Abstract

Aims—To evaluate a possible association between clinical outcome in patients with glioblastoma and expression of some immunohistochemical variables and apoptosis.

Methods—168 selected patients with cerebral glioblastomas were studied retrospectively. Tumour specimens were examined immunohistochemically with antibodies to proliferating cell nuclear antigen (PCNA), p53, bcl-2, and epidermal growth factor receptor (EGFR) to detect the intracellular receptor domain. Apoptosis was detected by in situ end labelling. Multivariate analysis was performed using the Cox proportional hazard model.

Results—On univariate analysis the PCNA labelling index, immunoreactivity of EGFR, and the apoptotic index were significantly related to glioblastoma outcome. Survival time was reduced as PCNA labelling index increased and apoptotic index decreased (p = 0.0073 and p = 0.00031, respectively). Survival time in patients with EGFR positive tumours was found to be reduced (p = 0.00024). Multivariate analysis showed independent prognostic value for the EGFR positivity and apoptotic index only (p = 0.0053 and p = 0.0039, respectively). There was no association between clinical outcome of glioblastoma and p53 or bcl-2 immunostaining.

Conclusions—EGFR immunoreactivity and apoptotic index were found to be useful for assessing prognosis of individual glioblastomas but it seems unlikely that p53 and bcl-2 immunohistochemistry will be of value in determining survival in such patients.

Keywords: glioblastoma; epidermal growth factor receptor; apoptosis

Glioblastoma is the commonest neuroectodermal tumour and the most malignant in the range of cerebral astrocytic gliomas. The patient’s course fully reflects the biological aggressiveness of the tumour. Thus in spite of multimodal treatment the median postoperative survival time does not exceed 12 months.

Few clinical factors have been identified as useful for assessing the prognosis of individual glioblastomas. Those that have are the patient’s age, the volume of tumour resected, Karnofsky performance status, and the use of radiotherapy. Attempts have been made to identify biological markers that could be useful in predicting prognosis in these tumours. The prognostic significance of proliferative markers, p53 gene and protein status, and epidermal growth factor receptor amplification and overexpression have been also tested, but the results obtained are regarded as controversial. This can be explained by the use of clinically unselected groups of patients, the simultaneous investigation of the whole spectrum of astrocytic gliomas, differences in experimental design, and so on.

In the present study, survival data on 168 cerebral glioblastoma patients receiving a standard treatment protocol were studied using univariate and multivariate analyses to evaluate a possible association between prognosis and expression of some immunohistochemical variables—including proliferating cell nuclear antigen (PCNA), p53 and bcl-2 oncoproteins, epidermal growth factor receptor (EGFR)—together with apoptosis, which was detected by in situ end labelling (ISEL).

Methods

PATIENT SELECTION

One hundred and sixty eight adult patients with newly diagnosed cerebral glioblastomas which had been treated in the Neurosurgical Burdenko Institute were selected for study. In all cases a pathological diagnosis was made according to the World Health Organisation histological classification of central nervous system tumours. The patients met the following criteria:

1. Tumour location was exclusively lobar without extension into the midline structures.
2. Tumours were untreated before operation.
3. All patients had undergone subtotal tumour resection, confirmed by contrast computed tomography within 48 hours after the operation. The volume of remaining tumour was less than 10% of the initial neoplasm.
4. Karnofsky performance status (KPS) score in the third week after the operation was 80–100 (not significantly handicapped and independent of assistance).
5. All patients received postoperative radiotherapy with a total dose of 60 Gy and chemotherapy with nitrosourea compounds.
6. Follow up data were received at least 15 months after operation with endpoint at May 1, 1998.
Tumour associated antigen immunoreactivity and apoptosis in glioblastoma

IMMUNOHISTOCHEMICAL ANALYSIS
Biopsy samples were immediately fixed in cold 10% buffered formaldehyde solution and embedded in paraffin (Histowax, Leica). Immunohistochemical investigation was performed in 5 µm serial sections with a minimum area of 1 cm², mounted on the poly-L-lysine coated slides (Menzel-Glaser). Monoclonal antibodies against the following antigens were used:

