Acta Neuropathol (1993) 86: 345 – 352

Acta Neuropathologica

Stabilization of c-*myc* protein in human glioma cells

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Received: 8 February 1993/Revised, accepted: 3 May 1993

Abstract. The regulation of c-*myc* protein, product of c-*myc*/genes, was studied in four glioma cell lines by Northern blot, pulse-chase dot blot, immunoblot and immunoprecipitation analyses. Northern blot analysis revealed no overexpression of c-*myc* transcript, and pulse-chase dot blot analysis showed normal turnover rate of c-*myc* transcript, suggestive of no evidence of aberrant regulation of c-*myc* at post-transcriptional level. The synthesis levels of c-*myc* protein were shown by immunoprecipitation and closely associated with the c-*myc* transcript levels demonstrated by Northern blot, suggestive of no evidence of aberrant translational control of c-*myc*, whereas they were dissociated from the accumulation levels of c-*myc* protein shown by immunoblot, suggestive of an evidence of aberrant regulation of c-*myc* at post-translational level. The mean (± standard deviation) half-lives of c-*myc* protein in four glioma cell lines were calculated from the pulse-chase immunoprecipitation analysis, and being 98 ± 8 to 143 ± 11 min, were about four- to sixfold longer than normal. In surgical specimens, the immunostain of c-*myc* protein was not found in normal astrocytes but localized heterogeneously in nuclei of reactive astrocytes and glioma cells, and increased in stained cell number in proportion to malignancy. Although this study was limited to four glioma cell lines, it suggests that the c-*myc* protein in glioma cells may be accumulated due to its prolonged half-life contributing to an uncontrolled proliferation.

Key words: Glioma – c-*myc* transcript – c-*myc* protein – Pulse-chase analysis – Immunohistochemistry

This work was supported in part by Research Grant from the Hyogo College of Medicine

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As a complementation oncogene, c-*myc* may cooperate with other oncogenes to induce complete transformation in primary cells and appear to function as a subtle and evanescent regulator of cellular proliferation [25, 27]. Steady-state levels of c-*myc* protein and transcript in untransformed cells appear to be very tightly controlled by a mechanism of autoregulation at levels much lower than those observed in tumor cells [28]. In contrast, deregulated expression of c-*myc* protein is a frequent observation in tumors and may lead to an uncontrolled proliferation [25, 27]. This implies that some form of lesion in c-*myc* autoregulation is responsible for the high expression levels of c-*myc* protein in tumor cells. Such lesions are obvious as amplification or rearrangement of c-*myc* in some cases, but the cause of uncontrolled c-*myc* protein expression is unknown in the majority of tumor cells.

The amplification, rearrangement or overexpression of c-*myc* has been rarely shown in human gliomas [2, 13, 37, 43, 46]. Nevertheless, Engelhard et al. [12], Shindo et al. [37], and Orian et al. [31] reported that the c-*myc* protein was highly expressed in human glioma cell lines and surgical glioblastoma specimens, suggestive of some change in the regulation of c-*myc* at the post-transcriptional, tanslational or post-translational level. In this report, we examine at what level the regulation of c-*myc* becomes aberrant in human glioma cells.

Materials and methods

Cell lines

Four glioma cell lines (GB-1, U-373MG, T98G and A-172) were examined, and a human colon carcinoma cell line (COLO320HSR) was used as a reference cell with c-*myc* amplification [1]. GB-1 was derived from a human glioblastoma [37], and other glioma cell lines and COLO320HSR were kindly supplied from Human Tumor Cell Bank (American Type Culture Collection, Rockville, Md., USA). Glioma cell lines were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics (penicillin G, 100 U/ml, and streptomycin,

100 μg/ml) whereas COLO320HSR was grown in RPMI 1640 medium supplemented with 10 % fetal calf serum and the antibiotics. The mean (± standard deviation) cell doubling times were 40 ± 6 h in GB-1, 36 ± 6 h in U-373MG, 32 ± 2 h in T98G, 32 ± 3 h in A-172, and 28 ± 2 h in COLO320HSR, respectively.

