial differentiation (data not shown).

**Migration and Differentiation of MDNCCs in Tumor Model.** To further confirm the migratory properties of the MDNCCs, we investigated their ability to migrate toward intracranial gliomas, as has been reported previously for a murine NSC line (2, 3). Adenovirus-transduced MDNCCs expressing GFP were injected distally (3.5 mm) into the ipsilateral cortical hemisphere of mouse brains harboring an intracerebral established U87-MG human glioblastoma. Nearly all of the GFP-positive cells migrated toward the established U87 glioma within 12–20 days of injection (Fig. 5). More than 90% of the labeled MDNCCs were found at the interface of the tumor mass and "normal" brain (an area known to contain infiltrative tumor cells) rather than within the center of the tumor (Fig. 5). A similar pattern of MDNCC migration was observed in the brains of animals harboring rat glioblastoma CNS-1 tumors (data not shown). We performed in vitro assays to better understand this pattern of migration. Normal brain lysates or lysates from U87-MG gliomas implanted intracranially or s.c. induced only minimal migration of MDNCCs and BDNSCs in vitro. Likewise, cultured U87-MG cells, vascular endothelial growth factor, and bFGF did not stimulate significant cellular migration. By contrast, lysates of brain tissue at the edge of the invading tumor border induced migration of both MDNCCs and BDNSCs in vitro by an order of magnitude greater than any other stimuli (Fig. 5A). Using a two-way ANOVA, we found a highly significant difference in the cell migration pattern between the different populations of cells (BDNSCs, MDNCCs, and fresh bone marrow) when all three groups were included (F = 12.02; df = 10,54; P = 0.001). There were no significant differences, however, when only BDNSCs (F = 1.21; df = 5,36; P = 0.33) and MDNCCs (F = 2.11; df = 4,1; P = 0.15) were analyzed, suggesting the similarity between BDNSCs and MDNCCs to chemotaxic stimuli. These data further demonstrate the similar migratory properties between MDNCCs and BDNSCs. In addition, the in vitro and in vivo data suggest that brain tissue adjacent
to or infiltrated by tumor results in the stimulus for migration of MDNCCs and BDNSCs rather than the tumor itself. The mechanism for this migratory stimulus remains unknown, although we suspect that the destructive and/or inflammatory environment at the edge of the advancing tumor might be the stimulus for migration, consistent with our demonstration that brain injury is a potent stimulus for MDNCC and BDNSC migration.

We performed immunohistochemical studies of the MDNCCs that had migrated toward the intracerebral glioma to reveal their differentiation fate (Fig. 5B). Whereas numerous tumor cells stained positively for the proliferation marker Ki67, GFP-expressing MDNCCs were Ki67 negative, suggesting that these cells are largely nonproliferative in vivo. MDNCCs were also negative for Glut1 and HuMac, markers for endothelial cells and human macrophage cells, respectively. Within the border between tumor and cerebral cortex, however, were large numbers of GFP-expressing MDNCCs that stained positive for GFAP. Consistent with these findings, the tumor-bearing hemispheres showed increased GFAP immunoreactivity characteristic of reactive gliosis. These cells constituted approximately 72% of all GFP-expressing cells. Some cells coexpressing GFP and GFAP were also positive for nestin, aquaporin 4, and S100β, consistent with a reactive astrocyte phenotype (33). These cells coexpressing GFAP and nestin constituted approximately 30–40% of all of the MDNCCs.

Approximately 2.5% of the MDNCCs stained positive for NeuN. Finally, approximately 20% of MDNCCs expressed no identifiable neural, migroglial, or hematopoietic markers (data not shown). The reactive astrocyte phenotype observed around the tumor was not detected in the brains of either normal adult mice or neonatal mice that received injection with MDNCCs. Taken together, these data suggest that MDNCCs not only migrate toward intracerebral gliomas but also respond to environmental cues.

