Tumor Young Investigator Award: Tropism and Antitumorigenic Effect of Endogenous Neural Precursors for Gliomas

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INTRODUCTION

Precursor cells are undifferentiated cells capable of proliferation, self-renewal, production of large numbers of differentiated progeny, and have an ability to regenerate tissues.[52] Mammalian adult neural precursor cells (NPCs) are capable of extensive cell division and self-renewal.[13,38,43,50] NPCs are restricted to two areas of the adult brain, i.e., the subgranular zone (SGZ) of the hippocampus and the subependymal zone (SZ) of the lateral ventricle (LV). Endogenous NPCs can migrate and home in specifically on sites of damage or regeneration[10,17] and on sites of brain tumors, such as glioma.[1] The existence of putative glial brain tumor stem cells driving tumorigenesis and generating tumor cell heterogeneity suggests that these tumors originate from transformed neural stem cells and/or from NPCs.[14,20,23,26,27,45,46,51] On the other hand, an apparent tropism and the migratory capacity of immortalized NPCs for glial brain tumors endorsed these cells as candidate carriers for brain tumor therapy,[1,5,9,36] although it was not clear whether the observed tropism was based on precursor cell features or their immortalization. The activation and attraction of endogenous NPCs located in the subventricular zone (SVZ) in response to glial tumor growth represents an intrinsic—albeit age-dependent—antitumorigenic response.

TROPISM OF ENDOGENOUS NPCS

NPCs contain multipotent cells that can be cultivated from embryonic, fetal, neonatal, or adult tissues and are capable of long-term, sustained, in vitro propagation and terminal differentiation. Several groups have demonstrated that exogenously added immortalized NPCs exhibit potent tropism for disseminating glioma cells in vivo. After injection into glioma-bearing rodents, NPCs migrate away from the primary site of injection and intersperse themselves into the tumor mass.[1,9] This recommends the use of NPCs as specific targeting tool for tumor mass and disseminated tumor microsatellites,[5,8,9] Further, immortalized cells from early postnatal NPCs, genetically modified to overexpress interleukin-4, improved survival after inoculation into glioblastoma-bearing mice.[5,36] Interestingly, however, the unmodified control cells in that study also enhanced survival. Using dsred-labeled glioma cells inoculated into transgenic mice expressing enhanced green fluorescent protein (GFP) under the control of the nestin promoter,[25] which specifically labels NPCs, we were able to distinguish whether precursor cells originated from the tumor or from the recipient's germinal zones. In the adult brain, nestin is only expressed in NPCs, and the GFP fluorescence served as a marker for this cell population (nestin/GFP-positive cells). Two weeks after glioma induction into the caudate putamen of adult mice (P25), glioblastoma had developed and nestin/GFP-positive cells abundantly enwrapped the tumor in areas unrelated to the rostral migratory stream (RMS) (*Fig. 40.1*). No precursor cell attraction was observed in the contralateral side. Within the tumor margin, NPCs and glioblastoma cells were intermingled. This observation was confirmed by glioblastoma induction into the anterior striatum and after xenotransplantation of fluorescently labeled U373 and F98 cells lines, supporting the idea of directly specific migration of endogenous NPCs activated by an intrinsic pathological event such as a glial brain tumor.[15]

SPECIFIC ENDOGENOUS NPC RESPONSE TO DIFFERENT PATHOLOGICAL EVENTS

The mobilization and recruitment and subsequent differentiation of NPCs from the SVZ after lesions has been assessed in several models that have been used to determine the contribution of NPCs in CNS repair of acute or chronic injury. Most of these models have involved rodents and have demonstrated that NPCs from the SVZ are reactivated in response to different insults. Indeed, the proliferation rate of SVZ cells is increased after seizure, ischemia, transaction, and demyelination.[3,7,24,39] To date, these models reveal the ability of NPCs from the SVZ to undergo increased proliferation, ectopic migration, and multipotential differentiation