Northern blot analysis

Total cellular ribonucleic acid (RNA) was isolated from exponentially growing cells by acid guanidinium thiocyanate-phenol-chloroform method [7]. Total RNA sample (15 μg) was analyzed by electrophoresis on 1 % agarose-0.66 M formaldehyde gel and transferred onto a nylon filter after two rinses of the gel in 10 × standard saline citrate buffer (SSC) at pH 7.0. The filter, after baked in a vacuum oven for 2 h at 80 °C, hybridized at 42 °C with hybridization cocktail [50 % formamide/5 × SSC/5 × Denhardt's solution/0.5 % sodium dodecylsulfate (SDS)/50 μg of denatured salmon sperm deoxyribonucleic acid (DNA) per ml/10 % dextran sulfate] containing ^{32}P-labeled human c-*myc* probe or human β-actin probe, as reported previously [37], washed in 0.1 × SSC at 61 °C, and exposed to Kodak X-Omat AR film with intensifying screen at −70 °C.

Human c-*myc* probe was a 2.2-kb fragment containing the full length of human c-*myc* complementary DNA and prepared by digestion of pSPT-c-*myc* plasmid with *Eco*RI. Human β-actin probe used was a 0.4-kb fragment containing exon 4 of human β-actin complementary DNA and prepared by digestion of pBA400 plasmid with *Eco*RI and *Hin*dIII. The c-*myc* and β-actin probes were labeled by random priming to a specific activity with 1×10^9 dpm/μg using [^{32}P] deoxycytidine triphosphate (3000 Ci/mmol, Amersham International plc, Buckinghamshire, UK). The pSPT-c-*myc* plasmid and pBA400 plasmid were kindly supplied by Japanese Cancer Research Resources Bank, Tokyo, and T. Kakunaga, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, respectively.

Dot blot analysis of c-myc transcript

To determine the turnover rate of c-*myc* transcript, exponentially growing cells were treated with 5 μg/ml of actino*myc*in D. The viability of the cells following the exposure of actino*myc*in D was tested by trypan blue dye exclusion method. Total cellular RNA was recovered by acid guanidinium thiocyanate-phenol-chloroform method [7] from the cells at indicated time period after the addition of actino*myc*in D, and spotted onto a nylon filter after incubation in 4 × SSC and 0.5 % formaldehyde for 5 min at 70 °C. The baked filter hybridized as described for Northern blotting and was autoradiographed with intensifying screen at −70 °C.

Immunoprecipitation analysis

Exponentially growing cells were incubated in methionine-free medium for 60 min and then metabolically labeled by the addition of [^{35}S] methionine (1.0 mCi/10^7 cells, E.I. du Pont de Nemours & Co., Boston, Mass., USA) to the culture medium. After the labeling period for 30 min, the cells were incubated for the indicated time period in the medium containing methionine, washed three times in phosphate-buffered saline (PBS) at 4 °C, and solubilized by sonication in Ab buffer (10 mM Tris-HCl buffer, pH 7.5/50 mM NaCl/0.5 % deoxycholate/0.5 % Nonidet P/-40/0.5 % SDS/2 mM phenylmethylsulfonyl fluoride/10 mM iodoacetamide) at 4 °C. The lysate was centrifuged at 12 000 *g* for 10 min at 4 °C. An equal number of counts from each supernatant was incubated with 2 μg of anti-human c-*myc* protein mouse monoclonal antibody IF7 [29] and 20 μl of anti-mouse IgG-agarose for 4 h at 4 °C to immunoprecipitate the c-*myc* protein, which was then washed five times with RIPA buffer (10 mM Tris-HCl buffer, pH 7.4/150 mM NaCl/1 % deoxycholate/1 % Nonidet P/-40/0.1 % SDS) and subjected to 10 % SDS-polyacrylamide gel electrophoresis after incubation in Laemmli's sample buffer [24] for 3 min at 95 °C. The gel was fixed with 30 % methanol/10 % acetic acid/10 % glycerol for 60 min, permeated with autoradiography enhancer, EN^{3}HANCE (E.I. du Pont de Nemours & Co.) to shorten the exposure time, and fluorographed with intensifying screen at −70 °C after incubation in cold water for 30 min. IF7 was kindly provided by C. Miyamoto, Nippon Roche Research Center, Kamakura, Japan.

For the measurement of counts of [^{35}S] methionine incorporated into the cells, the supernatant was mixed with bovine serum albumin and incubated for 30 min at 4 °C after the addition of ice-cold 10 % trichloroacetic acid. The sample was added dropwise to a glass fiber filter, which was then washed twice with 10 % trichloroacetic acid and twice with 95 % ethanol. The dried filter was counted with liquid scintillation analyzer, Model 2500TR (Packard Instrument Co., Meridan, Conn., USA), after the addition of scintillant.

