MOLECULAR AND CELLULAR BIOLOGY, Oct. 1986, p. 3537–3540
0270-7306/86/103537-04$02.00/0

Vol. 6, No. 10

Expression of c-*sis* and Platelet-Derived Growth Factor in In Vitro-Transformed Glioma Cells from Rat Brain Tissue Transplacentally Treated with Ethylnitrosourea

PETER F. LENS,[1]† BEP ALTENA,[1] AND ROEL NUSSE[2]*

Departments of Chemical Carcinogenesis[1] and Molecular Biology,[2] The Netherlands Cancer Institute (Antoni van Leeuwenhoekhuis), 1066 CX Amsterdam, The Netherlands

Received 13 March 1986/Accepted 21 June 1986

Long-term culturing of brain cells from neonatal BD-IX rats after transplacental treatment with *N*-ethyl-*N*-nitrosourea (ENU) results in malignantly transformed cells after a lag period of about 250 days. During culturing, the brain cells undergo a sequence of morphological changes. We examined oncogene expression in cultured cells from ENU-treated animals and found that transformed glioma cells differ from premalignant glial cells by containing high levels of c-*sis* transcripts. We also report that the transformed cells synthesize functional platelet-derived growth factor. Because glial cells have receptors for platelet-derived growth factor, we propose that an autocrine mechanism plays an important role in ENU-induced brain tumorigenesis.

Administration of the carcinogen *N*-ethyl-*N*-nitrosourea (ENU) to pregnant rats results in the development of malignant gliomas in the offspring after a latency period of 200 to 300 days (14). By long-term culturing of brain cells from neonatal rats after transplacental treatment with ENU, malignant transformation is also observed in vitro, after approximately the same latency period (16). During culturing, the brain cells undergo a sequence of morphological changes which are similar to alterations found during in vivo tumorigenesis. Thus, the contribution of specific oncogenes to progression can be studied in an in vitro system that is a faithful model of tumorigenesis in vivo (16). We examined oncogene expression in cultured cells from ENU-treated animals and found that transformed glioma cells differ from premalignant glial cells by containing high levels of c-*sis* transcripts.

Neonatal brain cells from normal rats and from animals treated transplacentally with a single dose of ENU (75 μg/g of body weight) were cultured by the method of Laerum and Rajewsky (16), under conditions that select for glial cells. Brains of newborn rats of one litter (6 to 10 per experiment) were dissected aseptically, cut into fragments, and passed through a stainless-steel grid. The resulting cells were cultured in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. The cells from untreated animals could be maintained in culture for periods up to 400 days and were phenotypically normal (B21 culture). In contrast to untreated control cells, dissociated brain cells from ENU-treated animals (B8 and B14 cells) transformed in culture after a period of 250 days. Two cultures from independently transformed cells were established, B8.34 and B14.91. The transformed cells were able to grow in soft agar, in contrast to premalignant stages of ENU-treated cultures or control cultures. In addition, only the transformed cells could be cultured in serum-free medium and were tumorigenic in syngeneic rats. The tumors resulting from injected cells were histologically classified as gliomas. Phase-contrast micrographs of premalignant and transformed cells are shown in Fig. 1.

Several markers were used to identify the cell type of the brain cells cultured under these conditions. In indirect immunofluorescence assays, the premalignant cells (from ENU-treated animals) and normal cells (from untreated animals) stained homogeneously positive with an antiserum against glial fibrillary acidic protein, an intermediate filament protein of glial cells (4). The cells were negative for neurofilament expression, a marker for neuronal cells (27), and negative for carbonic anhydrase and galactocerebroside, both markers for oligodendrocytes (20). These cells thus belong to the glial cell lineage.