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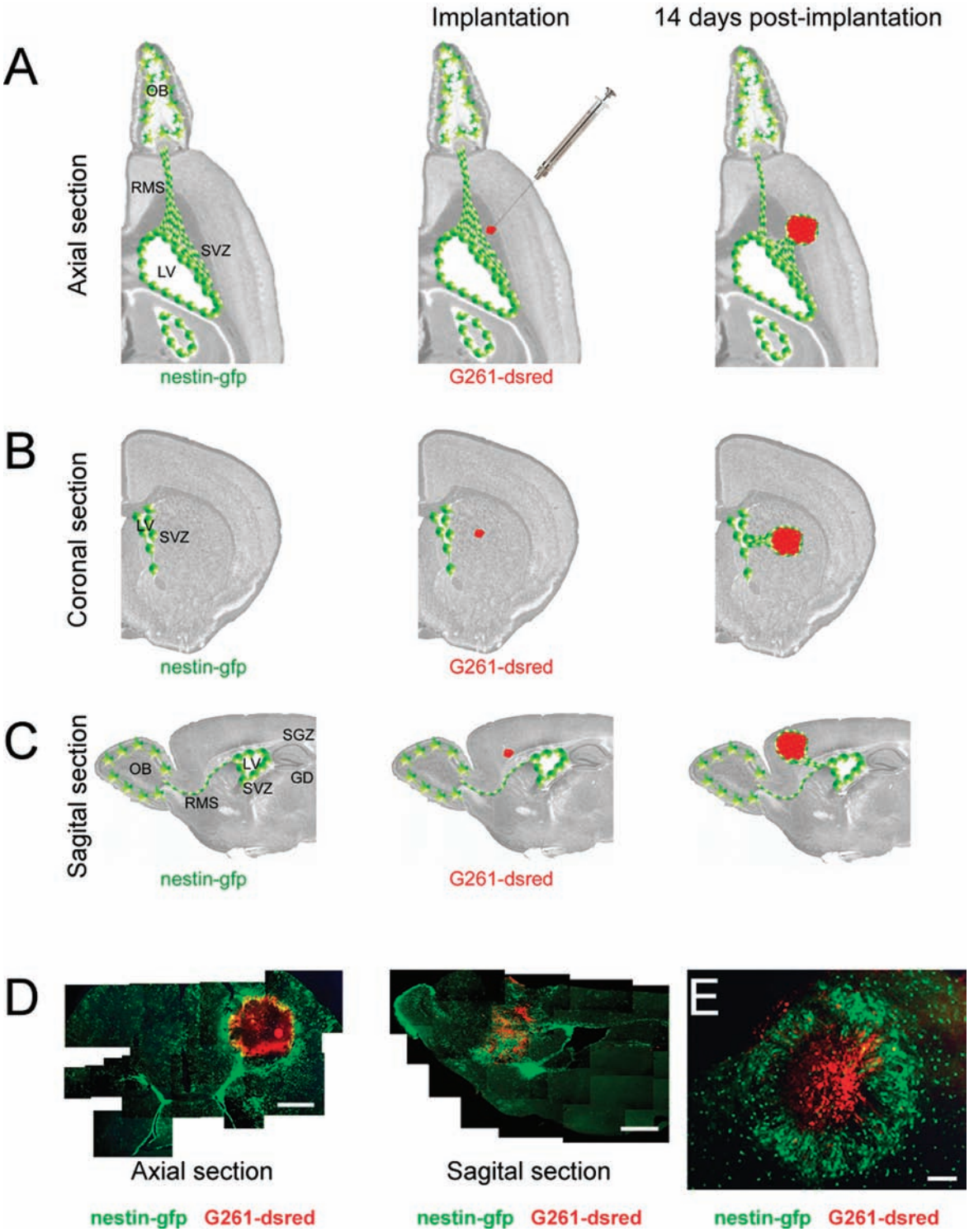

FIGURE 40.1. Tropism of NPCs. Nestin/GFP-positive NPCs (*green*) from the SVZ surround dsred-labeled glial tumors in nestin/GFP mice of P25. *A*, axial section through the brain indicating the LV/SVZ, RMS, and OB, and the location used for the implantation of tumors in the experiments. *B* and *C*, the analogous planes in coronal and sagittal directions. *D*, corresponding composite picture giving an overview on the distribution of nestin-positive cells around the glioma and in the SVZ. In all three planes, the occurrence of precursor cells at the border of tumor and parenchyma persists at this site, and the intensities for nestin/GFP can even be higher than in the SVZ, remarkably in coronal and sagittal plane. *E*, optical section through the glioma shows the presence of several layers of precursor cells enwrapping the tumor. GD,. Scale bars: *A*–*C*, 1 mm; *D*, 20 μm.

that is lesion specific insofar as trauma induces astroglial differentiation, a loss of neurons favors neuronal differentiation, and oligodendrogenesis occurs essentially in response to demyelination.

Studying the specificity and time course of NPC aggregation around experimental glioblastoma, we observed an acceleration in the extent of NPC attraction peaking at day 14, when the density of NPCs around glioblastomas had augmented so strongly that the tumor was completely surrounded by a layer of nestin/GFP-positive cells (*Fig. 40.2*). To distinguish whether the accumulation of NPCs is triggered by injury or is a specific response to a tumor, we compared the tumor-induced accumulation with sham operations (stab wounds). Accumulation of NPCs in animals receiving stab wounds peaked 7 days after application of the lesion and, in contrast to their association with glioma, NPCs gathered loosely around the stab wound. Additional controls for grafts of glioblastoma cells included the injection of a nontumorigenic fibroblast cell line (scrc-1008) and primary cultured astrocytes. NPCs showed only a minor accumulation at these control grafts. In summary, we were able to demonstrate mobilization and recruitment of NPCs from the SVZ, which was specifically induced by glial tumor growth. The accumulation of endogenous precursors is likely to resemble a normal intrinsic tissue response to tumors because the substantial tropism of precursor cells to the tumor could not be initiated by any other lesion, such as stab wounds or injection of nontumorigenic cells. Moreover, the tropism of NPCs for glioblastomas persisted in noninjured areas.