Immunoblot analysis

Exponentially growing cells were washed briefly with PBS and sonicated in Ab buffer. After centrifugation at 10 000 *g* for 10 min, protein content in supernatant was determined by Bradford's technique [5] and dissolved in Laemmli's sample buffer [24] and boiled for 3 min. The equal amounts of samples were subjected to 10 % SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane by electroblotting. The membrane was incubated with IF7 for 2 h at room temperature after blocking with a milk buffer (25 mM Tris-HCl buffer, pH 7.5/144 mM NaCl/5 % nonfat dried milk/0.5 % Tween 20) for 12 h at room temperature. The blots were washed with three changes of the milk buffer, then incubated with ^{125}I-labeled sheep anti-mouse Ig (1×10^6 cpm/ml, Amersham) for 1 h at room temperature, washed with five changes of the milk buffer, and autoradiographed with intensifying screen at −70 °C. Nonimmune serum was used instead of the primary antibody to assess the specificity of IF7.

Turnover rates of c-myc transcript and protein

Northern blot, dot blot, immunoblot and immunoprecipitation analyses were performed in triplicate, respectively, and the expression levels of c-*myc* transcripts and proteins were determined with Shimadzu dual-wavelength TLC scanner CS-900 (Shimadzu Co., Kyoto, Japan). The turnover rates of c-*myc* transcript and protein were evaluated from pulse-chase dot blot and immunoprecipitation by analysis of the natural logarithmic values for the expression levels of c-*myc* transcript and protein at indicated time divided by those at time zero, respectively.

Immunohistochemistry

Surgical glioma and lobectomy specimens as well as surgical specimens of optic nerve encircled completely by optic sheath meningioma were fixed with 4 % paraformaldehyde in 20 % sucrose and embedded with OCT compound (Tissue Tek II, Miles Laboratories, Naperville, Ill., USA). Also paraffin-embedded formalin-fixed surgical specimens were deparaffinized, and the sections were treated in 0.05 % protease for 10 min at room temperature. Both sections were washed with PBS and treated with 0.3 % H_2O_2 for 40 min at room temperature. After blocking with 10 % donkey serum for 2 h at room temperature, they were incubated in 30 μg/ml of IF7 overnight at 4 °C. Specifically bound

antibody was detected with 15 μg/ml of peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) by incubation for 1 h at room temperature followed by color reaction in the presence of 0.125 mg/ml of diaminobenzidine tetrahydrochloride in 0.015% H_2O_2 for 5 min. Finally, the specimens were counterstained with methylgreen. The specificity of stain was confirmed by omitting IF7 or using a nonimmune serum. Statistical significance of immunostained cell number was assessed by unpaired Student's *t*-test.

Results

Northern and dot blot analyses

The ^{32}P-labeled human c-*myc* and β-actin probes hybridized with 2.4-kb and 2.0-kb transcripts, respectively, in all cell lines, as shown representatively in Fig. 1. The c-*myc* transcripts, although variable in expression, were only expressed weakly in all glioma cell lines, but more markedly in COLO320HSR, whereas the β-actin was expressed more strongly in all glioma cell lines than in COLO320HSR, suggesting that the c-*myc* transcript is overexpressed in COLO320HSR and not overexpressed in all glioma cell lines.

All cell lines were viable following the treatment with actinomycin D for up to 45 min. Pulse-chase dot blots showed a decrease in density of c-*myc* transcript with the passage of time after the exposure to actinomycin D, as shown representatively in GB-1 and T98G in Fig. 2. In contrast, similar density in β-actin transcript during the pulse-chase period suggested that total RNA used was equal in amount. The mean (± standard deviation) half-lives of c-*myc* transcript were calculated from the turnover rate as shown representatively in Fig. 3, and were 15 ± 2 min in GB-1, 22 ± 3 min in U-373MG, 19 ± 4 min in T98G, 26 ± 4 min in A-172 and 27 ± 3 min in COLO320HSR.

