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SHORT COMMUNICATION

Complementary tumor induction in neural grafts exposed to *N*-ethyl-*N*-nitrosourea and an activated *myc* gene

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Using a combination of transplacental carcinogen exposure and retrovirus-mediated oncogene transfer into fetal brain transplants, we have studied complementary transformation by *N*-ethyl-*N*-nitrosourea (NEU) and the v-*myc* oncogene in the nervous system. Previous experiments had demonstrated that both agents will not induce tumors independently whereas simultaneous expression of v-H-*ras* and v-*gag/myc* exerted a powerful transforming potential in neural grafts. In order to identify other genetic alterations that co-operate with an activated *myc* gene, the neurotropic carcinogen NEU was used to generate mutations of cellular genes. On embryonic day 14 (ED14), pregnant donor animals (F344 rats) received a single i.v. dose of NEU (50 mg/kg). Twenty-four hours later (ED15), the fetal brains were removed, triturated and incubated with a retroviral vector carrying the v-*gag/myc* oncogene. Subsequently, these primary cell suspensions were transplanted stereotactically into the caudate-putamen of syngenic adult recipients. After latency periods of 3–6 months, 5 of 10 recipients harboring ED15 fetal brain transplants developed malignant, poorly differentiated neuroectodermal tumors in the grafts. No tumor development was observed in seven recipients harboring ED16 neural grafts. Cell lines were established from three tumors and the 110 kd gag/myc fusion protein encoded by the retroviral construct was identified in the tumors by Western blotting. Several candidate genes for mutational activation by NEU including the H-*ras*, K-*ras* and *neu* oncogenes were analyzed for specific point mutations by polymerase chain reaction (PCR) and direct DNA sequencing of the PCR products. However, no mutations were found in any of these genes. These findings lend further support to the multistep hypothesis of neoplastic transformation in the brain. The tumors induced in this model provide an interesting tool for the identification of genes that co-operate with an activated *myc* gene in neurocarcinogenesis.

We have recently established a novel strategy to introduce foreign genes into the central nervous system. This approach employs retrovirus-mediated gene transfer into neural and non-neuroectodermal cells of fetal brain transplants (1–3). In this model, the simultaneous expression of activated *ras* and *myc* genes yields highly malignant neoplasms in 100% of the grafts. Introduction of a retroviral construct containing the v-*myc* gene only or pretreatment of the donor cells with a single prenatal dose of the neurotropic carcinogen *N*-ethyl-*N*-nitrosourea (NEU*) does not result in a malignant phenotype (3,4). Since to date no mutationally activated *ras* genes have been detected in sporadic human brain tumors, the combination of *ras* and *myc* genes does not appear to play a role in human neurocarcinogenesis. In contrast, the *myc* oncogene family has been associated with the pathogenesis of a variety of neuroectodermal tumors (5,6). In order to identify other genes that co-operate with *myc* in neoplastic transformation, we have combined the expression of an activated *myc* gene with a second transforming agent, i.e. a single exposure of the donor cells to the alkylating carcinogen NEU.

For gene transfer into neural grafts, the replication-defective retroviral vector, DoK v-*myc*, which is based on the Moloney sarcoma virus genome, was used (Figure 1). This retroviral construct harbors the v-*gag/myc* gene of the MC29 avian myelocytomatosis virus and a bacterial phosphoribosyl transferase gene (*neo*^{r}) (7). Retroviral particles were produced in the ψ2 helper-free packaging cell line (8). ψ2 cells were kept in Iscove's modified Dulbecco's medium (IMDM) containing 10% newborn calf serum and virus containing supernatant was collected overnight at ~80% confluency.

Fetal CNS donor cells were prepared as described (1,9). Briefly, timed Fischer (F344) rats received a single i.v. dose of NEU (10 mg/ml in 3 mM sodium citrate, pH 5.6; total dose: 50 mg/kg) at days 14 or 15 of gestation. Twenty-four hours later, i.e. on embryonic day (ED) 15 or 16, the embryos were removed and the entire fetal brain was carefully dissected. Approximately 10 fetal brains were enzymatically dissociated (0.25% trypsin, 0.1% DNase in PBS, 10 min at room temperature) and gently triturated in order to obtain a single cell suspension. For retroviral infection the cells were incubated with the tissue culture supernatant of the ψ2 packaging cell line in the presence of polybrene (3 μg/ml) for 4 h at 37°C and 5% CO_2. After adsorption of the virus, the cells were washed with Hank's balanced salt solution to remove free retroviral particles. Cells were then pelleted and immediately used for transplantation. Host animals (adult male F344 rats, body wt 150–200 g) were anesthetized with 0.2 mg fentanyl and 10 mg droperidol/kg body wt and received a single stereotaxic injection of 5 μl cell suspension containing ~10^6 cells into the center of the left caudate-putamen. Control neural grafts with fetal donor cells exposed only to a transplacental dose of NEU or to the retroviral vector