AGE-DEPENDENT INDUCED RECRUITMENT OF ENDOGENOUS NPCS

The activity of the germinal centers declines with increasing age.[32] Unlike hematopoietic stem cells, the number of NPCs within the SVZ decreases during aging. It seems that the reserve capacity of the hematopoietic stem cell population may be greater and the rate at which stem cells are replaced seems to be higher for hematopoietic stem cells. The fact that neurogenesis within the brain preserved the normal CNS function, the loss of functioning NPCs can be expected to lead to age-related neurodegeneration.[44] This could be a potential contributing factor to the development of Parkinson's and Alzheimer's disease.

We were able to demonstrate that attraction of NPCs to glioblastomas declines with increasing age. Therefore, we

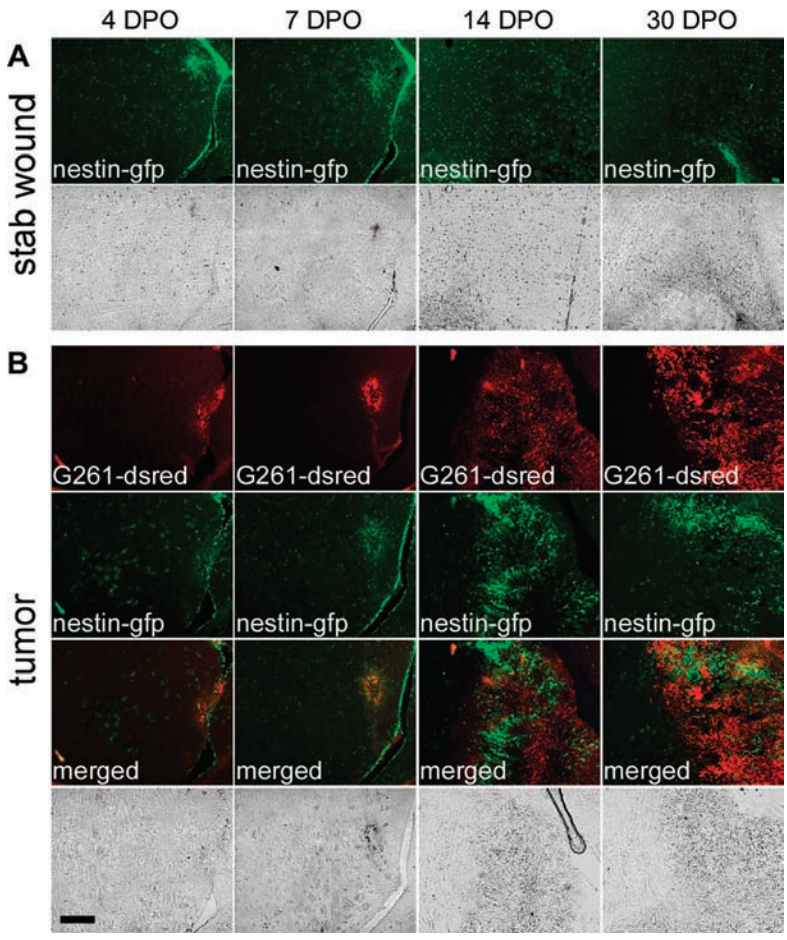

FIGURE 40.2. Specific responses of endogenous NPCs to different pathological events. Time course and intensity of the accumulation of nestin/GFP cells at G261/dsred-induced glioblastomas (*B*) or at stab wounds (*A*) are different in mice of P25. At 4 and 7 days postoperatively (DPO), nestin/GFP cells accumulated similarly at tumors and controls. This changed dramatically at 14 DPO. In stab wound controls of 14 DPO and 30 DPO, no nestin/GFP-positive cells remained at the lesion. At 14 DPO, G261/dsred-induced glioblastomas reached a diameter of more than 1 mm, closely encircled by a rim of NPCs forming a layer of at least 200 μm. In glioblastoma-bearing mice at 30 DPO, the continuous layer of nestin/GFP-positive cells around the tumor was absent and the tumors had largely increased in size. Scale bars for fluorescence images, 120 μm.

compared accumulation of nestin/GFP-expressing cells (NPCs) and glioblastoma tumor growth (induced by G261-dsred) 14 days after inoculation into mice at the age of P25, P100, P180, and P400 (*Fig. 40.3*). In mice at P25, the tumors were small and compact and we found significantly more aggregates of NPCs at the tumor margin compared with animals at ages of P100, P180, and P400. Concomitant with this decreasing accumulation of NPCs at the experimental glioblastomas, the tumor sizes increased significantly with age. At P400, the tumors extended over most of the brain hemisphere and we found only a few NPCs interspersed with the glioblastoma cells. This indicates that, in older subjects, precursor cells can no longer be activated to accumulate at the tumor and, in addition, tumor growth inversely correlates with the abundance of NPCs in the brain parenchyma.

