TIAZOFURIN MODULATES LIPOPOLYSACCHARIDE-ACTIVATED MICROGLIA IN VITRO

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Abstract - Tiazofurin is a purine nucleoside analogue, with a broad spectrum of antitumoral and anti-inflammatory properties. In the present study, we have investigated the effect of tiazofurin on microglial inflammatory response to lipopolysaccharide in vitro. The cytotoxic effect of the drug was examined by sulforhodamine B assay. The Griess method was used to quantify nitrite production. Microglial morphology was assessed by measuring cell body size. Release of the pro-inflammatory cytokines, tumor necrosis factor-α, interleukin-1β, interleukin-6, and the anti-inflammatory cytokine interleukin-10, were evaluated by enzyme-linked immunosorbent assay. Our data showed that tiazofurin decreased the number of activated microglia, lowered nitric oxide production and reduced the average cell surface of these cells. Tiazofurin reduced tumor necrosis factor-α, interleukin-6 and increased interleukin-10 secretion. Conversely, this drug promoted the release of interleukin-1β. Results obtained in this study indicate that TR displayed both anti- and pro-inflammatory modulation of activated microglia that could be relevant for its antitumor action within the central nervous system.

Key words: Tiazofurin; microglia; nitric oxide; cytokines; glioma

INTRODUCTION

Tiazofurin (TR) is a prodrug that potently inhibits inosine 5'-monophosphate dehydrogenase (Weber et al., 2003) that results in the reduction of cellular guanylate content, which is the basis of TR antitumor activity (Carney et al., 1985). TR is in phase II clinical trials as a potential treatment for leukemia (Grifantini, 2000). The fact that TR could penetrate the blood-brain barrier (Grygie et al., 1985), suggests a potential role in the treatment of central nervous system (CNS) tumors. Indeed, several studies have demonstrated TR-induced inhibition of proliferation in glioma cell lines (Piperski et al., 1998; Pesic et al., 2000). Glioblastoma is the most common malignant brain tumor in adults associated with poor prognosis.

In the normal mature nervous system, microglia persist as resting ramified cells that upon any danger to the CNS undergo morphological and functional changes to become activated amoeboid cells. Thus,
Microglia can acquire classical M1 activation, associated with pro-inflammatory molecules like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and nitric oxide (NO); or microglia can acquire alternative M2 activation that expresses anti-inflammatory cytokines, e.g. interleukin-10 (IL-10) (Rawji and Yong, 2013). Although these processes are meant to prevent damage to the brain, excessive or sustained activation of microglia may result in the promotion of neurodegenerative and neoplastic processes (Perry et al., 2010).

It was shown that microglia infiltrate glioma and that there is a positive correlation between the number of microglia and glioma malignancy (Wei et al., 2013). The role of microglia in astrocytic tumors is poorly understood. However, M1 microglia are associated with tumor cytotoxic effect, while M2 microglia promote tumor progression (Wei et al., 2013). Therefore, an agent that could shift the balance of M1/M2 functions of microglia in favor of the M1 type might be useful in glioma therapy.

To the best of our knowledge, there are no available data about TR influence on microglia properties in culture. Therefore, in this study we used a classical in vitro model of microglial activation with bacterial endotoxin lipopolysaccharide (LPS) to assess TR ability to modulate pro- (TNF-α, IL-1β, IL-6, and NO) and anti-inflammatory (IL-10) mediators of activated microglia. In addition, we addressed the question of TR toxicity and its influence on microglia morphology.

MATERIALS AND METHODS

Cell Culture and tiazofurin treatment

The preparation of microglia from rats up to 3 days postpartum was described previously (Giulian and Baker, 1986). All experimental procedures complied with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Siniša Stanković”, University of Belgrade. Briefly, glial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, from Gibco), supplemented with 10% heat-inactivated fetal calf serum (FCS, from PAA Laboratories) and 1% penicillin/streptomycin (PAA Laboratories). Rat microglial cells were obtained by shaking the primary mixed glial cultures between days 10 and 14. Microglial cells were then pelleted by centrifugation (5 min at 1500×g). For morphological analysis, cells were plated at 4×10⁴ on glass coverslip circles (14 mm) in 35-mm dishes (Sarstedt) with 2 mL astrocyte-conditioned medium (ACM). For the sulforhodamine B (SRB) assays, Griess assays and enzyme-linked immunosorbent assays (ELISA), 8×10⁴ cells per well were seeded in 96-well plates (Sarstedt) with 200 μL ACM. Cultured microglial cells were incubated for 48 h at 37°C in the presence (LPS-stimulated) or absence (non-stimulated) of 25 ng/mL LPS from Escherichia coli serotype 026:B6 (Sigma) and co-treated with 1, 5, 10, 20 μM TR (TR, ICN Pharmaceuticals).

