Primary cultures of microglial cells for testing toxicity of anticancer drugs

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Abstract

Toxicity of anticancer agents on normal neural cells during chemotherapy of primary or secondary brain tumors is a clinical problem of increasing relevance and concern. In this perspective, here we used primary cultures of rat cortical microglia as an in vitro paradigm of normal glia to investigate the neurotoxicity of anticancer agents. The effects of two compounds frequently used for treatment of brain tumors, methotrexate (MTX) and temozolomide (TMZ), were compared to those of a known microglial activator, bacterial lipopolysaccharide (LPS); cell viability and metabolism was assessed by the MTS assay.

We found that LPS, in the low-intermediate range of concentrations, strongly activates microglia cells, but a highly significant decrease in viability was observed from 100 ng/ml onward. TMZ has no effect at concentrations of clinical interest, whereas MTX significantly increases cell metabolism at 30 μM, a phenomenon possibly reflecting MTX neurotoxicity observed in patients.

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1. Introduction

The pharmacological treatment of primary or metastatic brain tumors is a cutting-edge field in modern medicine. The therapeutic approach to these disorders is biased by serious prognosis in the short period; criteria of drug choice are essentially limited to the consideration of pharmacokinetics parameters that may facilitate penetration and accumulation within the central nervous system (CNS). Drugs that easily cross the blood brain barrier by virtue of their liposolubility are more suitable for use. Among them are nitrosoureas, the first compounds shown to be active on brain tumors (Edwards et al., 1980), and temozolomide (TMZ), a novel methylating agent recently approved for the treatment of recurrent high grade gliomas (Yung et al., 1999). Moreover, TMZ has shown activity against CNS metastases from solid tumors and brain lymphoma (Biasco et al., 2001; Reni et al., 2000; Tentori et al., 2002). In addition, if
high doses are adopted or the drug is given via intracerebroventricular (ICV) infusion, other less lipophylic compounds can be used, i.e. methotrexate (MTX).

When an antineoplastic agent is chosen, generally the sensitivity of cerebral tissue to its actions is poorly considered, nor is the fact that cytotoxic properties of the drug may affect not only neoplastic cells but also normal tissues surrounding the neoplasm. Neurotoxicity induced by antineoplastic agents during chemotherapy of brain tumors (Shapiro and Young, 1984) has been rarely considered as a problem in clinical research; however, it can be foreseen that this problem might become more relevant in the future, as a consequence of increased cytotoxic activity of the antineoplastic drugs and the awaited prognostic improvements of these patients.

In this light, we have used a previously validated model, i.e. primary cultures of rat cortical microglia (Variano et al., 2002), as an in vitro paradigm of normal glial cell, and investigated the effects of treatments with anticancer agents frequently used in brain tumors, namely TMZ and MTX. Cell viability was assessed by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(4-sulfophenyl)2H-tetrazolium] test; MTS is a tetrazolium salt which is reduced to a colored formazan product by reducing enzymes present only in metabolically active, viable cells (Berridge and Tan, 1993). The effects of the above drugs in this paradigm were compared to that of bacterial lipopolysaccharide (LPS), a tool commonly used to activate microglia (Rivest, 2003).

2. Materials and methods

2.1. Reagents

Lipopolysaccharide from Escherichia coli, serotype 026:B6, and methotrexate were obtained from Sigma Chemicals Co. (St. Louis, MO, USA); temozolomide was a kind gift from Schering-Plough Research Institute (Kenilworth, NY, USA).

2.2. Cell cultures

Cultures of purified cortical rat microglial cells were prepared from the cerebral cortex of 1–2 days old Westar rats as previously described. The use of animals for this experimental work has been approved by the Italian Ministry of Health (licensed authorization to P. Navarra). Microglial cells were isolated by 11–14 days mixed glial cultures, in brief, after separation from astrocytes by gentle shaking of the flask, cells were resuspended in culture medium—Dulbecco’s MEM with Glutamax-I (DMEM; Gibco, Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated endotoxin-free fetal calf serum (FCS; Gibco, Gaithersburg, MD, USA), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Sigma)—and plated on plastic in 96-well plates at a density of 2 × 10^4 cells/100 μl per well and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h the medium was replaced with fresh culture medium (100 μl per well) containing or not the drugs under study. Microglial cultures were 95–98% OX42-positive.

