Indoleamine 2,3-dioxygenase as a new target for malignant glioma therapy

Laboratory investigation

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Object. Indoleamine 2,3-dioxygenase (IDO), a kynurenine pathway (KP) enzyme catalyzing oxidation of the essential amino acid tryptophan (Trp), is thought to be involved in the immune resistance of malignant tumors through T-cell inactivation caused by Trp depletion and metabolite accumulation. Human malignant gliomas may use this strategy to escape immune attack. The object of this study was to investigate the possibility of IDO-dependent Trp depletion by malignant gliomas and the practicality of using an IDO inhibitor together with anticancer drugs to reserve Trp without decreasing the cytotoxicity of the drugs.

Methods. The authors studied expression of IDO and other KP enzymes and the effects of an IDO inhibitor, 1-methyl L-tryptophan (1MT), on Trp metabolism and cytotoxicity of anticancer drugs, together with direct measurement of KP metabolites, in cultured human malignant glioma cells.

Results. Upon interferon-γ (IFN-γ) stimulation, the glioma cells greatly increased their IDO mRNA expression concomitant with depletion of Trp. The IDO inhibitor 1MT successfully prevented Trp consumption by the stimulated glioma cells. Combining 1MT with anticancer drugs (temozolomide, bischloroethyl nitrosourea [BCNU], etoposide and cisplatin) did not interfere with the drugs’ suppression of growth of LN229 glioma cells but rather increased their inhibitory effects on IDO activity.

Conclusions. These findings suggest that the robust IDO expression with rapid consumption of Trp in human glioma cells induced by IFN-γ could lead to immune resistance in glioma cells. Indoleamine 2,3-dioxygenase inhibitors that prevent Trp depletion could be used with anticancer drugs to improve therapeutic effects. (DOI: 10.3171/2008.10.JNS081141)

Key Words • immune escape • indoleamine 2,3-dioxygenase • malignant glioma • tryptophan • 1-methyl L-tryptophan

Malignant gliomas are aggressive and invasive tumors; they are also the most common tumors of the CNS. Gliomas have a poor prognosis because the tumors are difficult to resect completely and are resistant to radiation and chemotherapy. Strategies to overcome these difficulties have long been actively investigated. Among these strategies, immunotherapy for the treatment of malignant gliomas has been attempted, but there has been little success to date. One of the obstacles associated with immunotherapy is the ability of glioma cells to escape the immune system. Tumor cells are known to actively disable immune recognition through complex mechanisms, including loss of antigen-presenting machinery or the expression of inhibitory molecules that induce T-cell apoptosis or anergy. Recently, IDO, a KP enzyme that catalyzes oxidation of the essential amino acid Trp (Fig. 1 left) and is present in many tumors, including malignant gliomas, has been implicated in tumor immune escape. Activation of IDO results in the metabolism of its substrate Trp, which is present in the local environment, and the depletion of this essential amino acid together with the accumulation of downstream metabolites results in the suppression of T-cell activation and proliferation. The basis of this concept is essentially derived from maternal tolerance toward the fetus; maternal administration of IDO inhibitors.
Inducible immunosuppressive IDO and its inhibitor’s effect

IDO inhibitors could also be effective in defeating the IDO-dependent immune escape mechanism of tumors. In fact, an IDO inhibitor, 1MT, has been shown to have a synergistic effect with some chemotherapy drugs against cells of some extracranial tumors such as breast cancer or melanoma. Thus, IDO inhibitors could also be effective in defeating the IDO-dependent immune escape mechanism of tumors.

The application of the “IDO-dependent immune escape” hypothesis to the study of malignant gliomas (Fig. 1 right) could contribute to identifying possible mechanisms related to the characteristic resistance encountered during tumor immunotherapy and could help develop new therapeutic strategies that would strengthen the immune attack directed against gliomas. Expression of IDO in malignant glioma cells in the human brain could be triggered when the glioma cells are recognized by T cells or natural killer cells and are exposed to a key antitumor cytokine, IFN-γ, produced by these cells; the increased expression of the enzyme may catabolize Trp and lead to T-cell inactivation. This sequence of reactions would obstruct an effective immune attack against glioma cells. In this scenario, IDO inhibition would prevent the immune escape of the glioma cells from T-cells and be a useful adjuvant therapy for treating malignant gliomas.

In fact, high levels of IDO expression have been shown in human malignant glioma tissues, and it is known that IDO is present in human glial cells and that its activity is increased by cytokines such as IFN-γ. Furthermore, the IDO inhibitor 1MT has been shown to reach mouse brain after systemic administration, and systemically administered 1MT has been shown to inhibit IDO activity and lead to the exacerbation of T-cell-induced experimental autoimmune encephalomyelitis, an experimental model of multiple sclerosis. However, like the KP characteristics of human malignant gliomas, whether human malignant gliomas dissipate Trp in response to these stimuli and whether 1MT can effectively prevent Trp depletion by the glioma cells without unfavorable effects on other glioma treatments have also not been fully determined.

