Pretreatment Prediction of the Chemotherapeutic Response of Human Glioma Cell Cultures Using Nuclear Magnetic Resonance Spectroscopy and Artificial Neural Networks

Wael El-Deredy, Sally M. Ashmore, Neil M. Branston, John L. Darling, Steven R. Williams, and David G. T. Thomas

Department of Neurological Surgery, Institute of Neurology, London, WCIN 3BG, United Kingdom. The Royal College of Surgeons Unit of Biophysics, Institute of Child Health, London, WCIN 1EH [S. R. W.], United Kingdom

Abstract

Both tumor metabolism and its response to cytotoxic drugs are intrinsic properties of tumor cells. It is therefore likely that there is a relationship between the two properties, however subtle and complex, wherein the metabolic characteristics of tumor cells can reflect the inherent response (resistance or sensitivity) of these cells to cytotoxic drugs. We used artificial neural network analysis to show that it is possible to distinguish, prior to treatment, between drug-resistant and drug-sensitive human glioma cell cultures from their metabolic profiles, as given by high-resolution proton nuclear magnetic resonance spectra of the cell extracts, and to predict their cellular response to the chemotherapeutic drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in vitro. The results suggest that neural network analysis of tumor nuclear magnetic resonance spectra has potential as a prognostic tool for determining treatment of gliomas, ultimately noninvasively, and may be used to provide information about the metabolic pathways involved in drug response that may be helpful in developing novel treatments for these tumors.

Introduction

Gliomas are the most common primary brain tumor in adults, and most are malignant. Treatment of these tumors has centered around surgical excision and radiotherapy. However, because these tumors are diffusely infiltrative, surgery is rarely curative, and median survival of patients receiving surgery and radiotherapy is less than a year (1). The addition of adjuvant chemotherapy produces a modest but significant improvement in survival (2). However, it is not usually possible to determine how individual patients will respond to chemotherapy on the basis of clinical parameters alone. Because short-term cultures can be prepared with relative ease from almost all biopsy samples of malignant glioma, there has been considerable interest in developing in vitro assays to predict chemotherapeutic outcome on an individual basis using a variety of end points (3–6). Studies of the relationship between in vitro chemosensitivity and clinical outcome have shown that chemosensitivity acts as an independent prognostic variable (7–9). This indicates that among the several factors determining the response of a tumor to chemotherapy, the genetic characteristics of individual tumor cells and cellular metabolism are likely to be important (10). One would therefore expect that differences in cytotoxic drug response are likely to be reflected in differences in cellular metabolism.

NMR spectroscopy provides simultaneous access to a wide range of tissue metabolites (Fig. 1) without preselection and is used to investigate tissue biochemistry in a range of systems from cells and extracts through animal models to human cancers in vitro and in vivo (11). Brain tumor spectra reveal a number of differences in comparison to spectra from normal brain (12), and both tumor type and malignancy have been studied by analyzing in vivo spectra or spectra recorded from biopsy and cell culture extracts. In addition, pattern recognition techniques have been successfully used in the analysis of these noisy and complex spectra and have detected differences between types of brain tumors (13) or between different grades of histological malignancy (14).

Because both the metabolism of the tumor cells and their response to drugs are intrinsic characteristics of the tumor, we hypothesize that a relationship exists between them. This study investigates whether the pretreatment metabolic profile of glioma cell cultures (measured by high-resolution proton NMR spectroscopy of the cell extracts) can be used to infer chemosensitivity to CCNU, determined by an in vitro chemosensitivity assay. Because of the likely subtlety of such a relationship and the possibility that it is nonlinear, in addition to the complexity of NMR spectra, the analysis is performed using artificial neural network techniques.

Neural networks are adaptive multivariate signal analysis tools related to statistical pattern recognition (15). Their nonlinear activation function (Fig. 2) provides them with the flexibility to be trained to map any arbitrary function without explicit knowledge of the relationship between the input patterns and their associated outputs responses. After training, given a new pattern (whose output response is unknown), the network produces an estimate of what that output value should be. Theoretically, any statistical pattern recognition algorithm could be cast in neural computing terms. Neural networks have been successfully used in complex problems of data classification and regression (function approximation) and in a variety of biomedical applications. In NMR spectroscopy, they have been trained as classifiers to recognize the differences between normal and cancerous tissue (16) or between different tumor types (13). In this study, the network is trained as a nonlinear regression tool to predict a continuous function of chemosensitivity.

Materials and Methods

Training a neural network in the fashion described below requires a data set comprising the NMR spectra from untreated cell extracts, together with their associated experimental ID50 values (ID50 exp) evaluated using cells from the same population as those used to obtain the spectra.