- **Proliferating cell nuclear antigen**: clone PC10, prediluted; Dako, catalogue No N1519; positive control, human tonsil.
- **p53 Oncoprotein**: clone DO-7, 1:60; Dako, catalogue No M0776; positive control, human colon carcinoma.
- **bcl-2 Oncoprotein**: clone 124, microwave pre-treatment, 1:20; Dako, catalogue No M0887; positive control, human B cell lymphoma.
- **Epidermal growth factor receptor**: clone F4, 1:2000; Sigma, catalogue No E3138; positive control, human squamous cell carcinoma. This monoclonal antibody especially recognises the intracellular domain of receptor (product description).
- **Glyceraldehyde phosphate dehydrogenase**: clone 6F2, prediluted; Dako, catalogue No U7038; positive control, human brain tissue.
- **bcl-xL Oncoprotein**: clone 124, microwave pre-treatment, 1:20; Dako, catalogue No M0887; positive control, human B cell lymphoma.
- **Epidermal growth factor receptor**: clone F4, 1:2000; Sigma, catalogue No E3138; positive control, human squamous cell carcinoma. This monoclonal antibody especially recognises the intracellular domain of receptor (product description).

In addition, antibody against glial fibrillary acidic protein (clone 6F2, prediluted); Dako, catalogue No U7038; positive control, human brain tissue was used for confirmation of the astrocytic nature of tumour.

The sections were incubated with all the above mentioned antibodies overnight at 4°C. Immunostain visualisation was achieved with the standard streptavidin–biotin peroxidase technique (Dako LSAB kit, catalogue No K0675). The slides were stained with 3,3’-diaminobenzidine, counterstained with haematoxylin, and mounted. For the negative control procedure, primary antibodies were changed to mouse immunoglobulin G in order to reproduce the immunohistochemical findings.

STATISTICAL ANALYSIS
For statistical analysis contingency tables were used to examine the relation between expression of different antigens. The χ² test was performed to determine whether the relations were statistically significant. Non-parametric Spearman rank correlation coefficients were used to assess the degree of linear association between pairs of immunohistochemical and clinical variables. Survival analyses from the date of operation were estimated by the Kaplan–Meier method.

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into distinct survival groups was determined according to Segal. Comparisons among various patient subgroups were performed by the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. Probability (p) values < 0.05 were considered significant. A significant correlation between two variables was taken at the 95% confidence interval.

Results

Clinical Data
The 168 patients included 95 men and 73 women. The age of the patients ranged from 18 to 73 years, median 50 years. Forty two patients were under 40 years of age. At the end point of the follow up analysis 149 patients had died within the period 4 to 48 months after operation; their median survival time was 11 months. Survival time for the remaining 19 living patients ranged from 17 to 53 months; median survival time for this subgroup was 26 months. The median survival time for the entire cohort of patients was 13 months.

Ninety five patients survived more than one year after their operation, 15 patients more than two years, and seven patients more than three years.

Immunohistochemical Analyses
The PCNA labelling index for the 168 glioblastomas ranged from 25% to 66% with mean (SD) value of 46 (9.1)%. PCNA values of 40% or less were found in 52 tumours. Nuclear p53 immunostaining in more than 10% of tumour cells was observed in 84 glioblastomas. Thirty tumours had more than 50% immunostained nuclei.

All glioblastomas contained bcl positive cells with diffuse cytoplasmic immunostaining. Glioblastoma cells with abundant cytoplasm, especially tumour gemistocytes, were more intensively bcl positive. Quantitative evaluation of bcl-2 overexpression in individual tumours revealed a wide range of cell staining from 0.5% to 48%, with a mean (SD) value of 11 (9.5)%. Cytoplasmic bcl-2 immunostaining in more than 5% of tumour cells was found in 74 glioblastomas.

One hundred and two glioblastomas were immunopositive for EGFR and showed a diffuse or granular intracytoplasmic staining pattern (fig 1A). Usually clusters of immunopositive cells were scattered heterogeneously through the tumour section.

Apoptotic ISEL positive cells with strong nuclear immunostaining were found in all tumours examined; the mean (SD) apoptotic index for all tumours was 0.77 (0.81)%. Necrotic areas showed slight diffuse immunostaining. In 74 tumours with apoptotic index less than 0.5%, ISEL positive nuclei tended to be concentrated around the necrotic foci, whereas 94 glioblastomas with apoptotic index ≥ 0.5% showed staining nuclei scattered through the entire tumour specimen (fig 1B).