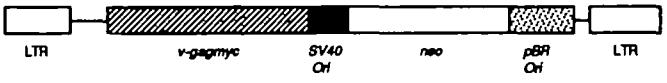

Fig. 1. Structure of the retroviral vector harboring cDNAs for the v-*gag/myc* gene of the MC29 avian myelocytomatosis virus and the *Escherichia coli* phosphoribosyl transferase gene (*neo*^{r}). Expression of the v-*gag/myc* gene is driven by the Moloney murine leukemia virus 5′ long terminal repeat.

*Abbreviations: NEU, *N*-ethyl-*N*-nitrosourea; IMDM, Iscove's modified Dulbecco's medium; ED, embryonic day; H–E, hematoxylin–eosin; GFAP, glial fibrillary acidic protein; NSE, neurone-specific enolase; PCR, polymerase chain reaction.

encoding v-*gag/myc* did not develop any pathology within an observation period of 12 months.

Postoperatively, recipient animals were closely monitored for signs of neurological impairment. Animals with severe neurological symptoms were anesthetized with diethyl ether and decapitated. Frontal brain slices were fixed in 4% buffered paraformaldehyde (pH 7.6) and embedded in paraffin. Sections from all animals were stained with hematoxylin–eosin (H–E). For immunocytochemical reactions, polyclonal antibodies to glial fibrillary acidic protein (GFAP; Dakopatts, Copenhagen, Denmark), neurone-specific enolase (NSE; Dakopatts), S-100 protein (Dakopatts), and monoclonal antibodies to synaptophysin (SY-38, Boehringer, Mannheim, Germany), neurofilament protein (Becton Dickinson), vimentin (Dakopatts) and the panepithelial antigen Lu-5 (F.Hoffmann-La Roche, Basel, Switzerland) were used. Polyclonal antibodies were visualized with a commercial avidin–biotin–peroxidase kit (Dakopatts), monoclonal antibodies with a peroxidase–anti-peroxidase reaction.