LESION-SPECIFIC RECRUITMENT OF NPCS FROM THE GERMINATIVE CENTERS

In the developing brain, cells born in the ventricular zone migrate to forebrain nuclei, whereas neuroblasts of the adult SVZ migrate to the olfactory bulb (OB) along the RMS. Within the RMS, neuroblasts are guided by a meshwork of astrocytes-like cells and move by mode of migration known as chain migration.[29] Several studies underline the notion that brain injury induces NPCs from the SVZ to migrate to non-OB areas, especially to the site of the lesion.[16] Even lesions that do not directly damage the SVZ can affect migration within the SVZ/RMS. To date, no studies exist that examine the induction of NPCs from the SVZ induced by glial brain tumors as glioblastoma. Thus, we labeled the dividing NPCs of the brain with the thymidine analog, BrdU, 24 hours before the inoculation of glioblastomas. Fourteen days after tumor cell injection, individual cells in the immediate surroundings of the tumor were immunoreactive for both nestin/GFP and BrdU (*Fig. 40.4*). To examine the tropism of NPCs in vivo, we administered the fluorescent dye, DiI, into the ventricle of the left brain hemisphere and, thereby, labeled all cells (ependymal and stem cells of both hemispheres) having direct contact with the fluid continuum of the ventricular system. Subsequently, we inoculated glio-