The abbreviations used are: NMR, nuclear magnetic resonance; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Cultured cells were shown to be free of infection by Mycoplasma spp. by cultures were derived from surgical biopsies diagnosed as grade III (n = 3) or IV (n = 12). The remaining two biopsies (IN1528 and IN2093) were diagnosed as grade III tumors. The tissues were finely sliced and disaggregated with 200 units/ml (w/v) Collagenase type IA (Sigma) in the culture medium Ham's F-10 (Life Technologies, Inc.) with 10% FCS at 37°C using methodology described previously (17). Disaggregated cells were washed and replated in fresh culture medium and passed using trypsin when confluent. All cultured cells were shown to be free of infection by Mycoplasma spp. by Hoechst staining and passaged until sufficient cells were obtained for both the chemosensitivity assay and the NMR measurements.

**Cell Culture.** Eighteen cell cultures from human gliomas were used; 17 were short-term cultures (passage levels 2–10) initiated in our laboratory, and I was an established grade IV cell line, U251MG, a kind gift from Dr. D. D. Bigner (Duke University Medical Center, Durham, NC). The short-term cell cultures were derived from surgical biopsies diagnosed as grade III (n = 3) or IV (n = 12). The remaining two biopsies (IN1528 and IN2093) were diagnosed as grade III/IV tumors. The tissues were finely sliced and disaggregated with 200 units/ml (w/v) Collagenase type IA (Sigma) in the culture medium Ham’s F-10 (Life Technologies, Inc.) with 10% FCS at 37°C using methodology described previously (17). Disaggregated cells were washed and replated in fresh culture medium and passed using trypsin when confluent. All cultured cells were shown to be free of infection by Mycoplasma spp. by Hoechst staining and passaged until sufficient cells were obtained for both the chemosensitivity assay and the NMR measurements.

**In Vitro Chemosensitivity Using the MTT Assay.** Response to the nitrosourea CCNU (Lundbeck) was determined using the MTT reduction assay on exponentially dividing cells in 96-well plates as described previously (18). Briefly, a 3-day drug exposure with a range of CCNU concentrations was used, followed by a 4-day recovery period in drug-free media. Cell growth was monitored to ensure that the cultures remained in the exponential phase throughout the assay. On the final day, 1 mg/ml MT1 (Sigma) was added to each well and incubated at 37°C for 4 h. The wells were aspirated, the formazan crystals were dissolved in DMSO (Sigma), and the absorbance was read at 570 nm using an MR600 plate reader (Dynatech). The average absorbance value for control wells was assumed to represent 100% cell survival. The average absorbance readings of the treated wells were then expressed as a percentage of the control, and the ID_{50 \text{EXP}} (the drug dose that inhibits MTT reduction by 50%) was determined.

**1H-NMR Spectroscopy.** Cells were cultured to confluence and harvested, and between 5 and 12 million cells were extracted with 12% perchloric acid (Merck) as described previously (19) and then freeze-dried and resuspended in 700 µl of deuterium oxide (D_{2}O) with 1 mM 3-trimethylsilyl-tetradecursodium propionate (TSP) as a chemical shift standard. Spectra were recorded at 30°C on a 500-MHz Varian Unity-plus spectrometer (Varian Associates, Inc., Palo Alto, CA) using a 45-degree single-pulse sequence with water presaturation and interpulse delay (TR) of 6.077 s.

**Neural Network Training.** A neural network with one hidden layer (Fig. 2) was trained with the pretreatment NMR spectra of the cell extracts in the range 0.5–4.2 ppm as its input patterns and with the corresponding experimentally determined in vitro ID_{50 \text{EXP}} responses to CCNU as its target outputs. In the context of this study, training was equivalent to nonlinear regression of the in vitro values on the NMR spectra to approximate a functional relationship between the spectra and the response to the drug. The training process, for which we used the commonly used back-propagation algorithm (20), finds an optimal set of values for the network connection weights that minimizes the mean squared error at the network outputs. This error is the difference between the quantity that the network estimates as its outputs (ID_{50 \text{EST}}) and the experimentally determined targets (ID_{50 \text{EXP}}). The network is tested by presenting it with patterns not included in the training set and checking how accurately the network can predict their corresponding outputs.

**Testing the Network Predictions.** For maximum utilization of the available data, we trained and tested the network using the “leave-one-out” method (21). In leave-one-out, all of the spectra except one form the training set and are used to generate the internal weights of the network, whereas the remaining spectrum is used for testing. In our case, the network, having no knowledge of the ID_{50 \text{EXP}} value associated with this test spectrum, was required to make an estimate of the ID_{50} value on the basis of its weights alone. We repeated the training and testing using leave-one-out such that each of the 18 spectra had been independently tested. Additionally, to incorporate the variation in the in vitro results (ID_{50 \text{EXP}}), the target output for each input spectrum during training was varied pseudo-randomly between three values: the mean value of ID_{50 \text{EXP}} and the mean ± 1 SD. Moreover, to insure the reproducibility of the network predictions and that no overfitting took place during training, the leave-one-out training and testing procedure was repeated 5–20 times for each test spectrum using different initial weights. A prediction was made from each network, and the average overall of the networks was then taken as the network estimate for the ID_{50} (ID_{50 \text{EST}}), whereas the SD across these networks was considered a measure of confidence in the prediction (22). The variations of the network predictions could then be compared to the corresponding variations in the experimental results as shown in Fig. 3. The reported results are those obtained for each spectrum when it was tested.