The expression of oncogenes in normal and transformed cells was studied by Northern blotting of poly(A)$^+$ RNA, followed by hybridization to several oncogene probes. Cellular RNA was isolated by LiCl precipitation essentially as described by Auffray and Rougeon (1). Total RNA was enriched for poly(A)-containing RNA by two cycles of oligo(dT)-cellulose affinity chromatography (18). Portions (10 μg) of poly(A)$^+$ RNA were subjected to electrophoresis in formaldehyde gels and transferred to nitrocellulose filters (18). Nitrocellulose filters were prehybridized for at least 4 h at 63°C in 3× SSC (450 mM sodium chloride, 45 mM sodium citrate)–5× Denhardt solution (bovine serum albumin, 1 mg/ml; Ficoll 400, 1 mg/ml; polyvinylpyrrolidone, 1 mg/ml)–dextran sulfate (10%) and subsequently hybridized for 16 h at 63°C in hybridization mix with different probes. The c-*sis* probe was a 1.7-kilobase *Bam*HI fragment prepared from a c-*sis*-containing cosmid clone (9) that was radioactively labeled by nick translation (18). Filters were washed three times in 3× SSC–0.1% sodium dodecyl sulfate (SDS) at 63°C and twice in 1× SSC–0.1% SDS at 63°C. Hybridization was detected by autoradiography for 3 days at −70°C with Kodak XS-1 film and an Ilford intensifying screen. The levels of c-*myc*, N-*ras*, and Ha-*ras* RNA did not significantly differ between premalignant and transformed cells (data not shown). However, a dramatic increase in the expression of c-*sis* was observed. In the two transformed cell cultures (B8.34 and B14.91), intense hybridization of the *sis* probe to a 3.8-kilobase RNA was found (Fig. 2), whereas in premalignant (B8.18 and B14.11) or in control (B21.20)

* Corresponding author.
† Present address: Hubrecht Laboratory, International Embryological Institute, 3584 CT Utrecht, The Netherlands.