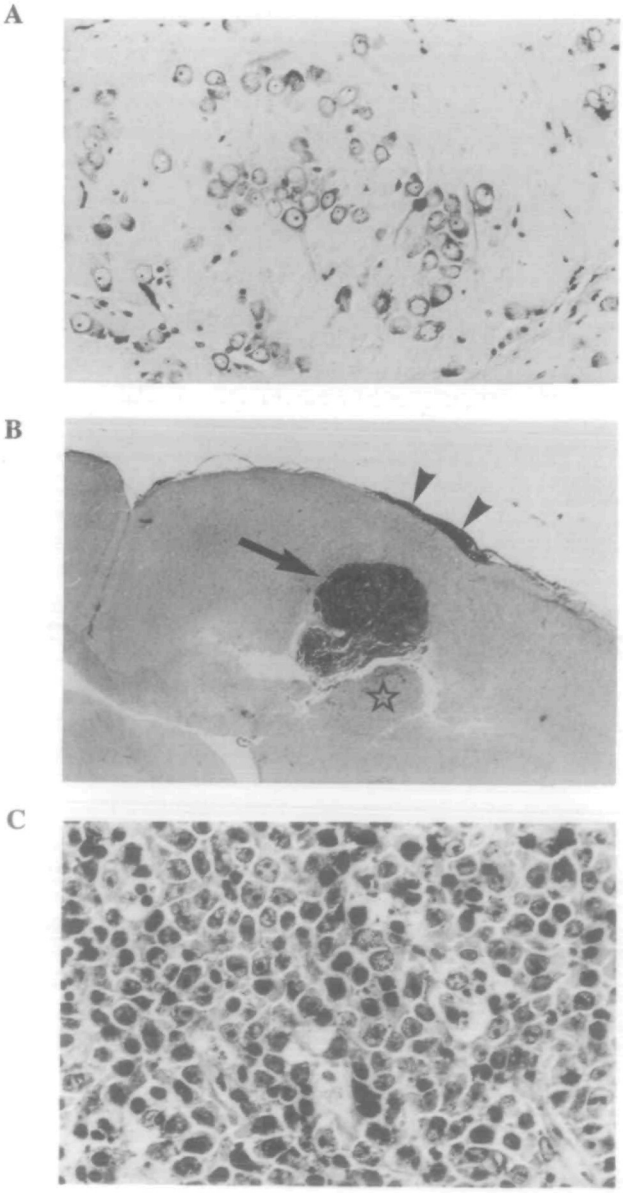

Fig. 2. Microscopic appearance of neural transplants exposed to NEU and the v-*gag/myc* gene. (**A**) Control transplant with a prominent population of mature neurones and glia. Note the focal pseudocortical arrangement of neuronal cells (H–E, ×250). (**B**) Solid tumor (arrow) in a transplant 3 months after exposure of the donor cells to NEU and a retroviral vector encoding the v-*gag/myc* gene. Note the infiltration of the tumor cells into the adjacent subarachnoid space (arrowheads). Unaffected graft tissue (asterisk) is located beneath the tumor (H–E, ×15). (**C**) At higher magnification, the undifferentiated tumor cells exhibit prominent nuclei and a small rim of eosinophilic cytoplasm. Numerous mitotic figures and areas of necrosis indicate malignancy (H–E, ×330).

Aliquots of tumor tissue were established as cell cultures and grown in IMDM containing 10% fetal calf serum. Starting 7 days after primary seeding, the cultures were propagated in selection medium containing geneticin (GIBCO, 0.8 mg/ml). *In vitro* differentiation of geneticin-resistant colonies derived from this experiment was assessed morphologically and immunocytochemically. In order to investigate the differentiation capacity of the established cultures *in vivo*, tumor cell lines were retransplanted into the brain of adult recipient animals as described above.

For Western blot analysis of the established tumor cell lines, cells were washed in cold PBS and incubated in lysis buffer (0.05 M NaCl, 50 μM leupeptin, 0.5% Nonidet P-40, 0.5% SDS, 0.5% sodium deoxycholate in 20 mM Tris–Cl, pH 7.4) for 15 min at 4°C. The suspensions were sonicated for 20 s and subsequently pelleted at 12 000 *g*, 4°C for 30 min. Each sample (10 mg) was separated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. A polyclonal rabbit antibody to the bacterially expressed exon 3 of the v-*myc* gene (Medac, Hamburg, Germany; dilution 1:500) and an immunoblot alkaline phosphatase assay kit (Bio-Rad Laboratories, Richmond, CA) were used to visualize the myc proteins.

Polymerase chain reaction (PCR)-mediated DNA amplification from paraffin sections and Sanger dideoxynucleotide sequencing of the PCR products were performed as described (10). Primer oligonucleotides for PCR were 5′-GCCTGCTGAAAATGACTGAG and 5′-CTCTATCGTAGGATCATATTC for exon 1 of K-*ras*, 5′-GACTCCTACAGGAAACAAGT and 5′-AGAAAGCCCTCCCCAGTTCT for exon 2 of K-*ras*, 5′-TCATTGGCAGGTGGGGCAGGA and 5′-GAGCTCACCTCTATAGTGGGA for exon 1 of H-*ras*, 5′-GACTCCTACCGGAAACAGGT and 5′-CTGTACTGATGGATGTCTTC for exon 2 of H-*ras*, and 5′-CAGCCCGGTGACATTCATCA and 5′-TCGTATACTTCCGGATCTTCTGTCT for the *neu* gene. Sequencing primers were 5′-TCTGAATTAGCTGTATCGTC for exon 1 of K-*ras*, 5′-GTAATTGATGGAGAAACCTG for exon 2 of the K-*ras*,

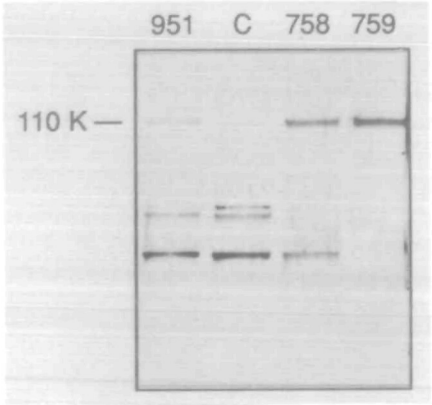

Fig. 3. Western blot analysis with an antibody to the bacterially expressed exon 3 of the v-*myc* gene. The 110 kd gag/myc fusion protein is detectable in the three cell lines derived from tumors induced in fetal neural grafts after exposure to NEU and v-*gag/myc* (951, 758, 759). Note the inverse correlation between the levels of v-gag/myc (upper band, 110 kd) and c-myc proteins (lower bands). C, Mock-infected fetal brain cells (negative control).

5'-ACCCCTGTAGAAGCGATGAC for exon 1 of H-*ras*, 5'-GTCATTGATGGGGAGACGTG for exon 2 of H-*ras* and 5'-TCGTATACTTCCGGATCTTCTGTCT for the *neu* gene.

