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Short communication

Statin expression in the untreated and SarCNU-exposed human glioma cell line, SK-MG-1

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Summary. Cytokinetic analyses of gliomas and other neoplasms rely exclusively on the use of proliferation-dependent markers such as [^{3}H]-thymidine and BuDR incorporation and the detection of growth-dependent proteins such as proliferating cell nuclear antigen (PCNA) and Ki-67. In normal tissues, the monoclonal antibody S-44 recognizes statin, a nuclear protein expressed only in nonproliferating cells. In the present study, indirect immunofluorescence microscopy using S-44 identified nuclear statin in 5.9% of a population of untreated human SK-MG-1 glioma cells in vitro. Incremental doses of the alkylating agent sarcosinamide chloroethylnitrosourea (SarCNU) induced a linear increase in the fraction of statin-positive SK-MG-1 cells. Labeling of nuclear statin with the monoclonal antibody S-44 may be a potentially useful marker of the cytotoxic effects of anticancer drugs in gliomas and other neoplastic tissues.

Introduction

A major research effort in the study of gliomas and other CNS neoplasms has involved the correlation of cytokinetic data with the histologic grade of malignancy and in situ tumour behaviour. Cytokinetic analyses rely almost exclusively on data derived from proliferation-dependent markers such as [^{3}H]-thymidine and BuDR incorporation [7, 18], which label only nuclei in the S-phase of the cell cycle, and from proliferating cell nuclear antigen (PCNA) [12] and the Ki-67 reactive protein [4, 5], which are expressed throughout the cell cycle with the possible exception of cells in the early G_1 phase [13]. Thus, unlabeled cells may still be undergoing cell-cycle traverse (e.g. early G_1 phase) or they may be truly quiescent (G_0 phase). Since the proportion of "cycling" cells (the growth fraction) rather than the cell-cycle duration determines rates of proliferation in vivo [10], development of a method to identify and quantitate the nonproliferating cell fraction (and, by subtraction, thereby determine the growth fraction) of a tumour would complement the above modalities and provide important information concerning the cytokinetics of malignant growth and the cytotoxic effects of chemotherapeutic agents.

Statin is a 57-kDa nuclear protein that was initially demonstrated in cultured human fibroblasts rendered quiescent (G_0 phase) by contact inhibition or serum deprivation [15, 17]. Statin-positive nuclei are readily visualized using the monoclonal antibodies (Mab) S-30 and S-44 in conjunction with standard immunolabeling techniques [15, 16]. The specificity of S-44 as a G_0-phase marker has been confirmed in a variety of tissues both in vitro (e.g. human fibroblasts [15] and rodent astrocytes [3]) and in situ (e.g. rodent gastrointestinal epithelia and lens epithelial cells [1, 9]. To our knowledge, studies of statin expression in transformed or neoplastic cells have not been reported. In the present study, we report low-level statin immunoreactivity in the untreated human glioma cell line SK-MG-1 and its induction with the alkylating agent sarcosinamide chloroethylnitrosourea (SarCNU). In SarCNU-treated glioma cells, statin expression varies inversely with growth in a clonogenicity assay [14], suggesting that statin may be a useful marker for dose-dependent cytotoxicity in neoplastic cells.

Materials and methods

Drugs. SarCNU was kindly provided by Dr. T. Suami, Keio University, Japan.

Cell line. SK-MG-1 cells were derived from an untreated human glioma biopsy and provided by Dr. G. Cairncross, University of Western Ontario. As previously described [14], cells were cultured on glass coverslips at an initial seeding density of 1.5×10^5 cells/60-mm dish in McCoy's 5A medium supplemented with 10% fetal calf serum and 4 µg/ml gentamicin (Schering, Pointe Claire, Quebec). Cells were grown