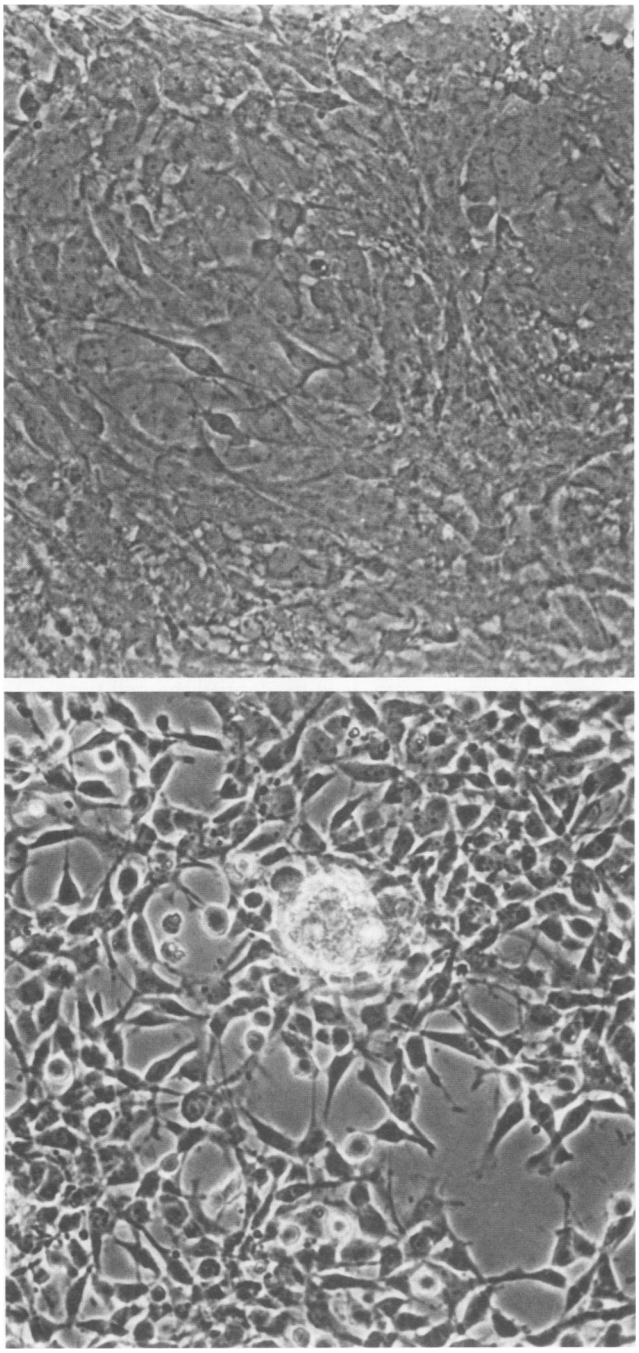

FIG. 1. Phase-contrast micrographs of premalignant and malignant rat brain cells. (a) Secondary culture (day 70 in culture) of premalignant flat epitheloid cells (B8.18). These cells are nontumorigenic, do not grow in soft agar, and show a high serum requirement. (b) Passage 13 of malignant cells. These cells are tumorigenic and are able to grow in soft agar and in 0.5% serum.

cells expression of c-*sis* was undetectable. The blots were hybridized afterwards with a probe for α-tubulin, showing that equal amounts of RNA had been loaded on the gels (data not shown). The rat c-*sis* transcript was approximately 0.4 kilobase shorter than the primate c-*sis* RNA, a difference that was reproducibly found but for which we have no explanation.

Since the c-*sis* gene encodes the B chain of platelet-derived growth factor (PDGF) (6, 26), a known mitogen for glial cells (10, 21, 23), we next investigated whether the transformed brain cells synthesized functional PDGF. An antiserum specific for PDGF was kindly provided by C.-H. Heldin (11) and used in radioimmunoprecipitation assays on medium from metabolically [^{35}S]cysteine-labeled transformed B8 cells. B8 cells were grown to subconfluency at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were then grown in serum-free linolea medium (13) for 1 week with two medium changes and then given 20 ml of cysteine-free RPMI medium supplemented with 1 mCi of [^{35}S]cysteine (1,300 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). After incubation for 36 h at 37°C, the medium was harvested and exposed to 50 μl of rabbit control serum. After incubation of the medium for 20 h at 4°C, protein A-Sepharose (Pharmacia, Uppsala, Sweden) was added (100 μl of packed beads), and the mixture was incubated for another 2 h. The beads were then collected by centrifugation, and the supernatant was incubated with 50 μl of anti-PDGF serum (11). After incubation for 20 h at 4°C, immune complexes were absorbed to protein A-Sepharose. The beads were washed four times with 0.5 M NaCl–0.01 M phosphate buffer (pH 7.4)–0.5% bovine serum albumin–0.1% Tween 80 and once with 10 mM Tris hydrochloride buffer (pH 7.4). The beads were then extracted with 100 μl of 3.6% SDS–80 mM Tris-hydrochloride buffer (pH 8.8)–0.01% bromophenol blue and heated for 3 min at 95°C. The supernatants were divided into two equal portions, one of which was reduced by incubation with 10 mM dithiothreitol for 3 min at 95°C and analyzed by SDS-gel electrophoresis with 14% polyacrylamide gels (15), followed by fluorography.

Under nonreducing conditions, the antiserum precipitated

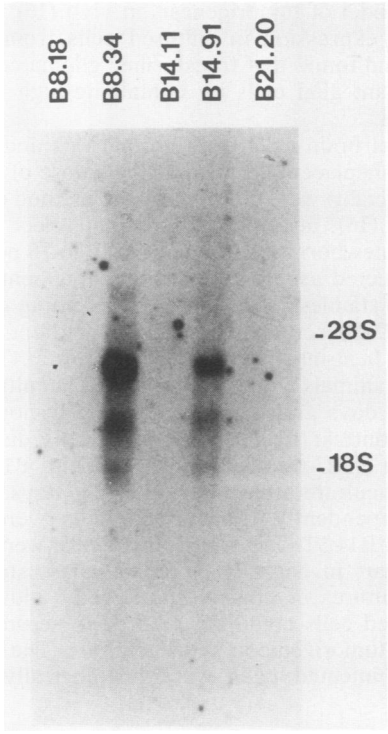

FIG. 2. c-*sis* transcripts in premalignant cells (B8.18 and B14.11), control cells from untreated animals (B21.20), and malignant glioma cells (B8.34 and B14.91), determined by RNA blot analysis.