**MDNCCs as a Therapeutic Cellular Vector.** We chose to explore the potential therapeutic implications of the MDNCC migratory properties by evaluating the ability of these cells to carry therapeutic genes to sites of intracerebral gliomas. The decision to genetically modify the MDNCCs to express antitumor transgenes that are nontoxic to normal cells was based on the migratory pattern of MDNCCs through the CNS, as well as the infiltrative nature of malignant gliomas into normal brain tissue. We have demonstrated previously that a secreted form of platelet factor 4 (PF4), an antiangiogenic protein, could be safely expressed within normal cerebral tissue, thereby inhibiting tumor angiogenesis and tumor growth (34). Thus, MDNCCs transduced by an adenoviral vector encoding the sPF4 transgene were stereotactically injected into an intracerebral site distant from the pre-established U87-MG glioblastomas. Animals treated with sPF4-transduced MDNCCs survived significantly longer than
mice treated with the MDNCCs transduced by a GFP-expressing control adenoviral vector or mice with tumor only (P < 0.001, log-rank test; Fig. 6A, a). The reduced microvascular density in the PF4-transduced MDNCC-treated animals is consistent with an antiangiogenic mechanism of tumor growth inhibition (Fig. 6A, b and c).

To further evaluate the potential utility of MDNCCs as a tumor-targeting vector, we examined the efficacy of transducing MDNCCs with a transgene encoding tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL induces apoptotic cell death in many tumor cell lines but generally spares normal cells (35, 36). Transduction of normal cells, including MDNCCs, by an adenoviral vector expressing a human TRAIL cDNA results in potent tumor cell killing through normal cell-mediated presentation of membrane-bound TRAIL to the tumor cell (36). Injection of either an excess or equal number TRAIL-transduced MDNCCs directly into the site of tumor cell implantation at the time of initial tumor cell implantation resulted in a very large and significant survival advantage compared with control vector-transduced MDNCCs (data not shown). Such experiments, however, do not reflect the reality of the clinical situation, where there generally exists a pre-established tumor with large numbers of distantly infiltrating tumor cells that far exceed the number of “therapeutic” or “targeting cells” that could be practically administered. We therefore stereotactically injected a log fewer TRAIL-transduced MDNCCs than tumor cells into the brain at a distance from a pre-established and growing intracerebral glioma to experimentally mirror the clinical scenario more precisely. TRAIL-transduced MDNCCs mediated a significant prolongation of survival compared with control-transduced MDNCCs, even under these most disadvantageous yet clinically relevant conditions (log-rank test: P = 0.017 for a and P < 0.001 for b; Fig. 6B). We observed increased tumor cell apoptosis in the MDNCC/TRAIL-treated tumors, consistent with a TRAIL-mediated antitumor effect (Fig. 6B, c and d). Furthermore, increasing the ratio of TRAIL-transduced MDNCCs to tumor cells resulted in an even longer prolongation of survival (Fig. 6B).

Finally, immunodeficient mice (n = 3) transplanted with MDNCCs remained tumor free and were clinically and histologically normal when sacrificed more than a year after transplantation, demonstrating the overall safety and lack of tumorigenic potential of the MDNCCs.

**DISCUSSION**

A number of groups have recently demonstrated that bone marrow cells could be differentiated into neurons and glial cells in vitro based on immunohistochemical studies (5, 6, 37, 38). We have furthered those observations by demonstrating that adult human bone marrow-derived neural progenitor-like cells can be propagated in vitro, can differentiate into different subtypes of neuronal and glial cells, and have gene expression profiles as well as migratory functions similar to those of endogenous brain-derived NSCs. Furthermore, we have demonstrated that these MDNCCs can be effectively used as cellular vectors to deliver therapeutic genes to intracranial tumors in an animal model of glioblastoma.