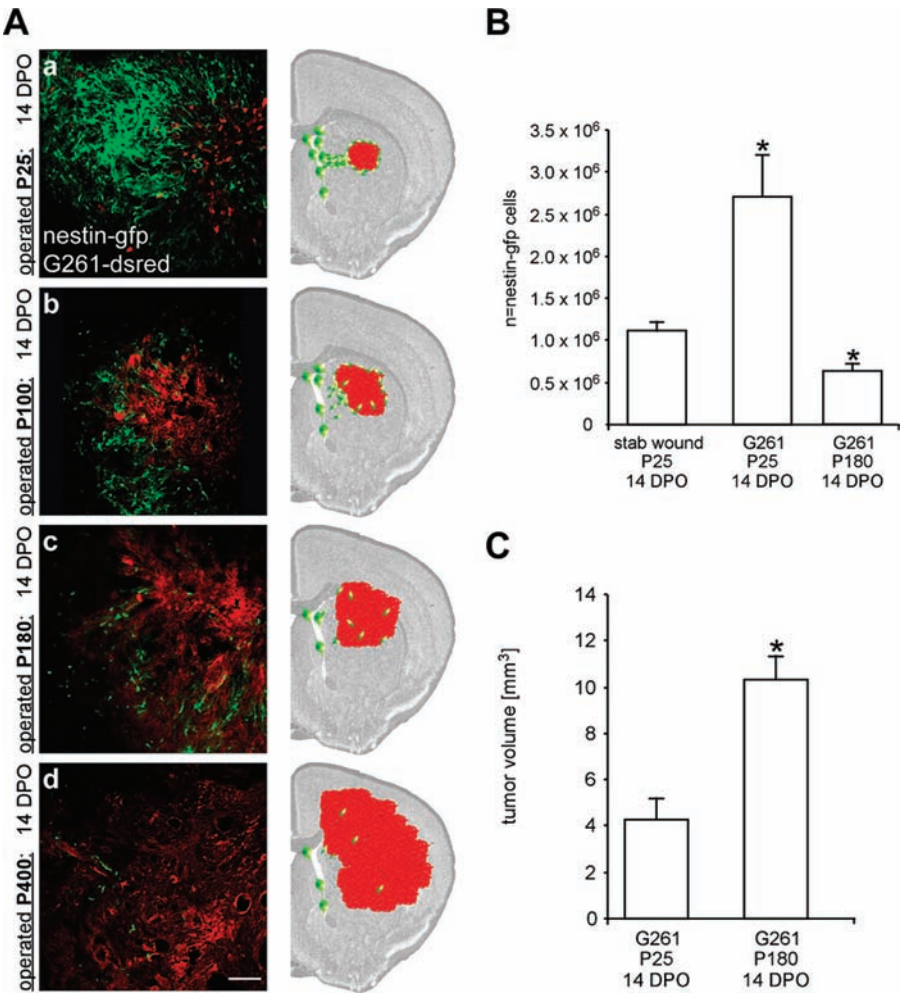

FIGURE 40.3. The induction of NPC recruitment is age dependent. *A*, a comparison between young (P25) and old (P400) animals shows that the significant rim of endogenous precursor cell surrounding gliomas in young animals 14 DPO is virtually undetectable in aged animals at P400. The severity of the pathological state augmented with increasing age and is illustrated by fluorescence images. G261-dsred induced glioblastomas in 100-day-old mice comprised a confined tumor mass, whereas in older mice, the G261 tumor cells invaded the brain parenchyma at P400, causing necrotic centers and local bleeding. Many nestin/GFP-positive cells surrounded the tumor in the 25-day-old animals, whereas at P100, only single, but nevertheless large, patches of NPCs were found. At P180 and P400, single scattered NPCs were deeply embedded within the tumors. *B*, stereological cell counting of serial horizontal sections was performed in the area where the main tumor mass was located, including the SVZ. The number of nestin/GFP-positive cells within the defined area was significantly ($P < 0.05$) larger in glioblastoma-bearing mice at P25 than in those of P180 or stab wound controls at P25. *C*, tumor volume in nestin/GFP mice injected with G261-DsRed cells was significantly ($P < 0.05$) smaller in young animals (P25) than in old animals (P180). Scale bars for fluorescence images, 50 μm.

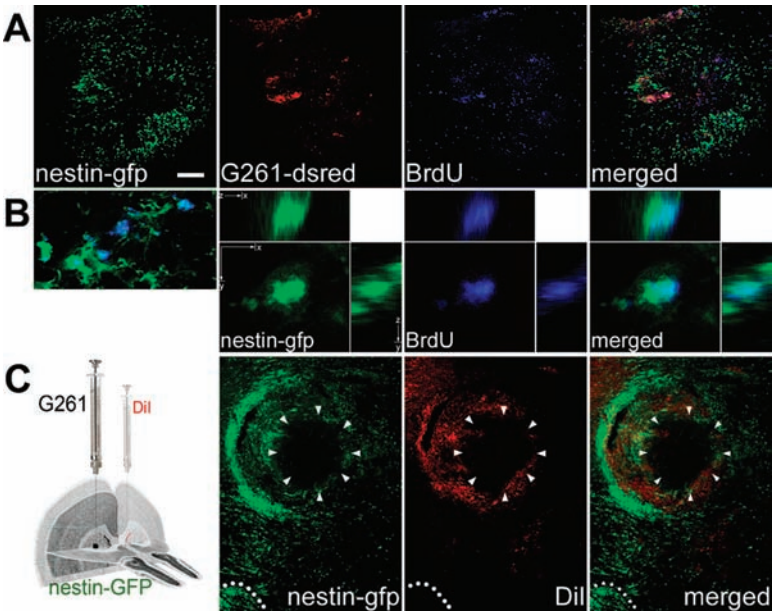

FIGURE 40.4. Lesion-specific recruitment of NPCs from the germinative centers. *A*, nestin/GFP-positive NPCs (*green*) were labeled preoperatively for BrdU (*blue*) and appear around a G261/dsred-induced glioblastoma (*red*). Many nestin/GFP cells at the tumor border are labeled for BrdU. *B*, colabeling for nestin/GFP and BrdU of a single cell was confirmed by three-dimensional reconstruction. *C*, illustration of the location and procedure for injections of unlabeled G261 cells and DiI into nestin/GFP mice. At 14 DPO, a glioblastoma has developed in the caudate putamen of a nestin/GFP mouse and green-labeled (nestin/GFP-positive) NPCs emanating from the SVZ (indicated by *dots*) accumulate around the tumor. The glioblastoma (*arrowheads*) is encircled by nestin/GFP-positive cells colocalized with the DiI staining.

blastoma cells into the caudate putamen of the right hemisphere (*Fig. 40.4*). Fourteen days after the operation, a significant co-labeling for both DiI and nestin/GFP was detected in the immediate vicinity of the tumor, suggesting that precursors had migrated in from the SVZ to the tumor. Triple labeling of nestin/GFP with doublecortin (DCx) and DiI demonstrates that the DiI-positive cells are migrating NPCs. In our view, these data indicate that proliferating NPCs from the SVZ were attracted by the glioblastomas. None of the experimental glioblastomas used for this study invaded into the SVZ, indicating that the attraction of NPCs toward the tumor was established over a distance without direct cell-to-cell contact. We are currently investigating which molecular mechanisms control such a recruitment of NPCs toward brain tumors.