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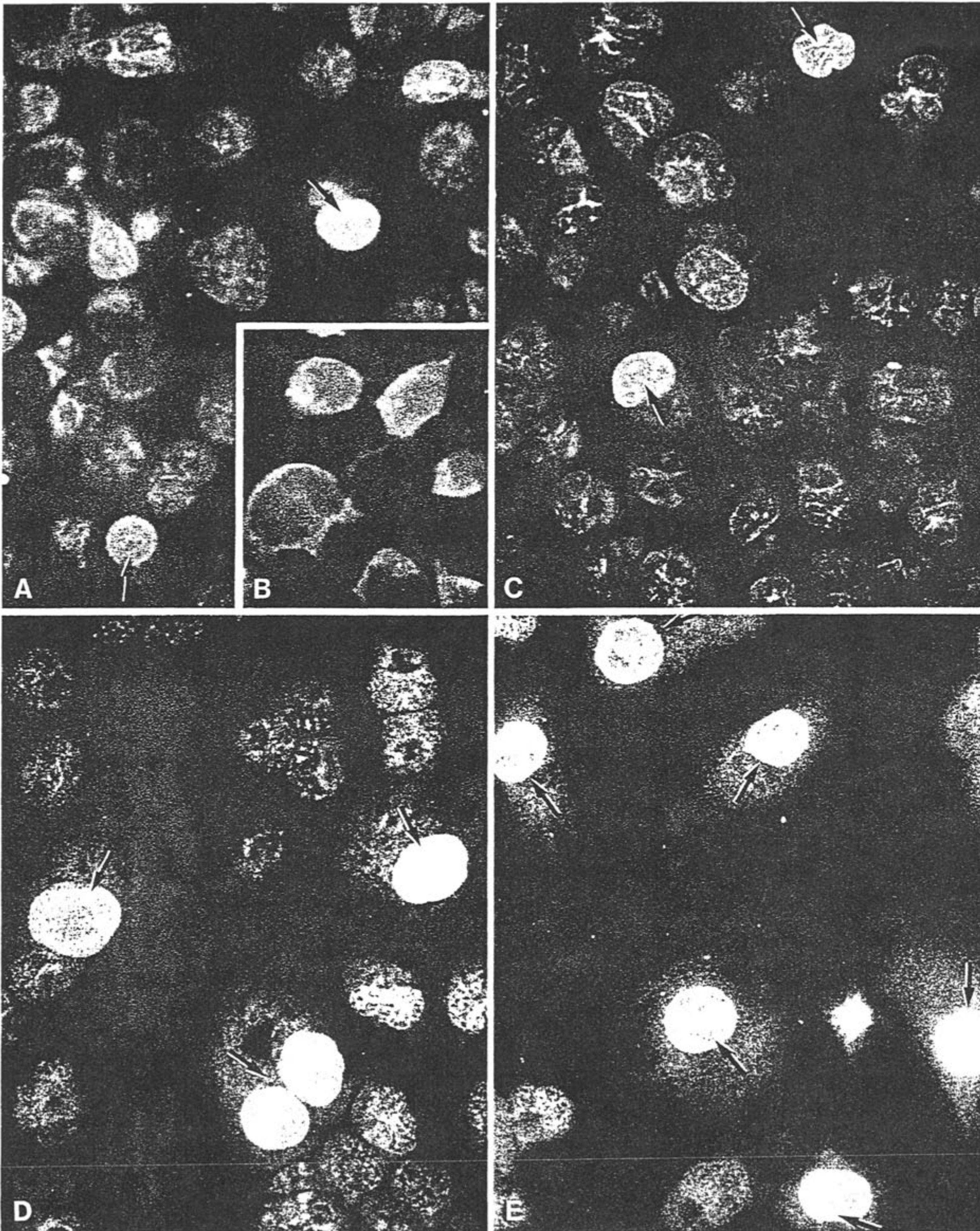

Fig. 1 A–E. Statin immunofluorescence in SK-MG-1 cells labelled with the monoclonal antibody S-44. Representative fields containing statin-positive nuclei (*arrows*) **A** in an untreated culture and in cultures exposed to **C** 3.6, **D** 21, and **E** 63 μg/ml SarCNU are depicted. Note the enlargement and binucleation of some statin-positive cells treated with **D** moderate and **E** high doses of SarCNU. Cytoplasmic background but not nuclear staining is illustrated **B** in a control culture incubated with pai ascites fluid instead of S-44. Original magnification, × 400

Table 1. Correlation of clonogenicity and statin expression with the dose of SarCNU in SK-MG-1 glioma cells

Concentration of SarCNU (μg/ml)	Clonogenicity assay (% of control)[a]	Statin-positive cells (%)
0	100	5.9 ± 1.80
3.6	37	7.3 ± 1.56
21	10	16.4 ± 3.02
63	0.5	33.2 ± 1.65

[a] The percentage of colonies that grew on drug-treated plates in comparison with untreated controls [14]

to sub-confluence (logarithmic growth phase) for 4 days at 37° C in a humidified atmosphere containing 5% CO_2.

SarCNU treatments. On day 4 after initial plating, cell cultures were rinsed with PAG (Dulbecco's modified phosphate-buffered saline, 0.026 m*M* phenol red, 13.9 m*M* dextrose and 26 m*M* $NaHCO_3$) and were either left untreated (control) or exposed to 3.6, 21, or 63 μg/ml SarCNU in buffer for 1 h at 37° C. These doses have previously been shown to inhibit growth of SK-MG-1 cultures by 63%, 90% and 99.5% of control values in a clonogenicity assay [14]. Following treatments, the plates were rinsed with medium to remove drug, resuspended in fresh medium and incubated for an additional 24 h in preparation for statin immunofluorescence microscopy.