Cell viability assay

Cell viability was determined by the SRB chemosensitivity assay. Following treatment with different concentrations of TR, the cells were fixed in 10% (w/v) trichloroacetic acid for 1 h at 4°C. Afterward, they were rinsed in tap water and stained with 0.4% (w/v) SRB in 1% acetic acid (100 μL/well) at room temperature for 30 min, and then rinsed three times in 1% acetic acid to remove the unbound stain. The protein-bound stain was extracted with 200 μL 10 mM Tris base (pH 10.5) per well. The optical density was read at 540 nm, with correction at 670 nm (LKB 5060-006 Micro Plate Reader).

NO assay

The generation of NO was determined indirectly, by nitrite measurement in the culture medium using Griess reagent (1% sulfanilamide (Sigma), and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride (Fluka), in 2% H3PO4). The culture supernatant and Griess reagent were mixed in equal volume and incubated at room temperature for 10 min. Absorption
was determined at 570 nm (Multiskan Spectrum, Thermo Electron North America LLC). Nitrite concentrations were calculated with reference to a standard curve generated with known concentrations of sodium nitrite in each assay.

**Morphological analysis**

For the morphological analysis, cells were first fixed in 4% paraformaldehyde at 4°C for 20 min followed by immunocytochemical labeling against Iba1 microglial marker. Briefly, after cell fixation endogenous peroxidase was neutralized through a hydrogen peroxide blocking step. Bovine serum albumin (5% for 30 min) was used as a blocker of nonspecific sites. Goat Anti-Iba1 primary antibody (Abcam, 1:700) was applied overnight at 4°C. Cells were then incubated in secondary anti-goat immunoglobulins/horseradish peroxidase (HRP) (DAKO, 1:200) for 2 h at room temperature. Peroxidase labeling was visualized by incubation in 0.05% diaminobenzidine (DAKO) and cells were analyzed under a light microscope. Morphological changes were quantified using the AxioVision Rel. 4.6 software (Zeiss). Five squares (500 x 500 μm) per slide were counted with three slides per individual microglial culture and three independent sets of experiments (n = 15 from one independent set).

**Enzyme-linked immunosorbent assay (ELISA)**

Cytokine levels (TNF-α, IL-1β, IL-6, IL-10) were measured in cell-free supernatants collected after 48 h of incubation with LPS (25 ng/mL) in the presence or absence of TR (5 μM), as well as in non-stimulated/untreated cultures. Quantification was performed using Quantikine ELISA systems (R&D Systems) according to the manufacturer's instructions. The absorbance was read at 450 nm, with wavelength correction at 540 nm using an automated microplate reader (LKB 5060–006 Micro Plate Reader). Released values were calculated as pg cytokine per mL and expressed as percentage of the release obtained in LPS-stimulated microglia. In addition, cytokine release was normalized to the protein content of the cell samples based on the SRB assay results.

**Data analysis**

The data were assessed from 3 independent experiments, each run in triplicate, and the values represent mean ± standard deviation (SD). Statistical significance was analyzed using ANOVA followed by Bonferroni's test and was considered significant if p <0.05. The EC50 value was calculated using the Origin 7.0 software based on a dose-response fitting function.

**RESULTS**

**Cytotoxic effect of TR and TR-induced reduction of NO**

The cytotoxic effect of TR on cultured microglia was examined by SRB assay. Cell viabilities in the LPS cultures co-treated with 1, 5, 10, 20 μM TR for 48 h were 95.2±15.5%, 80.0±15.3%, 73.6±14.7%, 69.3±10.7%, respectively (Fig. 1A). TR from 5-20 μM was significantly cytotoxic (p <0.05 for all). Maximum cytotoxicity (30%) was obtained at 20 μM concentration, with the experimental value of EC50 being 3.9 μM (Fig. 1B). Regarding the NO production, LPS markedly increased nitrite levels (11.4±1.6 μM) in comparison to non-stimulated cells (3.3±0.5 μM) (p <0.05, Fig. 1A). TR significantly reduced NO release from LPS-stimulated microglia at dosages of 10 and 20 μM, while the decrease in nitrite level induced with 5 μM TR was slightly outside the limits of statistical significance (p = 0.052).

Based on the obtained cytotoxicity measurements and calculated EC50 value, the concentration of 5 μM TR was chosen for further experiments.

**TR alters morphological features of microglial cells**

Activation of microglia is associated with morphological changes that include retraction of the processes with simultaneous enlargement of the cell body. To visualize the effects of 5 μM TR on LPS-induced changes of microglia morphology we used immunocytochemical labeling against the Iba1 microglial marker (Fig. 2A). LPS stimulation increased
the average cell surface area by 2-fold compared to non-stimulated cultures (p <0.05, Fig. 2B). Treatment of LPS-stimulated microglia with TR for 48 h significantly reduced cell surface area (p <0.05, Fig. 2B).