GLIOBLASTOMA-INDUCED DIFFERENTIATION OF GENUINE NPCS

Previous reports indicated that pathological events such as stroke initiated migration of NPCs toward the site of injury, where the NPCs then differentiated in a manner appropriate for the affected brain region.[3,24]

We could verify that 30% of SVZ-derived NPCs around the tumors were actively dividing, as identified by co-labeling with Ki67 (*Fig. 40.5*). These NPCs expressed markers of genuine NPCs, such as Musashi to 35%, NG-2 to 35%, polysialylated neural cell adhesion molecule (PSA-NCAM) to 30% and DCx to 10%. Glial fibrillary acidic protein (GFAP) is a classic marker for astrocytes and, in the context of nestin expression, is also a marker for putative resident stem cells, and was found in 60%. This does not imply that they are stem cells in the sense of the neurogenic regions, but sets them apart from other astrocytes, labeled by S100β as a marker for mature astrocytes or calbindin as a neuronal marker, which were not expressed in SVZ-derived NPCs. We detected coexpression of markers for the oligodendrocyte lineage, such as the glycolipid O4 or the myelin protein CNP, only in a few SVZ-derived NPCs (10%). Labeling for markers for endothelial cells (isolectin B4 and von Willebrand factor) was absent from SVZ-derived NPCs.

ANTITUMORIGENIC IMPACT OF ENDOGENOUS NPCS

During the last years, several studies have proven that genetically modified mouse, rat, and human NPCs are able to promote tumor regression and prolong survival of recipients animals.[5,8,9,12,47] We recently showed that, in old mice, the pool of endogenous precursor cells is no longer recruited to experimental brain tumors. At the same time, glioblastoma in these older mice grow more vigorously, suggesting that endogenous NPCs have an influence on glioblastoma growth. To directly determine the influence of NPCs on tumor growth, we injected tumor cells either alone or together with exogenous NPCs into brains of older mice, which retain little capacity to respond to the tumor with the activation of their endogenous NPCs. We then compared the survival times after inoculation in three groups of nontransgenic recipients (*Fig. 40.6A*): one group of young (P25) and older mice (P180) received G261 tumor cells alone, whereas a third group of P180 mice obtained a 3:1 mixture of cultured adult NPCs and G261 cells. Our results show that the cumulative survival of younger mice significantly exceeded that of older animals when only glioblastoma cells were administered. However, when NPCs were co-injected with the glioblastoma

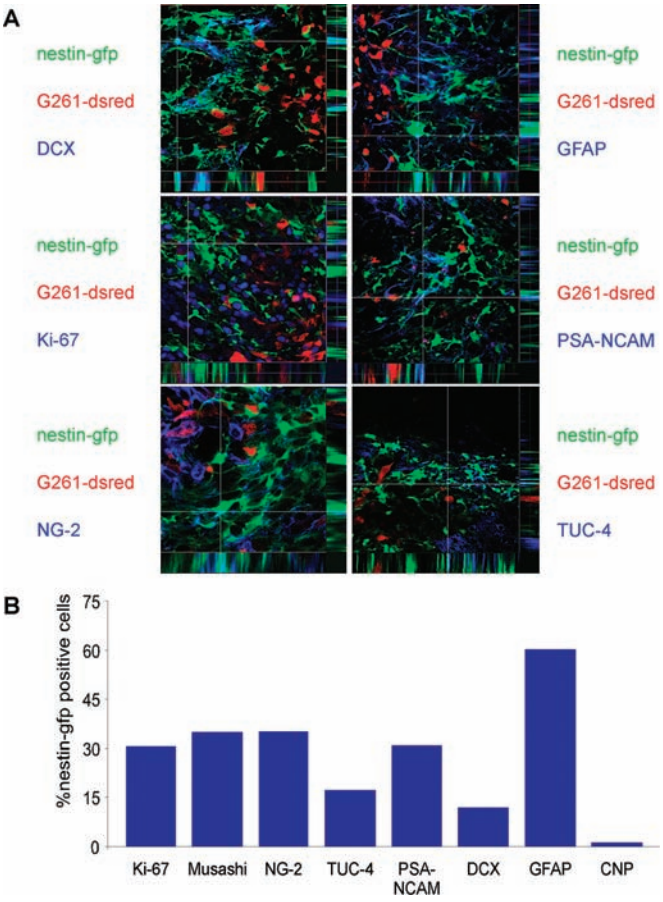

FIGURE 40.5. Glioblastoma-induced differentiation of genuine NPCs. *A*, images represent three-dimensional reconstruction along the *yz* axis (*right narrow panel*) and *xz* axis (*bottom narrow panel*) of a z series through P25 nestin/GFP-positive cells (green) in the tumor border of G261/dsred-induced glioblastomas (*red*) 14 DPO, which expressed cell type-specific markers (*blue*). Colocalization of these markers with nestin/GFP cells is demonstrated by the *crosshair*, which appears *cyan* in the corresponding planes. Illustrated markers are DCx, which controls migration in NPCs and was only detected in the cytoplasm of nestin/GFP-positive cells in the germinal centers and around tumors; the intermediate filament GFAP, which is present in neural stem cells, radial glia, and astrocytes, primarily colabeled with nestin/GFP in areas close to tumors. Few cells expressing only GFAP but not nestin/GFP were observed in the area bearing G261/dsred cells; the cell cycle protein Ki67 (a marker for cell proliferation) labeled nuclei of numerous glioblastoma cells and many nestin/GFP cells; the NPC marker PSA-NCAM, which was almost exclusively detected on nestin/GFP-positive cells and was not found on glioblastoma cells; NG-2, a marker for neoplastic cells, was abundant on the plasma membranes and in the cytosol of nestin/GFP-positive cells and glioblastomas; and TUC-4, a marker for postmitotic neuronal precursors, was well detectable in the area close to the experimental glioblastoma and colabels with a subpopulation of nestin/GFP cells. *B*, quantification of colabeled nestin/GFP-positive cells in the vicinity of G261/dsred-induced glioblastoma in P25 mice 14 DPO. Scale bars, 200 μm.