A correlation between EGFR immunoeexpression and apoptosis was found: the mean apoptotic index in EGFR positive tumours was significantly lower than in EGFR negative tumours, at 0.52% v 0.95% (p < 0.0001, χ² test). No associations were found between the remaining pairs of immunohistochemical markers.

Patients with EGFR negative glioblastomas were significantly younger: mean age 46 v 54 years (p < 0.001, χ² test). There was no correlation between age and remaining immunohistochemical variables.

Correlation between Immunohistochemical Findings and Survival
The Kaplan–Meier survival curves for the patient subgroups are presented in fig 2. Numerically continuous values for PCNA, bcl-2, and apoptosis were grouped according to the Segal method. Cut off points were 40% for PCNA, 5% for bcl, and 0.5% for apoptotic rate. Values for p53 and EGFR were grouped as positive v negative. The data obtained showed that survival time was significantly reduced as PCNA labelling index increased and apoptotic index decreased (p = 0.0073 and p = 0.00031, respectively, log rank test).

Figure 1 Photomicrographs showing expression of prognostically significant immunohistochemical markers. (A) Glioblastoma with strong cytoplasmic immunostain for EGFR F4. (B) A large number of apoptotic ISEL positive nuclei (mean apoptotic index 4.2%) within the "viable" small cell area of glioblastoma. EGFR, epidermal growth factor receptor; ISEL, in situ end labelling.
Moreover, survival time in patients with EGFR positive tumours was found to be significantly shorter (p = 0.00024). We found no difference in survival times in patients with or without p53 immunoreactivity (p = 0.12) or in cases with different grades of bcl-2 immunostaining (p = 0.69). In addition we found no difference in survival times between patients with p53 immunoreactivity when tumours with more than 50% of cells expressing p53 were compared with those with less than 50% or absence of p53 expression (p = 0.36, log rank test).

Multivariate analysis was performed using the Cox model and the results are presented in table 1. The single variable model shows that survival time in glioblastoma patients was significantly associated with age, PCNA labelling index, number of the apoptotic cells, and EGFR expression. Risk of dying increased with greater age and PCNA labelling index and with the presence of EGFR positivity, while increasing apoptotic index was a favourable factor and risk of dying was significantly reduced. The multivariate Cox model showed that survival time in glioblastoma patients was significantly associated only with EGFR immunoreactivity and apoptotic rate (p = 0.0053 and p = 0.0039, respectively).

**Discussion**

This study included a large cohort of patients with cerebral glioblastomas selected for similarity of various clinical features. The careful patient selection ensured that evaluation of the significance of immunohistochemical variables in predicting the clinical outcome was likely to be objective and reliable.

Studies of proliferative potential measured by flow cytometry, BrdU, Ki-67, and PCNA indicate that high indices of the “growth fraction” of the astrocytic gliomas are usually strongly associated with tumour grade, but their prognostic value is considered controversial.2 5 8 10–12 14 15 17 20 21 However, several studies have shown that PCNA and MIB-1 immunoreactivity is an independent prognostic factor for the whole spectrum of astrocytomas.17 21 According to previously reported studies, mean PCNA values for glioblastomas are vary broadly and very often overlap with the indices for anaplastic astrocytomas. Therefore some investigators have expressed scepticism over the reliability of PCNA in determining the proliferative potential of astrocytic gliomas.8 17 20

There are many reasons for the variability of the PCNA immunoreactivity in different studies. Among these are prolonged formalin...
fixation and other methodological failures, application of a various clones of the antibodies, small biopsy samples, differences in the intensity of nuclear immunostain, and subjectivity in evaluation.

In this study we applied a uniform experimental design for PCNA identification and we were able to show that the PCNA labelling index was significantly related to glioblastoma outcome in the univariate statistical model, with a cut off value of 40%. Our data suggest that this proliferative antigen is a relatively useful prognostic marker in cerebral glioblastoma. Nevertheless, we agree with the widely expressed view that there is little association between immunoreactivity of any of the proliferative markers and the true rates of tumour cell kinetics.