2.3. MTS assay

Cell viability was assessed by MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation assay, Promega Corporation, Madison, WI, USA). Briefly, purified microglial cells were seeded in 96-well plates after the second subculture, and then incubated for 24 h at 37°C and 5% CO₂ thereafter, cells were exposed for 24 h to graded concentration of LPS or to the indicated drugs. At the end of incubation, 20 μl of CellTiter 96® AQueous One Solution Reagent containing a tetrazolium compound (MTS) were added to each well and the cells were incubated for 4 h at 37°C. MTS was bio-reduced into a colored formazan product by reducing enzymes present only in metabolically active, viable cells (Berridge and Tan, 1993). This compound has an absorbance peak at 492 nm, that was measured in a spectrophotometric microplate reader (Perkin-Elmer Inc., MA, USA).

2.4. Protein assay

The total protein content of cell cultures was assessed using the bicinchoninic acid method (BCA Protein Reagent, Sigma), and determination of absorbance at 570 nm (A570 nm) in a spectrophotometric microplate reader (Perkin-Elmer Inc., MA, USA).
lysis of cells was obtained through 2 h incubation in Tris–HCl 50 mM (Sigma), pH 8.00, containing 0.1% Triton-X 100, 40 IU/ml of aprotinin (Trasylol®, Bayer, Germany) and 0.09% NaNO₃. The amount of protein was measured by extrapolation from a standard curve using BSA as standard (30–1000 μg/ml).

2.5. Statistical analysis

Experiments were done at least in triplicate. Data were expressed as the means ± standard error of the mean (S.E.M.) of six replicates for experimental group. Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by Newman-Keuls or Dunnett’s post tests using GraphPad Prism® for Windows, GraphPad Software, San Diego California USA. Differences were considered statistically significant if P < 0.05.

3. Results and conclusions

Fig. 1A shows that LPS activates microglial cells with a unique dose-response pattern: a marked metabolic activity is associated to relatively low concentrations, whereas the metabolic activity of cells is reduced at higher concentrations, and is significantly lower than controls at 100 and 1000 ng/ml LPS. These variations do not appear to be associated to changes in cell proliferation, as the total protein content (Vairano et al., 2002) is not modified by LPS treatments. Moreover, the activation by LPS, 1 ng/ml

Fig. 1. The effects of LPS (panel A), TMZ (panel B) and MTX (panel C) on microglia viability evaluated by MTS assay. Cells were treated with graded concentrations of the test substances and tested after 24 h using the MTS assay. Results are expressed as absorbance at 492 nm, the means ± S.E.M. of six replicates per group, the right Y-axis shows the corresponding levels of protein content, expressed as μg/ml. *P < 0.05, **P < 0.01 and ***P < 0.001 respectively, versus controls.
or less, seems to be specific to microglia, since astrocytes do not undergo any change in viability after exposure to LPS up to 10 μg/ml (not shown). In contrast, TMZ in the range of 30–240 μM, including concentrations of clinical interest, does not induce any change in cell viability, but a significant increase in total protein content is observed after treatment with 240 μM TMZ (Fig. 1B). Enhanced protein content has been frequently observed in a number of human tumor cell lines upon TMZ treatment (Tentori and Graziani, unpublished results). This effect might be attributed to DNA hypomethylation and consequently increased transcription, as previously reported for erythroleukaemia cells which undergo enhanced production of haemoglobin after TMZ exposure (Tisdale, 1986). MTX causes more than 100% increase in cell metabolism at 30 μM, a concentration in the range of toxicity which, however, might be reached in the CNS after doses of MTX higher than 1.5 g/m2 followed by leucovorin rescue. After exposure to 30 μM MTX, a significant increase in total protein content is also observed (Fig. 1C).

Thus, primary cultures of microglial cells can be used to test the effects of anticancer drugs on cell viability and metabolism. The activation of microglia, regardless of the aetiology, plays a crucial role in the so-called ‘innate’ immune response, i.e. a reaction independent from the recruitment of immune-competent lymphocytes, which represents a common patho-physiological background to different CNS disorders (Aloisi, 2001). In this paradigm, a high concentration of MTX causes microglial activation to the same extent of that induced by a known microglial activator, bacterial LPS. The present finding that MTX markedly stimulates microglial activation, together with the observation of MTX toxicity on cultured astrocytes (Gregorius and Soucy, 1990; Gregorius et al., 1991), suggests that the effects of MTX on glial cells may play a role in the action mechanism of MTX neurotoxicity in vivo.

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References