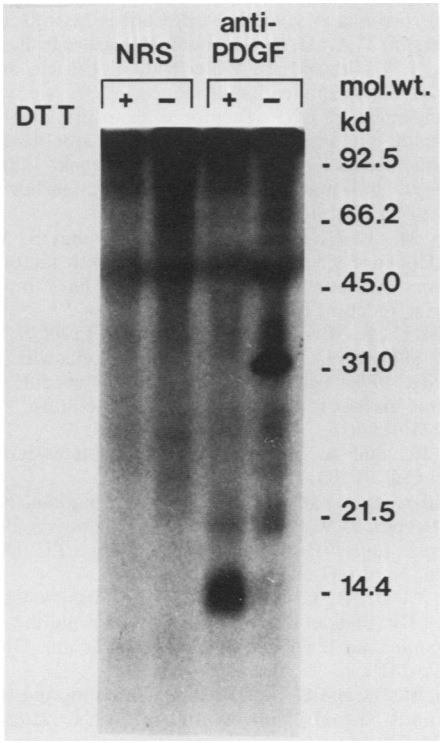

FIG. 3. Immunoprecipitation of PDGF from medium conditioned with malignant B8 cells after biosynthetic labeling with [^{35}S]cysteine. Immunoprecipitates were fractioned by electrophoresis on an SDS-polyacrylamide gel followed by fluorography. NRS, Normal rabbit serum; kd, kilodaltons; DTT, dithiothreitol.

a 31-kilodalton protein, which, after reduction, migrated as a diffuse band at 14 to 17 kilodaltons (Fig. 3). The sizes of these proteins are characteristic for the dimer molecule of mature PDGF (2). Since the antiserum used here was raised against PDGF isolated from human platelets, we cannot exclude the possibility that the precipitated protein is actually PDGF A_2 rather than B_2.

The presence of PDGF in the medium of the transformed B8 cells was also detected in a mitogenic assay on NR6-6 cells, a 3T3 mutant lacking epidermal growth factor receptors (permitting a selective assay for PDGF [25]). Malignant B8 cells were grown in linolea medium (13), and the medium was collected, dialyzed extensively against 1% acetic acid, and subsequently lyophilized. The freeze-dried material was dissolved in 1/10th of the original volume of linolea medium. Monolayers of NR6-6 cells were grown in Dulbecco modified Eagle medium containing 10% newborn calf serum in 24-well plates. Cellular growth was arrested by changing the medium for Dulbecco modified Eagle medium containing 1% newborn calf serum in linolea medium. Then [^{3}H]thymidine was added (50 μl; 1 μCi per well), and after incubation for 5 h at 37°C, the monolayers were washed sequentially with 1 ml of phosphate-buffered saline three times, with 1 ml of cold 10% trichloroacetic acid twice, and with 1 ml of ethanol-ether (2:1, vol/vol) twice. The trichloroacetic acid-insoluble material was dissolved in 1 ml of 0.4 N NaOH for measurement of incorporated radioactivity. Serum-free conditioned medium from B8.34 cells stimulated incorporation of [^{3}H]thymidine into the cellular DNA of the NR6-6 cells in a dose-dependent manner (Table 1).