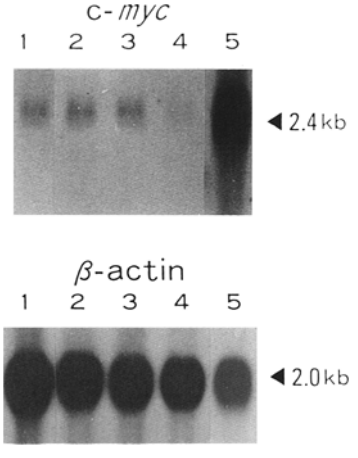

Fig. 1. Northern blot analysis. Total cellular RNA was isolated from exponentially growing cells, and hybridized with human c-*myc* and β-actin probes labeled with [^{32}P] deoxycytidine triphosphate by random priming. Ribosomal RNA was used as size standard. *Lane 1*, GB-1; *lane 2*, U-373MG; *lane 3*, T98G; *lane 4*, A-172; *lane 5*, COLO320HSR; GB-1, U-373MG, T98G and A-172, human glioma cell lines; COLO320HSR, human colon carcinoma cell line; kb, kilobase pairs

Immunoprecipitation analysis

Immunoprecipitation showed two immunoreactive c-*myc* protein species, a major form at 60 kDa and a minor one at 64 kDa in all cell lines, as demonstrated representatively in Fig. 4A–D. The immunoprecipitation profiles immediately after the labeling with [^{35}S] methionine indicate generally the synthesis levels of c-*myc* protein. The total synthesis levels of two c-*myc* protein species were well correlated to the expression levels of c-*myc* transcript in all cell lines.

Pulse-chase immunoprecipitation profiles showed a decrease in density of two c-*myc* protein species with the passage of time, as shown representatively in GB-1 and U-373MG as well as COLO320HSR in Fig. 4B–D, respectively, and the major component of c-*myc* protein appeared to be decreased in density earlier than the minor form in all cell lines. The mean (± standard deviation) half-lives of total c-*myc* protein were calculated from the turnover rate as shown representatively in Fig. 5, and were 143 ± 11 min in GB-1, 120 ± 12 min in U-373MG, 98 ± 8 min in T98G, 135 ± 10 min in A-172, and 16 ± 3 min in COLO320HSR, respectively, suggestive of longer half-life of c-*myc* protein in all glioma cell lines than in COLO320HSR.

Immunoblot analysis

Immunoblot analysis showed two immunoreactive c-*myc* protein species in all cell lines, similar in size to those detected by immunoprecipitation, as shown representatively in Fig. 6. The immunoblot profiles show usually the accumulation levels of c-*myc* protein. The total accumulation levels of two c-*myc* protein species, although highest in COLO320HSR, were relatively high in all glioma cell lines compared to the expression levels

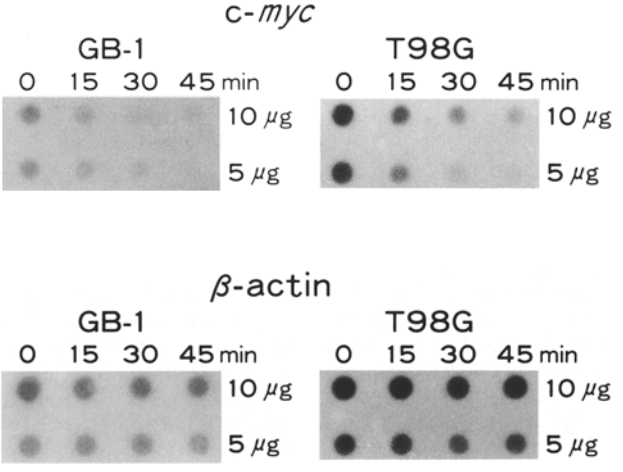

Fig. 2. Dot blot analysis. Exponentially growing cells were treated with 5 μg/ml of actinomycin D for the indicated time periods. Total cellular RNA (10 μg in *first line* and 5 μg in *second line*) was subsequently isolated and hybridized as described for Northern blot analysis. GB-1 and T98G, human glioma cell lines