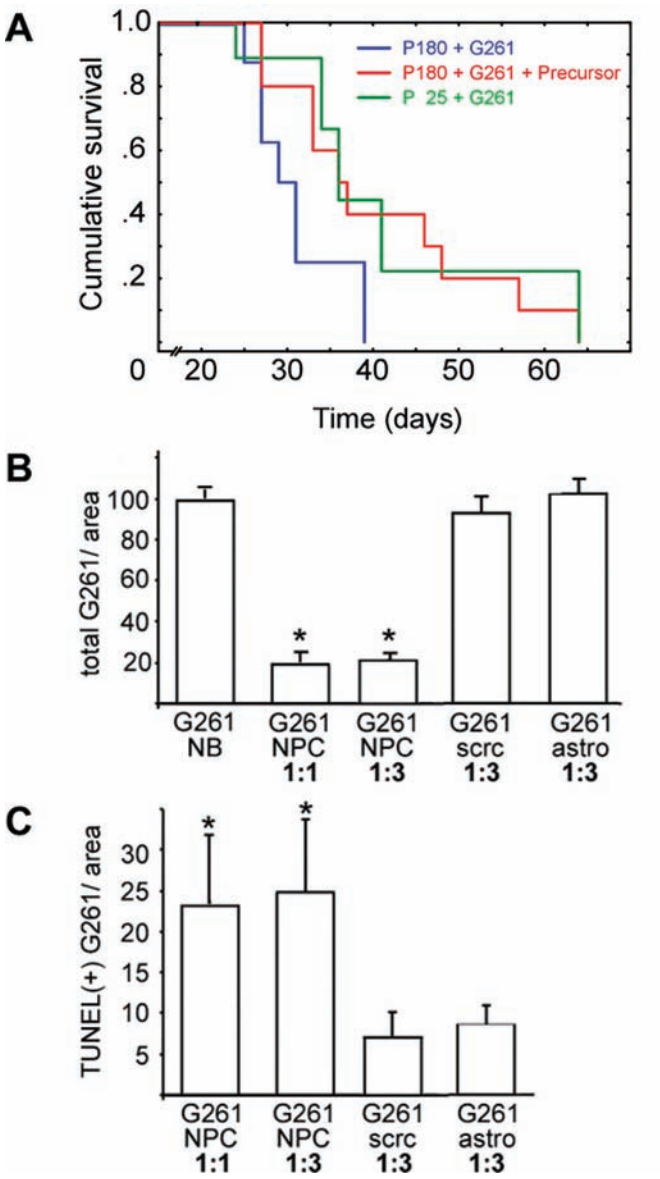

FIGURE 40.6. Antitumorigenic impact of endogenous NPCs. *A*, survival curves of young (P25 + G261; n = 8) and old (P180 + G261; n = 9) animals after G261 cell inoculation demonstrate that the young animals survived significantly longer than the older but identically treated animals. However, when old mice (P180) were injected with a mixture of cultured adult NPC and G261 cells (P180 + G261 + precursor; n = 10), the old animals survived better, similar to the young mice. *B*, glioblastoma cells (G261) were either cultured in unconditioned precursor cell medium (NB) or cocultured with NPCs in different ratios (1:1 or 1:3); G261 cells were also cocultured with scrc-1008 fibroblasts (scrc) and astrocytes (astro); after 72 hours, the number of G261 cells detected in cocultures with NPC was five times lower than in the controls. *C*, after 72 hours of coculturing, the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-positive (apoptotic) G261 cells was four times greater in cocultures with NPC than in controls.

cells, the older mice reached the same survival time as the young animals. Further, we determined whether the NPCs mediated a direct effect on glioblastoma cell numbers and whether the precursors were able to induce apoptosis of tumor cells. Co-culturing of G261 cells together with NPCs for 72 hours mostly (by 80%) and specifically (compared with controls) abrogated the increase in cell number normally observed with these highly proliferative tumor cells (*Fig. 40.6B*). The rate of apoptosis was increased approximately four times in co-cultures of glioblastomas and NPCs compared with controls within the 72-hour observation time (*Fig. 40.6C*).