**Results**

ID_{50 \text{EXP}} values for the 17 short-term cultures ranged between 1.65 and 23.06 µg/ml, and there was no statistically significant evidence of a relationship between histological grade or doubling time and the in vitro chemosensitivity, which is consistent with previously published results (9). The ID_{50 \text{EXP}} value for the cell line U251MG was 5.47 ± 1.60 µg/ml (the median of the whole set was 5.455 µg/ml).

There was marked overlap between the range of ID_{50} values determined for each culture using the in vitro assay and those estimated by the network in 16 of 18 spectra (89%; Fig. 3). A paired t test showed no significant difference between the mean experimental values of drug responses and the mean values of the network predictions of those responses. In addition, the experimental and predicted values were significantly correlated (r = 0.81; P < 0.001). The large deviation from experimental results in the culture IN1728 could be attributed to the fact that the in vitro response of this tumor was 23.06...
mu g/ml, 1.5 times the largest value in the training session in which this
culture was being tested. To predict the response of this tumor, the
network was operating outside its training range and was therefore
extrapolating rather than interpolating the approximated function,
consequently with greater possibility of error. The spectrum from
culture IN1461 showed no obvious differences from the other spectra
that might explain the deviation of the prediction from the experi-
mental results (see “Discussion”). Overall, however, it is clear that
despite the limited number of training samples, the network was able
to predict the response of previously unseen test patterns by producing
values consistent with those obtained from the corresponding in vitro
assay.

Discussion
The correlation in Fig. 3 substantiates our hypothesis of a relation-
ship between the pretreatment tumor metabolism measured by NMR
spectroscopy and the tumor response to drugs. This relationship is
evident in spite of the fact that the process of extracting cells using
perchloric acid results in the loss of most of the macromolecule
resonances, the lipids, and some metabolites. Perhaps the most im-
portant of these losses is that of glutathione, which has been suggested
to play an important role in the mechanism of response to alkyla-
ting agents (22). Some of this missing information may explain the devi-
ation in the result of IN1461 in Fig. 3.
In this study, the spectra were obtained before treatment from the
same population of cultured cells as those treated by the drug. There-
fore, regardless of the extent to which culturing the heterogeneous
tissue might have been selective, the metabolic profile of the cultured
glioma cells predicted the way these cells responded to the drug
CCNU. The choice of nitrosoureas, being non-phase-specific drugs,
for this study was deliberate to avoid any possible correlation between
chemosensitivity and doubling time that might influence the neural
network training and the predicted results. Nitrosoureas are effective
against resting cells as well as dividing cells, and their lack of phase
specificity has been comprehensively documented (24). The possibil-
ity of a relationship between doubling time and chemosensitivity
would be more relevant to studies using phase-specific drugs like
vincristine and would then have to be incorporated in the network
training.

Although NMR spectroscopy is being used to assess and monitor
tumor progression and response to treatment and to identify markers
involved in determining the response of tumors to drugs (25), this
study demonstrates that together with pattern recognition analysis,
NMR spectroscopy can be taken a step further to act as a pretreatment
predictor of drug response, thereby suggesting a potential role for this
type of analysis to operate in a clinical setting. It should therefore be
possible to demonstrate that the relationship established in this study
also holds for spectra acquired in vivo and for other chemotherapeutic
drugs.

Despite their reduced resolution in comparison to in vitro spectra,
in vivo spectra reveal important additional information about tissue
metabolism including pH, macromolecules, and lipids. The latter have
been suggested to play a role in response mechanisms (26). With
NMR imaging already part of routine clinical diagnosis and spectro-
copy becoming more available and widely used on clinical scanners,
it would be advantageous if the pretreatment metabolic profile given
by NMR spectroscopy could provide prognostic information about the
response of the tumor to chemotherapy. These clinical goals should
not, however, diminish the value of in vitro studies in the future.
High-resolution NMR spectroscopy reveals metabolites not accessible
using the relatively low field clinical scanners, and although in vivo
NMR spectra may prove useful in predicting response and recom-
mending chemotherapy, the analysis of in vitro NMR spectra of tumor
extracts and cultures may still be useful for studying the metabolic
pathways involved in the response of tumors to drugs and for the
design of new drugs.

This study provides a further example of the power of neural
networks as a computational tool for detecting subtle or implicit
relationships among data with little or no requirement for prior knowl-
edge of the nature of these relationships. Unlike other methods of
interpreting NMR spectra obtained from cancer patients, which have
focused on finding tumor diagnostic and prognostic factors in the
spectra, the neural network techniques used in the present study
require no such factors but observe the spectra overall as patterns. The
network extracts its own discriminating factors through the training
process. In addition, neural network techniques are now available (27,
28) that allow identification of those regions of the spectrum playing
the most significant role in classification of tumor types or, as in the
present study, prediction of tumor cellular response. Such regions,
although they may contain only small or complex peaks, are likely to be directly related to the metabolites characterizing differences between these tumor attributes.

References
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