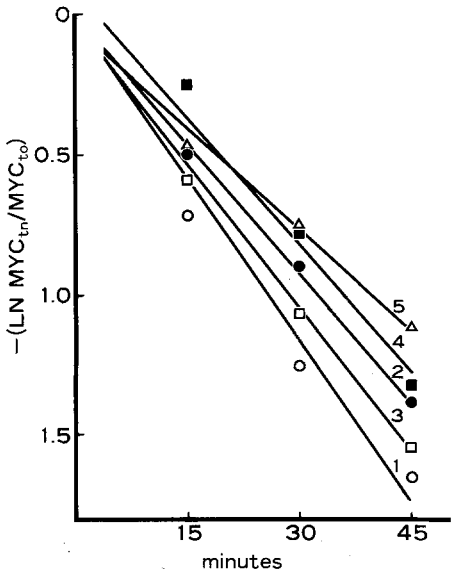

Fig. 3. Turnover rate of c-*myc* transcript. Turnover rate of c-*myc* transcript was derived by analysis of the natural logarithmic (*LN*) values for the expression levels of c-*myc* transcript at time n (*MYCtn*) divided by those at time zero (*MYCto*) in dot blot analysis. *Open circles* and *line 1*, GB-1; *closed circles* and *line 2*, U-373MG; *open squares* and *line 3*, T98G; *closed squares* and *line 4*, A-172; *open triangles* and *line 5*, COLO320HSR; GB-1, U-373MG, T98G and A-172, human glioma cell lines; COLO320HSR, human colon carcinoma cell line

of c-*myc* transcript. In addition, the total accumulation levels of two c-*myc* protein species were dissociated from their synthesis levels in all glioma cell lines and associated with their synthesis levels in COLO320HSR. No immunoblot was detected with nonimmune serum.

Immunohistochemistry

Gliomas were classified and graded according to the World Health Organization guidelines [22, 47]. The surgical glioma specimens were separated into astrocytoma (grade II), anaplastic astrocytoma (grade III) or glioblastoma multiforme (grade IV). The immunostain of c-*myc* protein was not found in normal astrocytes in lobectomy specimens, but localized heterogeneously in nuclei of reactive astrocytes in optic nerves involved circumferentially by optic sheath meningioma and of glioma cells, as shown representatively in Fig. 7A,B, respectively. Since all constituent cells were not always immunostained, the percentage of stained cells was determined by counting at least 1000 cells per preparation. The mean (± standard deviation) percentages of staining were 32 ± 8 % in reactive astrocytes in 4 gliotic optic nerves, 66 ± 8 % in 5 astrocytomas, 80 ± 6 % in 10 anaplastic astrocytomas, and 92 ± 8 % in 22 glioblastomas, respectively. Statistical significances of the immunostained cell numbers between reactive astrocytes and each grade of glioma cells and between different grades of glioma cells were shown in Fig. 8. In addition, the immunostain was also observed in nuclei of some

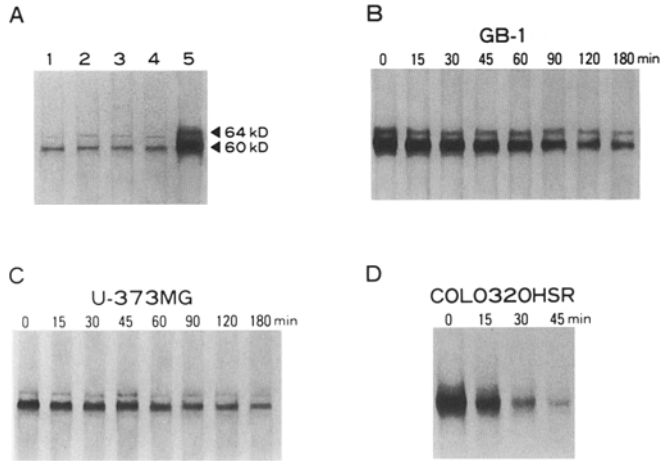

Fig. 4A–D. Immunoprecipitation. Exponentially growing cells were metabolically labeled with [^{35}S] methionine and incubated for the indicated time periods in the presence of methionine. Equal amounts of radioactivity were immunoprecipitated by anti-human c-*myc* protein monoclonal antibody and analyzed by 10 % sodium dodecylsulfate-polyacrylamide gel electrophoresis. High molecular weight markers were used as size standard. **A** Immunoprecipitation profile immediately after the labeling. *Lane 1*, GB-1; *lane 2*, U-373MG; *lane 3*, T98G; *lane 4*, A-172; *lane 5*, COLO320HSR. **B–D** Pulse-chase immunoprecipitation analyses for the indicated time periods. GB-1, U-373MG, T98G and A-172, human glioma cell lines; COLO320HSR, human colon carcinoma cell line; kD, kilodalton