Monoclonal antibody production. The S-44 antibody was developed using a mouse hybridoma technique after immunization of animals with cytoskeletal extracts derived from nonproliferative, senescent human fibroblasts (for details see [15]). S-44 labeling of nuclear statin was performed with indirect immunofluorescence microscopy to evaluate the anti-proliferative and cytotoxic effects of SarCNU on the SK-MG-1 cultures.

Statin immunofluorescence. Media were removed and cultures were washed twice with phosphate-buffered saline (PBS) (×5 min each). Cultures were then fixed in methanol: acetone (1 : 1, v/v) for 10 min at -20° C. Cells were washed in PBS and incubated overnight with S-44 (at a dilution of 1 : 250 in monoclonal ascites fluid) at room temperature. Following three 15-min washes in PBS, cultures were incubated with fluorescein isothiocyanate-labeled goat anti-mouse serum (1 : 100, v/v) (Jackson Laboratories, Inc.) for 30 min at room temperature and then mounted with PBS-glycerol (1 : 1, v/v). Control cultures were incubated with control hybridoma ascites fluid instead of S-44. Slides were examined using a Nikon Labophot microscope equipped with epi-fluorescence optics and appropriate filters.

Quantitation and statistics. For each culture, >200 glioma cells were randomly counted (by a single individual unaware of the treatment groups) and the mean percentage of statin-positive cells was determined. Occasional binucleate cells, observed in cultures exposed to SarCNU, were counted once. Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) and linear regression analysis.

Results

Statin-positive glioma cells were readily identified in S-44-labeled cultures by the presence of bright nuclear fluorescence (Fig. 1). The pattern of staining and the antibody concentration required to obtain a clear nuclear signal (1 : 250 dilution) were analogous to those observed in quiescent, normal human fibroblasts [15] and nonproliferating rodent astrocytes [3] in vitro. Nuclei of statin-negative cells remained unstained. All cells exhibited faint cytoplasmic background fluorescence, enabling accurate cell counting in the sub-confluent cultures. Representative fields derived from each treatment group are depicted in Fig. 1.

In untreated (Fig. 1 A) and low-dose (Fig. 1 C) SarCNU-exposed cultures, statin-positive and negative cells were morphologically indistinguishable. In cultures treated with moderate (Fig. 1 D) and high (Fig. 1 E) doses of SarCNU, many statin-positive cells appeared to be considerably enlarged and were occasionally binucleate. In cases of the latter, both nuclei were always statin-positive. Replacement of the S-44 antibody with control ascites fluid eliminated all statin immunoreactivity (Fig. 1 B). In the untreated cultures, 5.9% of cells were statin-positive. In the treated cultures, 7.3%, 16.5% and 33.3% of cells were immunoreactive for statin following exposure to 3.6, 21, and 63 μg/ml SarCNU, respectively (Table 1).

Dose-related increments in numbers of statin-positive cells were statistically significant by one-way ANOVA [$F = 24.9$, (df) = 3,6; $P < 0.01$]. Linear regression analysis revealed that the dose-response curve was highly linear over the drug concentrations tested [$r = 0.998$, where (m) = 0.434 and (b) = 6.20 in $y = mx + b$; $P < 0.01$] (df = degrees of freedom; m = slope; b = Y-intercept). The correlation of statin expression and cytotoxicity as determined by clonogenicity assay in SarCNU-treated SK-MG-1 cells is shown in Table 1. The linear increase in the fraction of statin-positive cells following SarCNU exposure varied inversely with the proportion of cells capable of forming colonies in the clonogenicity assay under identical treatment conditions.

Discussion

Using the S-44 monoclonal antibody and indirect immunofluorescence microscopy, we demonstrated the presence of statin in nuclei of approximately 6% of untreated SK-MG-1 glioma cells. The pattern and intensity of nuclear staining in these tumour cells were similar to results obtained with the same methodology in quiescent human fibroblast [15] and dibutyryl cyclic AMP-treated rodent astrocyte [3] cultures.

In the SK-MG-1 cells, statin expression was induced in a linear fashion with incremental doses of SarCNU. Like other chloronitrosoureas, SarCNU is a bifunctional alkylating agent that produces interstrand DNA cross-links and non-cell-cycle-specific cytotoxicity in glioma cells both in vivo and in culture [14]. The SarCNU regimen used in this study was previously shown to inhibit growth of SK-MG-1 colonies in a dose-dependent manner in a clonogenicity assay [14]. The substantial enlargement and binucleate appearance of statin-positive, but not statin-negative, SK-MG-1 cells exposed to moderate and high doses of SarCNU probably represent morphologic manifestations of cytotoxicity and arrested cell division, which correlates with the cells' nonproliferative status in the clonogenicity assay.