Thus, in transformed glioma cells cultured from brain cells treated in vivo with ENU, expression of c-*sis* and functional

TABLE 1. Growth factor activity in conditioned medium from malignant glial cells

Addition to cultures of NR6 cells	[^{3}H]thymidine incorporation (×10^3 dpm/well)
Linolea medium	23 ± 4
Serum-free conditioned medium	
1:5	426 ± 42
1:10	277 ± 41
1:20	204 ± 24
1:40	86 ± 20
1:80	77 ± 14
10% newborn calf serum	69 ± 10

PDGF is markedly elevated compared with that in premalignant cells. Glial cells have receptors for PDGF (12); hence, the synthesis of this growth factor can generate an autocrine loop (24), causing rapid cell proliferation. It is not clear whether activation of c-*sis* is a direct consequence of ENU treatment, i.e., whether the c-*sis* is structurally mutated. By means of Southern blotting, we looked for gross rearrangements of the gene, but none were found, nor could we detect amplification. Structural changes could be examined more closely by molecular cloning of c-*sis* from transformed cells, but in view of the fact that high expression of normal c-*sis* is already capable of transforming cells (5, 8), it would be difficult to discriminate the normal c-*sis* allele from its possibly mutated counterpart.

Expression of c-*sis* and PDGF has also been observed in a number of human tumor cell lines, including gliomas, osteosarcomas, and fibrosarcomas (3, 7, 19). Interestingly, in rat neuroblastoma induced by ENU, a unique transforming oncogene has been detected: the *neu* gene (22), which is related to *erbB*, whereas the *erbB* gene itself is activated in human gliomas (17). It is at present not clear to us whether the glioma cells that we studied also contain an activated oncogene of the *erbB* family. If this is the case, the activation of at least two oncogenes, *sis* and *neu*/*erbB*, would be implicated in ENU-induced transformation. It would be interesting to correlate the sequential phenotypic changes observed in culture and in in vivo tumorigenesis to the successive activation of several oncogenes.

We thank A. Riethorst for technical assistance, E. Kriek and E. Matthews for discussions, C. Heyting for advice during the initial stages of this work, and G. Grosveld for the c-*sis* probe. C.-H. Heldin and E. J. J. van Zoelen kindly provided reagents and cells.

P.F.L. and B.A. were supported by a grant from the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

LITERATURE CITED

1. **Auffray, D., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. **107**:303–314.
2. **Betsholtz, C., C.-H. Heldin, C. Nister, B. Ek, A. Wasteson, and B. Westermark.** 1983. Synthesis of a PDGF-like growth factor in human glioma and sarcoma cells suggests the expression of the cellular homologue to the transforming protein of simian sarcoma virus. Biochem. Biophys. Res. Commun. **117**:176–182.
3. **Betsholz, C., B. Westermark, B. Ek, and C.-H. Heldin.** 1984. Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line; implications for autocrine receptor activation. Cell **39**:447–457.
4. **Bignami, A., L. Eng, L. D. Dahl, and C. T. Uyeda.** 1972. Localisation of the glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res. **43**:429–435.