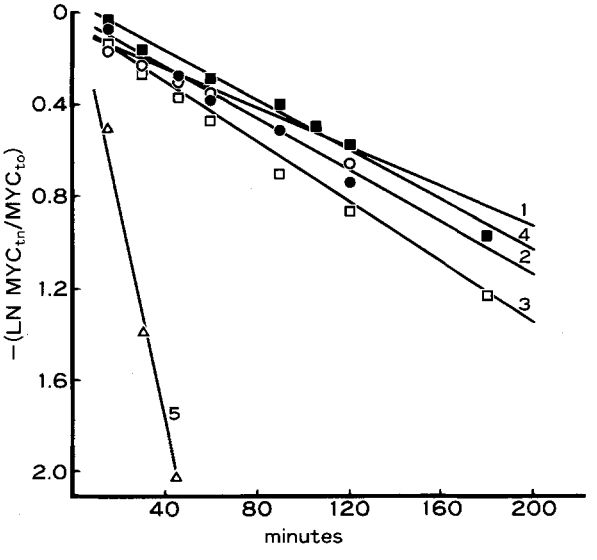

Fig. 5. Turnover rate of c-*myc* protein. Turnover rate of c-*myc* protein was estimated by analysis of the natural logarithmic (*LN*) values for the expression levels of two c-*myc* proteins at time n (*MYCtn*) divided by those at time zero (*MYCto*) in pulse-chase immunoprecipitation. *Open circles* and *line 1*, GB-1; *closed circles* and *line 2*, U-373MG; *open squares* and *line 3*, T98G; *closed squares* and *line 4*, A-172; *open triangles* and *line 5*, COLO320HSR; GB-1, U-373MG, T98G and A-172, human glioma cell lines; COLO320HSR, human colon carcinoma cell line

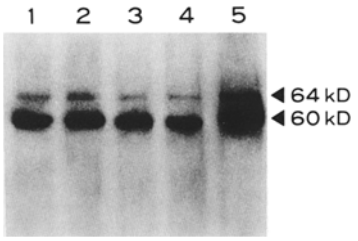

Fig. 6. Immunoblot analysis. Proteins were prepared from exponentially growing cells and examined by immunoblotting with anti-human c-*myc* protein monoclonal antibody. High molecular weight markers were used as size standard. *Lane 1,* GB-1; *lane 2,* U-373MG; *lane 3,* T98G; *lane 4,* A-172; *lane 5,* COLO320HSR; GB-1, U-373MG, T98G and A-172, human glioma cell lines; COLO320HSR, human colon carcinoma cell line; kD, kilodalton

proliferating endothelial cells in glioblastomas. There was no marked difference in immunostain between OCT- and paraffin-embedded specimens. No immunostain was found in glioma cells with nonimmune serum or without IF7.

Discussion

Analysis of c-myc transcript

Amplification and rearrangement of c-*myc* were reported in glioblastoma cells from an 8-year-old boy [43], but they have not been found usually in malignant human gliomas [2, 13, 37, 43, 46]. The previous Southern blot analysis showed no evidence of amplification or rearrangement of c-*myc* in GB-1 and U-373MG [37]. The present study demonstrates the lack of overexpression of c-*myc* in all glioma cell lines, and in addition, the expression levels of c-*myc* transcript are similar among GB-1, U-373MG and T98G, and less in A-172, suggestive of neither amplification nor rearrangement of c-*myc*.

In the orthodox view, the role of transcript is to produce protein, and any causal relationship to a change in transcript, therefore, must be mediated by a change in the level of the protein product of that transcript [15, 35]. Consequently, the concentrations of c-*myc* protein may be predicted to parallel very closely the changes in c-*myc* transcript and regulated at the post-transcriptional, translational and post-translational levels. Swartwout et al. [40] indicated that HL60 cells produced a population of poly(A^-) c-*myc* transcript which is considered more stable than poly(A^+) c-*myc* transcript, and an increase in the expression of such a population could conceivably be responsible for the maintenance of the high c-*myc* protein level. However, the mean half-lives of c-*myc* transcripts in all cell lines were similar to those in other human normal and neoplastic cells [6, 10, 39], suggestive of no evidence of aberrant post-transcriptional regulation of c-*myc* in glioma cell lines. In addition, the synthesis levels of c-*myc* protein detected by immunoprecipitation analysis were well correlated to the expression levels of c-*myc* transcript shown by Northern blot analysis in all cell lines, suggestive of no evidence of aberrant translational regulation of c-*myc* in glioma cell lines.