**Effect of TR on cytokine production by LPS-stimulated microglial cells**

Next, we analyzed the effect of TR on the production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and anti-inflammatory cytokine IL-10 by ELISA (Fig. 3). Compared with the non-stimulated/untreated microglia, stimulation with 25 ng/mL of LPS for 48 h strongly increased the production of all three examined pro-inflammatory cytokines and slightly, but significantly decreased secretion of the anti-inflammatory cytokine IL-10 (p <0.05 for all, Fig. 3). After treatment with 5 µM TR for 48 h, LPS-stimulated microglia produced a significantly lower level of TNF-α and IL-6 (by 8% and 23%, respectively, both p<0.05, Fig. 3). In contrast, TR additionally increased the secretion of IL-1β (by 32%, p <0.05, Fig. 3). Regarding IL-10, TR treatment of LPS-stimulated
microglia increased the production of this cytokine (by 10%, p <0.05, Fig. 3).

**DISCUSSION**

Data presented in this study are the first to demonstrate TR influence on microglia properties in culture. We provide evidence that TR has both anti-inflammatory and pro-inflammatory modulatory effects on LPS-activated microglia. TR anti-inflammatory modulation of microglia activity included a decrease in viability and NO production, alteration of the LPS-induced morphological profile, reduction in TNF-α and IL-6 release, as well as an increment in IL-10 secretion. On the other hand, pro-inflammatory modulation implied an enhanced release of IL-1β, pointing to TR as a potential drug against brain tumors (Piperski et al., 1998; Pesic et al., 2000). Hence, all TR effects on activated microglia presented here must be interpreted with this limitation in mind.

We have shown that TR reduced the number of activated microglial cells. TR maximum cytotoxicity on activated microglia was 30% with the EC₅₀ value of 3.9 μM. Reported TR dosages that have been applied to rat glioma cell line ranged from 15-60 μM (Piperski et al, 1998; Pesic et al, 2000). It seems plausible that activated microglia are more sensitive to TR than tumor cells. Previously, it was shown that depletion of microglia with M1-like characteristics from glioma enhanced tumor growth (Askew and Walker, 1996). Therefore, if TR could display the same cytotoxic effect on M1 microglia within a tumor, this would probably be an adverse action.

In addition, we have shown that TR reduced NO levels in activated microglia. Samardzic et al. (2001)
demonstrated that TR inhibited the activation of inducible nitric oxide synthase (iNOS) in fibrosarcoma cell line. After normalization of NO production to the number of cells using crystal violet staining (data not shown), we confirmed that the decrease in NO levels obtained in the presence of TR was due to the reduced cell number, which means that TR probably did not influence the activation of iNOS in LPS-stimulated microglia. This is an important finding because it was revealed that microglia in glioma retain iNOS function in order to display anti-glioblastoma activity (He et al., 2006).

We also demonstrated that TR inhibited LPS-induced changes in microglia morphology. It was shown that low-grade diffuse astrocytomas contain highly ramified microglia, while high-grade glioma has been associated with an amoeboid spherical

Fig. 3. Effects of TR on cytokine release in LPS-stimulated microglia. Release of TNF-α, IL-1β, IL-6 and IL-10 into microglia culture medium was determined by ELISA, and normalized to the protein content of the cell cultures. Cytokine release is expressed as percentage of the release obtained in LPS-stimulated microglia. Results represent mean ± SD for triplicate measurements from 3 independent experiments. Letter a – significantly different compared to LPS-stimulated microglia, (p<0.05); significance of the difference between groups was analyzed using ANOVA followed by the Bonferroni’s test.
shape (Graeber et al., 2002). It is certain that microglial cells react to brain tumors, but it is still to be determined whether these morphological changes are associated with anti-tumor activity or with tumor promotion.

Furthermore, we have shown that the exposure of activated microglia to TR led to the suppression of TNF-α and IL-6 release and promoted IL-10 production. It has been reported that the anti-inflammatory environment in glioma promotes tumor supportive actions (He et al., 2006; Wu et al., 2010). Therefore, TR suppression of the M1 microglia phenotype would not have a beneficial effect in glioma. On the other hand, TR promoted IL-1β secretion by activated microglia. It was reported that IL-1β released from LPS-stimulated microglia induced iNOS/NO production in astrocytomas (Kim et al, 2006). However, iNOS-mediated NO synthesis in tumors could have autocytotoxic effects (Xu et al., 2002). Therefore, we speculated that TR-induced induction of IL-1β production in activated microglia could send a tumor-suppressive signal to glioma. It should be emphasized that TR induced the strongest effect on IL-1β secretion compared to the other examined cytokines.

In conclusion, TR displayed modest anti-inflammatory action on activated microglia that could enable a tumor-pressive environment. However, the fact that TR promoted potent pro-inflammatory cytokine release (i.e. IL-1β) from activated microglia makes this drug a good candidate for further investigation that will include the co-culture of microglia and glioma cells.

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REFERENCES