DISCUSSION

There is substantial evidence that neurogenesis in the adult brain can be further stimulated by lesions or injury.[4,19,28,30,33,34,48,54–57] Seizures have been shown to trigger additional proliferation of NPCs in the dentate gyrus.[6,40] Pathological insults such as cerebral hypoxia/ischemia induce enhanced cell proliferation and recruitment in the injured regions of the adult brain to harbor NPCs, whereas this phenomenon rarely occurs in the contralateral noninjured site.[3] The mechanisms regulating migration of NPCs are not well defined. Factors that attract NPCs to lesions such as a tumor, although not yet known, are likely to include chemokines, trophic factors, and components of the extracellular matrix. Examples of trophic factor attraction for endogenous NPCs include stromal cell-derived factor 1, which promotes migration of cerebellar NPCs.[41] Transforming growth factor-α, which can attract NPCs from the SVZ to the striatum, is another well-evaluated candidate[11] for this lesion-specific induction.

There is ample evidence that brain tumors result from the aberrant, uncontrolled propagation of single clones of NPCs. This clonal origin of gliomas was suggested after identifying common mutations in two different cell types within the same tumor—i.e., oligodendroglial and astrocytic cells in oligoastrocytomas, and glial and sarcomatous cells in gliosarcomas.[31] Only neural stem cells retain the ability to form all of these different cell types and a conserved genetic lesion within differentiated cells indicated that they stem from an identical source. Therefore, it was concluded that transformed (genetically lesioned) neural stem cells are the point of origin of brain tumors. Further, many human gliomas express a truncated form of the epidermal growth factor (EGF) receptor that is constitutively active even in the absence of ligand binding, mimicking the ability of EGF receptor to promote NPCs proliferation.[35] Other genetic changes can also promote NPC proliferation, including activation of sonic hedgehog signaling, which can activate N-myc transcription in granule cell precursor cells, which are thought to be the cell of origin of pediatric medulloblastomas.[37] Neural stem cells have been identified as a small subpopulation in human brain tumors with the capacity for proliferation, self-renewal, and differentiation.[45,46] These cells may represent the regenerating core of the tumor, with other cells in the tumor derived from them being in the process of differentiation.[42]

Our own studies indicate that endogenous NPCs have a strong tropism for glioblastomas in vivo.[15] This is in concordance with observation that SVZ cells can undergo increased proliferation, ectopic migration, and multipotential differentiation, thereby specifically responding to certain lesions. Insofar as trauma induces astroglial differentiation, a loss of neurons favors neuronal differentiation, and oligodendrogenesis occurs essentially in response to demyelination. Thus, it seems that local cues originating either from the lesion or from the environment in which cells migrate are important to direct their differentiation into the appropriate cell fate. Further, the abundance of NPCs at the tumor is associated with reduced tumor size and increased survival. Such antitumorigenic effects have been described previously for exogenously added immortalized NPCs from newborn mice,[5,47] but, as mentioned above, no relationship to endogenous, non-immortalized precursor cells could be established. Glioblastomas can originate from NPCs undergoing misregulated proliferation or differentiation,[22] and a recent series of studies demonstrated that glioblastomas contain a highly aggressive subpopulation of cancer stem cells.[14,20,45,46] Our results extend the knowledge on the relation between NPCs and brain tumors from the previously suggested origin of gliomas from NPCs and the recently discovered brain tumor stem cells to the notion that endogenous NPCs also respond to existing tumors and mediate effects beneficial for the tumor host. Here we could prove that the accumulation of NPCs at a glioblastoma is age dependant and discovered a transient increase of precursors at the tumor, which peaked at 14 days after tumor inoculation. Progressive differentiation of NPCs could be initially responsible for the reduction of precursor cell numbers at the tumor. NPC differentiation may be initiated by the growth factors secreted from the tumor, such as insulin-like growth factor I,[21,49] and may promote NPC differentiation.[2] The secretion of such factors may be necessary for the glioblastoma to prevail over the precursors surrounding the tumor, and, with increasing tumor size, more growth factors are released. EGF and fibroblast growth factor-2 both trigger proliferation of NPCs, whereas the latter has also been shown to trigger differentiation.[18,53]

We conclude that the impairing effect on tumor growth observed in our study is mediated by an interaction between precursor and tumor cells rather than by a replacement of lost brain cells by the precursors. This response can be interpreted as an attempt of intrinsic antitumorigenic activity, or even regeneration, that ultimately succumbs to the invasiveness of the tumor. In young animals with a large endogenous precursor cell pool, this response might be more prominent than in

older animals that have a decreased precursor cell population. Finally, our data suggest that a strong intrinsic precursor cell response can control glioblastoma growth, and this control fails once the number of endogenous precursor cells decreases with increasing age.

ACKNOWLEDGMENTS

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