Analysis of c-myc protein

The specificity of monoclonal antibody IF7 used was confirmed by both immunoblot and immunohistochemical analyses. Two immunoreactive c-*myc* protein species, 60 and 64 kDa, were shown in both immunoblot and immunoprecipitation probed with IF7 in all cell lines, and similar in size to those reported in other cells [15, 29, 32, 35]. Nucleotide sequence analysis of the cloned c-*myc* reveals only one long translational open reading frame, which begins near 5′ end of exon 2 and extends through approximately two thirds of exon 3. Thus, the normal c-*myc* transcript that contains exon 1 would be expected to possess a substantial untranslated leader region preceding the open reading frame [8, 9]. Hann et al. [17] have shown that two c-*myc* protein

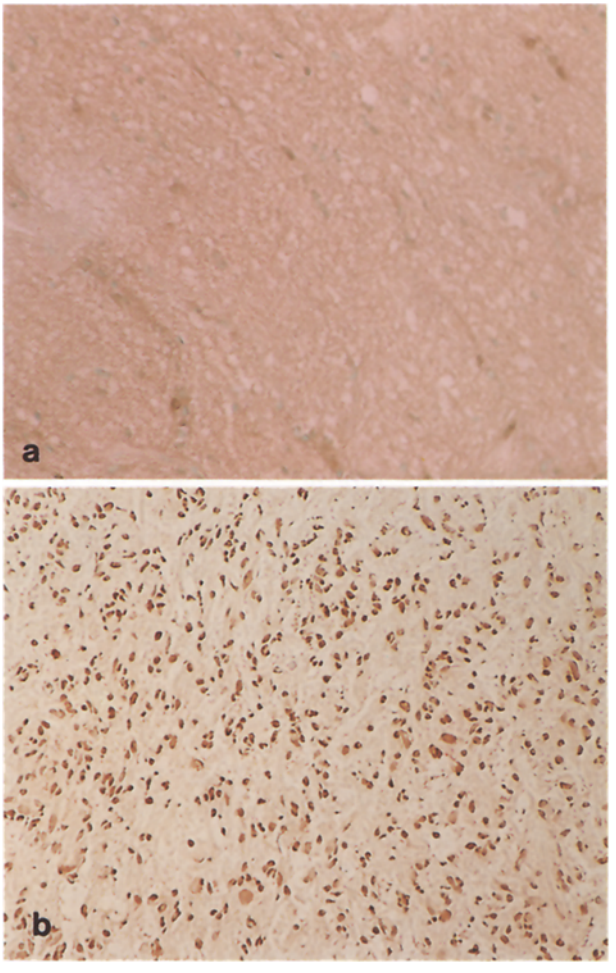

Fig. 7a,b. Immunohistochemistry. The c-*myc* protein is shown characteristically in nuclei of reactive astrocytes (*a*) and glioblastoma cells (*b*). The specimens were counterstained with methyl-green. Paraffin tissue sections. × 190

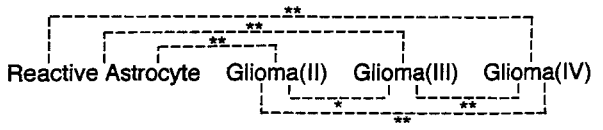

Fig. 8. Statistical significance of immunostained cell number. II, grade II; III, grade III; IV, grade IV. $*P < 0.005$, $**P < 0.0005$

species are derived from alternative translational initiations at exon 2 AUG and at a non-AUG codon near the 3′ end of exon 1, resulting in the production of proteins with distinct N termini.

The carboxyl terminus of c-*myc* protein has similarity to the helix-loop-helix leucine zipper motif and recognizes a six-nucleotide-long DNA sequence [3, 14]. Thus, the c-*myc* protein acts as a DNA-binding protein and may transform the cells by activating transcription of genes required for cell division. The half-life of the minor form of c-*myc* protein was rather longer than that of the major form, but the functional basis for the c-*myc* protein heterogeneity is not yet to be solved, mainly because of unavailability of antibody specific for each c-*myc* protein species. The significant variability in the relative ratio of the two c-*myc* protein species in different malignant cell lines [16] suggests that the presence of either form is sufficient for cell growth, but does not negate the possibility that one isoform is enzymatically more active than the other. Immunohistochemical analysis of c-*myc* protein in human reactive astrocytes and glioma cells reveals a heterogeneous distribution throughout the nuclei. Similar nuclear localization of c-*myc* protein was reported previously in human glioma cell lines [37]. Confocal microscopy suggests some evidence of colocalization with other known nuclear markers such as small nuclear ribonucleoprotein [38].