The animals harboring ED16 transplants were killed after a survival period of 9 months without any signs of neurological impairment. All seven recipients exhibited regular grafts with the characteristic histological appearance of embryonal brain transplants, i.e. an irregular distribution of neurones and glial elements and formation of pseudocortical architectures (Figure 2A). Immunohistochemically, NSE, synaptophysin and neurofilament protein were readily detectable in neurones of the graft. Astrocytes were identified with antibodies to GFAP. No evidence for an abnormal phenotype or tumor formation was noted in these transplants. In contrast, 5 of 10 recipient animals carrying ED15 transplants developed severe neurological symptoms 3–6 months after grafting. Post-mortem examination showed highly malignant tumors originating from the graft in all five animals (Figure 2B and C). The tumor cells exhibited round to oval nuclei and a small rim of eosinophilic cytoplasm. Histopathological criteria for malignancy included numerous mitotic figures and focal necroses. Metastatic spread along the CSF into the cerebral ventricles and the subarachnoid space was noted in three recipients. Immunohistochemical analysis of the neoplasms revealed no expression of GFAP, synaptophysin and neurofilaments, i.e. antigens that indicate advanced glial or neuronal differentiation. The tumor cells showed no immunoreactivity for epithelial or mesenchymal antigens, i.e. the panepithelial antigen Lu-5 and vimentin. In all five animals, remnants of unaltered graft tissue were found adjacent to the neoplasms (Figure 2B).

Geneticin-resistant permanent cell lines were obtained from three tumor specimens. In culture, the tumor cells exhibited round to oval nuclei and a small rim of cytoplasm that frequently extended into short bipolar processes. In contrast to the primary tumors, the cell lines showed a weak expression of NSE and S-100 protein. Expression of the retrovirally encoded myc protein was confirmed by Western blot analysis. The 110 kd gag/myc fusion protein was readily detectable in extracts of the cell cultures but was absent in the spent media and in protein extracts respectively from cultured fetal brain cells not exposed to the retrovirus. The antibody showed cross-reactivity with endogenous c-myc products. An inverse correlation between c-*myc* expression and expression of the introduced v-*gag/myc* gene was noted (Figure 3). Intracerebral retransplantation of the tumor cell lines into adult recipient animals yielded rapidly growing neoplasms within 2 weeks of transplantation. These tumors showed histopathological features indistinguishable from the primary tumors. Immunohistochemically, there was no evidence for advanced glial or neuronal differentiation.

DNA sequence analysis of the *neu* transmembrane region revealed wild-type sequences in all five tumor specimens. No alterations were detected in codon 659, which is consistently mutated in NEU-induced schwannomas of the peripheral nervous system (11). Sequence analysis of exons 1 and 2 of the H- and K-*ras* genes showed no abnormalities within the mutational hot spots, i.e. codons 12, 13 and 61. Therefore, it is unlikely that neoplastic transformation is due to complementation with an activated *ras* gene.

Our findings indicate that NEU has the potential to mutationally activate or inactivate cellular transforming genes, which can cooperate with *myc* in neurocarcinogenesis. However, the respective target genes of NEU remain unknown. Attempts to identify by transfection assay a transforming gene involved in the induction of CNS gliomas by NEU have failed (11). This might indicate that NEU-induced carcinogenesis is mediated via a recessive mechanism, e.g. inactivation of a tumor suppressor gene. Mutational inactivation of the p53 tumor suppressor gene has been implicated in the pathogenesis of several neuroectodermal tumors (12–14). Recently, a high incidence of p53 mutations was found in rat nephroblastomas induced by transplacental exposure to NEU (15). Preliminary results suggest that the neoplasms induced in neural transplants exposed to NEU and the v-*gag/myc* gene do not carry p53 mutations (unpublished data).

Interestingly, tumorigenesis was restricted to ED15 grafts; no neoplastic lesions were noted in transplants derived from ED16 and ED14 (data not shown) donor cells. This indicates that the susceptibility of the respective target cell population to the transforming effect of NEU and an activated *myc* gene is restricted to a specific developmental stage. The fact that integration of retroviral vectors into the host genome declines significantly after ED14 (unpublished data) may further support such a temporal restriction.

The absence of preneoplastic lesions within the transplants and the considerable latency periods between transplantation and tumorigenesis suggest that combined exposure to NEU and v-*gag/myc* initiates neoplastic transformation in the donor cells but is not sufficient to produce a malignant or premalignant phenotype. Acquisition by these initiated cells of yet additional genetic alterations appears to be necessary for tumor formation.

In conclusion, our results provide *in vivo* evidence for a multistep development of brain tumors. Expression of the v-*gag/myc* gene in fetal brain transplants previously exposed to NEU represents an approach for identifying cellular genes that co-operate with activated *myc* in neurocarcinogenesis.

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