We believe that our data have several interesting implications for autologous stem cell-based therapies, potentially avoiding many of the difficult issues surrounding the use of adult brain, fetal, or embryonic stem cell-derived NSCs (39–41). For purposes of exploring one such potential therapeutic application, we have focused our initial experiments on the use of MDNCCs as a cellular targeting vector. Cell-based gene transfer offers the potential advantage of controlled in vitro cellular transduction, thereby increasing the possibility for high cellular transduction efficiency while lessening the potential toxicity associated with direct vector administration to patients. MDNCCs appear particularly well suited as a cellular vector for CNS gene delivery given (a) their ability to be expanded in vitro, (b) their ability to respond to environmental cues based on three different in vivo models (the developing, the injured, and the tumor-containing brain), (c) their apparent lack of neurotoxicity or tumorigenicity, (d) the ease with which they are transduced by genetic vector in vitro, and (e) the ready availability of a nearly limitless supply of autologous MDNCCs for every patient. In particular, MDNCC-based gene transfer may be particularly promising for therapeutic strategies aimed at malignant gliomas, given their ability to migrate to sites of tumor infiltration within the brain, thereby potentially overcoming the current limitation of most direct gene transfer approaches. Furthermore, the ability of MDNCCs to migrate toward sites of brain injury around tumor or sites of trauma and differentiate in vitro into both glial and neuronal subtypes raises the intriguing prospect of using MDNCCs for CNS repair, although many more studies remain to be done before such a...
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Fig. 5. Continued.
prospect can be realistically entertained. These studies will need to address important questions such as whether the neurons generated by the MDNCCs form appropriate and functional synaptic connections in vivo, whether MDNCC-generated oligodendrocytes contribute to the re-myelination of de-myelinated neurons, and whether MDNCC-generated astrocytes are capable of constructing an in vivo niche conducive to neuronal regeneration and repair of a functional blood-brain barrier (42–44).

A number of groups have demonstrated that cells from a specific differentiated tissue type can be induced to express phenotypic markers and/or have functional properties consistent with differentiated cells from a different lineage (5–15). One potential interpretation of these observations is that tissue-specific stem cells possess the ability to “transdifferentiate” toward other fates. Several recently published reports, however, have failed to duplicate these observations, raising the possibility that the apparent plasticity of the tissue-specific stem cells may be a function of a contaminating, yet to be characterized rare multipotent cell population (45–47). We were unable to convincingly demonstrate the presence of NSC-like cells from fresh, uncultured human bone marrow. By contrast, we do demonstrate that adult human bone marrow-derived neural progenitor-like cells can be propagated in vitro under selective growth conditions. These data are consistent with the presence of and in vitro selection for an otherwise rare bone-marrow derived cell with neural lineage competent properties. Formal testing of this hypothesis, however, awaits the prospective isolation and identification of the clonal cell of origin.

Experimental approaches addressing issues regarding stem cell fate determination have been largely based on morphological and immunohistochemical studies to date (42–47). We believe the results of our gene expression profiling, although preliminary, demonstrate the power of a genetic approach for determining similarities between stem cell populations and lineages and may be useful as a starting point for understanding the genetic program responsible for the induction of NSC lineage commitment. The potential for these genetic approaches has been demonstrated in recent studies describing transcriptional profiling of embryonic, neural, and hematopoietic stem cells (48–50). These reports demonstrate that NSCs and hematopoietic stem cells share some of the same “stem cell” genes, yet not unexpectedly, express many distinct genes. Our data showing that the gene expression profiles of MDNCCs are much closer to those of BDNSCs than those of hematopoietic stem cells (CD34-positive cells) and mesenchymal stem cells (stroma cells) are of interest and further attest to the neural lineage-like properties of the MDNCCs.

In conclusion, we have demonstrated that human neural progenitor-like cells, generated from adult bone marrow cells, can be partially purified and propagated in vitro and used as cellular targeting vectors
within the CNS. The data presented here suggest that this nearly unlimited reserve of autologous cells might one day prove useful for the treatment of malignant brain tumors and possibly even other neurological diseases, should the MDNCC-derived cells ultimately be demonstrated to have physiological functions comparable with those of cells derived from NSCs of the CNS.

REFERENCES


Cellular and Genetic Characterization of Human Adult Bone Marrow-Derived Neural Stem-Like Cells: A Potential Antiglioma Cellular Vector


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