Stabilization of c-myc protein

To determine whether any aberrant post-translational regulation of c-*myc* protein occurs, we examined the synthesis and degradation of c-*myc* protein by immunoprecipitation in the same population of cells used for the immunoblot analysis. The synthesis levels of c-*myc* protein were greatly dissociated from its accumulation levels in all glioma cell lines. The turnover rate of c-*myc* protein was estimated by the standard [^{35}S] methionine pulse-chase immunoprecipitation, and indicated the normal half-life in COLO320HSR as reported in other cells [15, 34] and the prolonged half-life in all glioma cell lines. Since equal counts were used in each immunoprecipitation, the increase in half-life does not reflect a nonspecific change in the overall rate of synthesis or degradation of glioma cell protein, but a specific for the c-*myc* protein. Thus, the prolonged half-life of c-*myc* protein was well correlated to its high accumulation level in all glioma cell lines, suggestive of occurrence of aberrant post-translational regulation of c-*myc*.

The c-*myc* protein is also reported to bind to the product of retinoblastoma gene, a tumor suppressor gene, to control the cell cycle [36]. Careful quantitative analyses of the c-*myc* protein levels during mitogenic induction have been made in untransformed fibroblasts [30]. Fewer than 300 molecules of c-*myc* protein are present in quiescent fibroblast. Following the addition of growth factors, c-*myc* protein levels rise over about 2 h to a maximum of some 10000 molecules per cell. Thereafter, the level per cell falls within later G_1 to about 4000 molecules, a level which then remains constant throughout the cell cycle as long as the cell remains in logarithmic phase [16, 18, 42, 45]. Consequently, the c-*myc* protein levels are higher in the cells with the shorter cell-doubling time than in those with the longer cell-doubling time. The mean cell-doubling times were similar between glioma cell lines and rather shorter in COLO320HSR than in glioma cell lines. Nonetheless, the c-*myc* protein levels in COLO320HSR, although the c-*myc* transcript was overexpressed, were not so high compared to those in glioma cells, suggestive of no great effect of the cell cycle on the present c-*myc* protein levels.

Significance of c-myc protein in gliomas

The c-*myc* protein is a nuclear phosphoprotein generally thought to act as regulatory component of cell proliferation [15, 32, 35] by the control of transcription [3, 14] and cell cycle [36]. The rapid induction of c-*myc* transcript and protein following the addition of growth factors to quiescent cells [4, 11, 21, 30] suggests that they are sensitive and continuous indicators of external stimuli, consistent with a role in signal transduction. The c-*myc* protein was immunohistochemically found not in normal astrocytes but in reactive astrocytes and glioma cells. In addition, the immunostained cell number was increased in proportion to the malignancy of gliomas. Similar observations were also reported in human gliomas by Orian et al. [31], although the immunostained cell numbers were less than ours. The difference in the immunostained cell number may be mainly due to the difference in antibody used. The expression levels of c-*myc* protein in gliomas are well correlated to those of fibroblast growth factor [41], and to the scores of bromodeoxyuridine, Ki-67, nucleolar organizer region, DNA polymerase α or proliferating cell nuclear antigen [19, 20, 23, 26, 33, 41]. A small number of nucleolar organizer regions were found in human gliosis [26], but the other proliferation markers were not studied in details in reactive astrocytes.

The present study suggests that the high expression levels of c-*myc* protein in gliomas are induced not by the amplification, rearrangement or overexpression of c-*myc* but by the prolonged half-life of c-*myc* protein. This deregulated expression of c-*myc* protein may induce an uncontrolled proliferation and also acts as a complementary component of the multistage carcinogenic process [44]. It is unknown whether the c-*myc* protein in reactive

astrocytes or proliferating endothelial cells is accumulated by a similar mechanism to that in glioma cells or produced by the other mechanism.

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