5. **Clarke, M. F., E. Westin, D. Schmidt, S. F. Josephs, L. Ratner, F. Wong-Staal, R. C. Gallo, and M. S. Reitz.** 1984. Transformation of NIH 3T3 cells by a human c-*sis* cDNA clone. Nature (London) **308**:464–467.
6. **Doolittle, R. F., H. W. Hunkapiller, L. E. Hood, S. G. Devare, K. C. Robbins, S. A. Aaronson, and H. N. Antoniades.** 1983. Simian sarcoma virus onc gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science **221**:275–277.
7. **Eva, A., K. S. Robbins, P. R. Andersen, A. Srinivasan, S. R. Tronick, E. P. Reddy, N. W. Ellnore, A. T. Galen, J. A. Lantenberger, T. S. Papas, E. H. Westin, F. Wong-Staal, R. C. Gallo, and S. A. Aaronsen.** 1982. Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. Nature (London) **295**:116–119.
8. **Gazit, A., H. Igarashi, I. M. Chin, A. Srinivisan, A. Yaniv, S. R. Tronick, K. C. Robbins, and S. A. Aaronson.** 1984. Expression of the normal human *sis*/PDGF-2 coding sequence induces cellular transformation. Cell **39**:89–97.
9. **Groffen, J., N. Heisterkamp, J. R. Stephenson, A. Geurts van Kessel, A. de Kleine, G. C. Grosveld, and D. Bootsma.** 1983. c-*sis* is translocated from chromosome 22 to chromosome 9 in chronic myelocytic leukemia. J. Exp. Med. **158**:9–15.
10. **Heldin, C.-H., and B. Westermark.** 1984. Growth factors: mechanism of action and relation to oncogenes. Cell **37**:9–20.
11. **Heldin, C.-H., B. Westermark, and A. Wasteson.** 1981. Demonstration of an antibody against platelet-derived growth factor. Exp. Cell. Res. **136**:255–261.
12. **Heldin, C.-H., B. Westermark, and A. Wasteson.** 1981. Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proc. Natl. Acad. Sci. USA **78**:3664–3668.
13. **Ijssel, H., J. E. de Vries, M. Koken, W. van Blitterswijk, and H. Spits.** 1984. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J. Immunol. Methods **72**:219–227.
14. **Ivankovic, S., and H. Druckrey.** 1968. Transplacentare Erzeugung Maligner Tumoren des Nervensystems. I. Äthylnitrosoharnstoff (ÄNH) an BD IX-Ratten. Z. Krebsforsch. **71**:320–360.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Laerum, O. D., and M. F. Rajewsky.** 1975. Neoplastic transformation of fetal rat brain cells in culture after exposure to ethylnitrosourea in vivo. J. Natl. Cancer Inst. **55**:1177–1187.
17. **Libermann, T. A., H. R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, M. D. Waterfield, A. Ullrich, and J. Schlessinger.** 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature (London) **313**:144–147.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Nister, M., C.-H. Heldin, A. Wasteson, and B. Westermark.** 1984. Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line; implications for autocrine receptor activation. Cell **39**:447–457.
20. **Raff, M. C., R. Mirsky, K. L. Fields, R. P. Lisak, S. H. Dorfman, D. H. Silberberg, N. A. Gregson, S. Leibowitz, and M. C. Kennedy.** 1978. Galactocerebroside is a specific cell surface antigenic marker for oligodendrocytes in culture. Nature (London) **274**:813–816.
21. **Ross, R., and A. Vogel.** 1978. The platelet-derived growth factor. Cell **14**:203–210.
22. **Schechter, A. L., D. F. Stern, L. Vaidyanathan, S. J. Decker, J. A. Drebin, M. I. Greene, and R. A. Weinberg.** 1984. The *neu* oncogene: an *erb*-B-related gene encoding a 185,000-M_r tumour antigen. Nature (London) **312**:513–516.
23. **Scher, C. D., R. C. Shephard, H. M. Antoniades, and C. D. Stiles.** 1979. Platelet-derived growth factor and the regulation of the mammalian fibroblast cell cycle. Biochim. Biophys. Acta **560**:217–241.
24. **Sporn, M. B., and G. J. Todaro.** 1980. Autocrine secretion and malignant transformation of cells. N. Engl. J. Med. **303**:878–880.
25. **van Zoelen, E. J. J., D. R. Twardzik, T. M. J. van Oostwaard, P. T. van der Saag, S. W. de Laat, and G. J. Todaro.** 1984. Neuroblastoma cells produce transforming growth factors during exponential growth in a defined hormone-free medium. Proc. Natl. Acad. Sci. USA **81**:4085–4089.
26. **Waterfield, M. D., G. T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.-H. Heldin, J. S. Huang, and T. F. Deuel.** 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28 sis of simian sarcoma virus. Nature (London) **304**:35–39.
27. **Yen, S. H., and K. L. Fields.** 1981. Antibodies to neurofilament, glial filament, and fibroblast intermediate proteins bind to different cell types of the nervous system. J. Cell Biol. **88**:115–126.