Mutation of the p53 gene and p53 protein overexpression are found in 30–60% of astrocytic gliomas regardless of the tumour grade. We suggest that p53 overexpression is a questionable prognostic factor for the whole spectrum of astrocytomas, regardless of the undisputed importance of p53 gene mutation for oncogenesis and the progression of these tumours. Although overexpression of p53 protein quite often occurs in the astrocytic gliomas, there is controversy over the association between p53 immunoreactivity and patient survival. Some investigators claimed prognostic significance of p53 immunoreactivity for high grade astrocytic gliomas, but numerous studies, including our own, have failed to show a clear association between p53 overexpression and outcome. Discrepancies in the reported data may reflect variation in the antibodies used, differences in the evaluation of p53 immunostaining, variability in the number and grades of tumours investigated, and heterogeneity in the clinical data, including treatment regimens. Studies of representative series of glioblastoma patients received similar treatment protocols have been unable to show any prognostic value for p53 immunostaining, in agreement with our present data.

The lack of prognostic value of p53 overexpression in glioblastomas contrasts with the findings of numerous studies that have shown p53 expression to be of value in assessing the prognosis of various human malignancies outside the central nervous system. It is hypothesised that p53 gene mutation confers resistance to both radiotherapy and chemotherapy in the tumour cells. Nevertheless, recent experimental studies on the radiosensitivity of glioblastoma cell lines showed a dominant role of reproductive cell death rather than p53 dependent apoptosis or p53 mediated G1 cell arrest. This could partly explain the absence of association between p53 status in glioblastoma and clinical outcome after combined tumour treatment.

The bcl-2 protein is a 26 kDa protein localised in the mitochondrial and perinuclear cell membranes. It blocks apoptosis. Overexpression of bcl-2 has been found in numerous human tumours, including astrocytic gliomas. Recently, the prognostic value of bcl-2 immunoexpression for the various human malignancies has come under intensive examination. However, a clear relation between bcl-2 immunoreactivity and outcome has not yet been demonstrated.

Krishna et al investigated bcl-2 overexpression in the entire spectrum of astrocytic tumours and found that the number of immunopositive cells did not alter significantly in the various tumour grades. On the basis of the data obtained, these investigators hypothesised that bcl-2 expression is an early event in astrocytoma genesis and that astrocytoma cells are initially resistant to apoptosis.

From the data in our present study there is no close correlation between the number of bcl positive cells and the apoptotic rate in glioblastomas. This is in concordance with data published earlier. We also failed to show that bcl-2 immunoreactivity had any prognostic significance in cerebral glioblastomas. In our opinion, a clear relation between bcl-2 expression and therapeutic resistance of the astrocytic gliomas remains to be shown.

EGFR is a membrane glycoprotein possessing an extracellular amino terminal end, a transmembrane region, and an intracellular domain. Increased expression of EGFR has been correlated with poor prognosis in various epithelial human tumours. Amplification of the c-erbB1 gene encoding the EGFR, together with protein alteration, has been found in up to 50% of astrocytic gliomas, predominantly in the glioblastomas. It is well known that mutant EGFR amplification confers increased proliferative activity and reduces apoptosis in human glioblastoma cells. EGFR immunoreactivity in glioblastomas suggests more aggressive biological behaviour, and in the present series a statistically proven increased risk of dying in patients with tumours having EGFR overexpression came as no surprise.

However, previously reported data on the possible correlation between EGFR status and survival have not been in uniform agreement. Rainov et al found that EGFR immunoreactivity was absent in glioblastomas, while Waha et al studied EGFR immunoreactivity in 125 astrocytic gliomas, including 62 glioblastomas, and showed a strong correlation between EGFR immunopositivity and survival for the entire group of patients; nevertheless, for glioblastomas alone no differences in outcome were noted between immunopositive and immunonegative tumours. Zhu et al showed that EGFR immunopositivity was a significant and independent prognostic factor for overall survival for 71 irradiated patients with astrocytomas. The discrepancies in these data may be explained as follows: the patients examined were clinically unselected and did not receive treatment according to a
standardised regimen; there was major variability in the number and grades of astrocytic gliomas studied; and various different types of EGFR antibody were applied.

In present study we used EGFR F4 antibody and showed its independent prognostic significance for similarly treated cerebral glioblastomas. EGFR F4 antibody is distinguished by the following characteristics. First, it is a formalin resistant antibody that can readily be used in paraffin embedded specimens. Second, it recognises wild and mutant forms of the intracellular EGFR domain only (Sigma Chemical Corporation, product specification sheet).