Our results suggest that, as in the case of non-transformed tissues, detection and quantitation of statin may be

a useful marker in determining the non-proliferating and/or killed fraction of a tumour population. This hypothesis is further supported by our recent observations in solid tumours: in human gliomas and other CNS neoplasms (Tsanaclis et al., in preparation) and in infiltrating ductal carcinomas of the breast [11], the degree of statin immunolabeling correlated inversely with the expression of the proliferation-dependent markers, Ki-67 and a cyclin-like nuclear antigen. In all likelihood, statin expression in glioma cells exposed to SarCNU is not specific to this agent and simply reflects a loss of proliferative potential. However, the present data do not exclude the possibility that SarCNU directly activates the statin gene prior to cell death, analogous to the pre-morbid induction of various heat-shock proteins [2, 6] and proto-oncogenes [8] in normal tissues following injury.

Whether statin expression in SarCNU-treated glioma cells primarily represents reversible growth arrest or commitment to cell death remains to be determined. In either case, we demonstrated a strong correlation of statin expression with dose-dependent cytotoxicity as measured by the clonogenicity assay. If this relationship can be verified with other chemotherapeutic agents, S-44 antibody labeling may prove to be useful as a rapid, alternative method of assessing the cytotoxic and antiproliferative effects of anticancer drugs.

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References

1. Bissonnette R, Wang E (1989) Characterization of statin, a non-proliferation dependent protein, as a marker for terminal differentiation in intestinal epithelial cells. Abstract 146b, 29th Meeting of the American Society of Cell Biology, Houston
2. Brown I, Rush S, Ivy A (1989) Induction of a heat shock gene at the site of tissue injury in the rat brain. Neuron 2: 1559
3. Fedoroff S, Ahmed I, Wang E (1990) The relationship of expression of statin, the nuclear protein of non-proliferating cells, to the differentiation and cell cycle of astroglia. J Neurosci Res 26: 1–15
4. Gerdes J, Schwab U, Lemke H, Stein H (1983) Production of a mouse monoclonal antibody reactive with human nuclear antigen associated with proliferation. Int J Cancer 31: 13
5. Gerdes J, Lemke H, Baisch H, Wacker H, Schwab U, Stein H (1984) Cell cycle analysis of a cell prolification – associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133: 1710
6. Gonzalez M, Shiraiski K, Hisanaga K, Sagar S, Mandabach M, Sharp F (1989) Heat shock proteins as markers of neural injury. Mol Brain Res 6: 93
7. Gratzner H (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218: 474
8. Jorgensen M, Deckert J, Wright D, Gehbert D (1989) Delayed c-fos proto-oncogene expression in the rat hippocampus induced by transient global cerebral ischemia: an in situ hybridization study. Brain Res 484: 393
9. Muggleton-Harris A, Wang E (1989) Statin expression associated with terminally differentiating and postreplicative lens epithelial cells. Exp Cell Res 182: 152
10. Pardee A (1989) G_1 events and regulation of cell proliferation. Science 246: 603
11. Pollak M, Schipper HM, Richard M, Wang E (1990) Immunocytochemical detection of statin, a marker associated with G_0 cell cycle in primary human breast cancers (abstract). Proc Am Assoc Cancer Res (in press)
12. Robbins B, De La Vega D, Ogata K, Tan E, Nakamura R (1987) Immunohistochemical detection of proliferating cell nuclear antigen in solid human malignancies. Arch Pathol Lab Med 111: 841
13. Sasaki K, Murakami T, Kawasaki M, Takahashi M (1987) The cell cycle associated change of the Ki-67 reactive nuclear antigen expression. J Cell Physiol 133: 579
14. Skalski V, Rivas J, Panasci L, McQuillan A, Feindel W (1988) The cytotoxicity of sarcosinamide chloroethylnitrosourea (SarCNU) and BCNU in primary gliomas and glioma cell lines: analysis of data in reference to theoretical plak plasma concentrations in man. Cancer Chemother Pharmacol 22: 137
15. Wang E (1985) A 57,000-mol-wt protein uniquely present in nonproliferating cells and senescent human fibroblasts. J Cell Biol 100: 545
16. Wang E, Krueger JA (1985) Application of a unique monoclonal antibody as a marker for nonproliferating subpopulations of cells of some tissue. J Histochem Cytochem 33: 587
17. Wang E, Lin S (1986) Disappearance of statin, a protein marker for nonproliferating and senescent cells, following serum-stimulated cell cycle entry. Exp Cell Res 167: 135
18. Yoshii Y, Maki Y, Tsuboi K, Tomono Y, Nakagawa K, Hoshino T (1986) Estimation of growth fraction with bromodeoxyuridine in human central nervous system tumours. J Neurosurg 65: 659