The data we obtained showed that the intracellular EGFR domain was expressed in glioblastoma cells as diffuse or granular cytoplasmic immunostaining. Possibly this staining pattern reflects various c-erbB1 gene abnormalities, including errors of amplification and pattern reflects various c-erbB1 gene abnormalities of the entire receptor only. Thus EGFR F4 antibody appears to be more reliable in identifying a wide spectrum of the EGFR protein alterations, in contrast to antibodies to the extracellular EGFR domain that usually show both membrane and cytoplasmic immunoexpression. In our opinion, the latter staining pattern may be restricted to the single variant of EGFR alteration that is accompanied by the synthesis of the entire receptor only.

Apoptosis (programmed cell death) occurs in numerous normal and pathological conditions, including neoplastic processes. Immunohistochemical techniques for identification of apoptosis have been demonstrated in several series of CNS tumours, including astrocytic gliomas. To our knowledge, the predictive value of apoptotic rate in relation to outcome in the astrocytic gliomas is still unclear.

According to previously published data, the number of ISEL positive cells in glioblastomas is relatively small. This is compatible with our findings. In addition, we defined two subtypes of distribution of ISEL positive nuclei in glioblastomas: perinecrotic, where the apoptotic index was found to be less than 0.5%, and broadly scattered, where the apoptotic index varied from 0.5% to 6.0%.

Some investigators have expressed doubt about the reliability of the ISEL technique in detecting apoptosis because of the possibility of non-specific staining and other technical failures. Morphological identification of the apoptotic nuclei after staining with basic dyes has been proposed as an alternative to immunohistochemical methods. However, in our opinion, the ISEL technique is a unique method for studying intensity of cell loss in routinely processed glioblastoma specimens. The reliability of ISEL in the accurate detection of apoptosis in brain neoplasms has been proved by its correlation with the immunoexpression of apoptosis related gene products such as APO-1/Fas and p21/WAF1. Moreover, apoptosis detection on routine pathological examination is fraught with difficulty in glioblastoma cases owing to polymorphism and variable nuclear abnormalities of tumour cells. The latter are well known as a distinctive glioblastoma pattern.

We showed in both univariate and multivariate analysis that an increased number of ISEL positive cells was a strongly favourable factor for glioblastoma outcome. We believe that for glioblastomas with an apoptotic index of > 0.5%, some of the tumour cells appear to be initially programmed for apoptosis owing to activation of the responsible genes or to growth factor deprivation. We speculatively suggest that postoperatively the remaining tumour cells preserve a high apoptotic capacity which can be important for a successful therapeutic response and for the rate of tumour regrowth. Data on inhibition of glioblastoma cell growth after stimulation of apoptosis by pharmacological agents or viral gene transfer partly prove our suggestion.

Nevertheless, the results we obtained contrast with previously published reports that have shown a definite correlation between a high apoptotic index count and poor prognosis for breast, lung, and ovarian neoplasms. This discrepancy may be explained as follows. The studies cited above showed an association between an increased number of ISEL positive cells and tumour metastatic capacity. Moreover, some experimental research has demonstrated a close relation between increasing metastatic potential and intensity of apoptosis in the primary tumour. For example, Matsuda et al have observed that apoptosis is significantly different for various stages of primary and metastatic tumour growth of a rat mammary adenocarcinoma. As the extent of spontaneous apoptosis decreased, the primary tumour increased in size. In contrast, increased apoptosis in the primary tumour was associated with growth of metastases in the lymph nodes and lung.

It is well known that cerebrospinal dissemination occurs in about 5–10% of glioblastoma cases and systemic metastases alone are rare. Survival in glioblastoma patients is determined by the intensity of local growth, and 80–95% of glioblastoma deaths were attributed to tumour progression at the primary site. Therefore a statistically significant association between a high apoptotic index and favourable glioblastoma outcome appears to be theoretically logical.

CONCLUSIONS

Immunohistoexpression of EGFR and the number of ISEL positive nuclei were found to be the strongest predictors of glioblastoma outcome and they seem to be useful for assessing individual tumour prognosis in routinely processed biopsy specimens. It seems unlikely that p53 and bcl-2 immunohistochemistry will be of value in determining survival from cerebral glioblastomas.
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