We have recently developed a highly sensitive and accurate procedure to analyze KP pathway metabolites with liquid chromatography–mass spectrometry. Employing this method, we have examined the amounts of KP metabolites, together with the expression of IDO and the other KP enzymes, in several cultured human malignant glioma cell lines and found that exposure of these glioma cells to IFN-γ steeply decreases the level of Trp in the culture medium concomitant with greatly increased IDO expression of the cells. Furthermore, we demonstrated that the IDO inhibitor 1MT effectively prevented Trp depletion, had no proliferative effect—by itself—on glioma cell viability, and did not interfere with the cytotoxic effects of anticancer drugs when used together. These results suggest that increased IDO expression in the glioma cells, possibly induced by IFN-γ secreted from T cells in vivo, could lead to Trp depletion and thus to immune resistance in gliomas, and that IDO inhibitors maintain the Trp levels required by T cells, implying a possible role in immune adjuvant therapy with anticancer drugs to obtain better effects on glioma therapy.
Methods

Human Cell Lines

Cells from LN229 (glioblastoma), U251 (astrocytoma), T98G (glioblastoma multiforme), U87 (glioblastoma), HepG2 (hepatocellular carcinoma), and HEK (human embryo kidney) cell lines were cultured in DMEM supplemented with 10% FCS. In addition, SY5Y (neuroblastoma) cells were cultured in a mixture of MEM and Ham’s F-12 medium with 10% FCS. For the individual experiments, the cells were washed with phosphate-buffered saline and then further cultured under the conditions indicated.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from the cells using the QuickPrep micro mRNA purification kit (Amersham Biosciences). The amount of RNA was determined based on the A_{260} absorbance of RNA solutions. Complementary DNA was synthesized from 2 μg of RNA in a final volume of 20 μl using the First-Strand cDNA Synthesis Kit (Amersham Biosciences).

Polymerase chain reaction was performed using 2 μl of cDNA mixture in a 20-μl reaction mixture containing Taq-polymerase, 10 × Mg^{2+}-containing buffer, a 2 mM dNTP mixture, and specific primers (see below). Individual sets of primers specific for mRNA transcripts of human IDO, kynurenine 3-monooxygenase (KMO), kynureninase (KYNase), 3-hydroxyanthranilate 3,4-dioxygenase (3HAO), quinolinate phosphoribosyltransferase (QPRT), and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed to include at least 1 intron to distinguish amplification of cDNA from genomic DNA. The sequence of each PCR product was confirmed. Sequences of each primer set were as follows: IDO (sense: 5′-AGT GTT TCA CCA AAT CCA CG-3′, antisense: 5′-AAA ATG TGC TCT TGT TGG GTT ACA-3′), KMO (sense: 5′-GAC TAT TCC ACC TAA GAA CGG AGA-3′, antisense: 5′-ACA GGA AGA CAC AAA CTA AGG TCG-3′), KYNase (sense: 5′-GAT GAG GAA GAT AAG CTG AGG C-3′, antisense: 5′-CTT AAG GTT TCT TCC CCC TCT C-3′), 3HAO (sense: 5′-GTC ATG TTC ATC GGA GG-3′, antisense: 5′-TCA TAG GTG TCC CCA AAC AG-3′), QPRT (sense: 5′-CTC AAC TAC GCA GCC TTG-3′, antisense: 5′-CAG GCT GCT CAT GCA GCC TTG-3′), and G3PDH (sense: 5′-ACC ACA GTC CAT GCC ATC AC-3′, antisense: 5′-TCC ACC ACC CTG TTG CTG TA-3′). The PCR program was as follows: for IDO, KMO, and 3HAO, (2 min at 95°C) × 1, (35 sec at 95°C, 35 sec at 50°C, 2 min at 72°C) × 35, and 7 min at 72°C; for KYNase and QPRT, (5 min at 92°C) × 1, (1 min at 92°C, 1 min at 60°C, 1.5 min at 72°C) × 35, and 7 min at 72°C; for G3PDH, (2 min at 95°C) × 1, (35 sec at 95°C, 35 sec at 50°C, 2 min at 72°C) × 24, and 7 min at 72°C.

Measurements of Metabolites by Liquid Chromatography–Tandem Mass Spectrometry

Perchloric acid was added to each culture medium (final concentration 0.25 N), which was kept on ice for 10 min and then centrifuged (15,000 g for 10 min). The supernatant was diluted with an equal volume of 1.0 M ammonium formate and then subjected to LC/MS/MS.24

Anticancer Agents

Temzolomide, BCNU, and CDDP were purchased from Sigma Chemical Co., and etoposide from Calbiochem. These reagents were dissolved in dimethylsulfoxide. The final concentrations of dimethylsulfoxide in culture media for glioma cells did not exceed 0.2%.
Measurements of Cell Growth

The concentration of living cells was determined using the cell proliferation reagent WST-1 (Roche Molecular Biochemical). To each 100-μl culture volume in the well, 10 μl of reagent was added. After incubation for 2 hours in the cell incubator, the absorbance at 440 nm was measured.

Statistical Analysis

Differences in mean values were assessed with the Bonferroni test for intergroup comparison. Probability values < 0.05 were regarded as indicating significant differences.

Results

Expression of KP Enzymes in Human Glioma Cells and the Effect of IFN-γ on KP Enzyme Expression and Trp Catabolism

The expression of KP enzymes in glioma cell lines (LN229, U251, T98G, and U87) with or without IFN-γ treatment was examined using reverse transcription–PCR. Indoleamine 2,3-dioxygenase mRNA, together with KMO, KYNase, and QPRT mRNA, was detected in all glioma cell lines, even in the absence of IFN-γ. In these cell lines, IFN-γ treatment strongly increased the IDO mRNA level and weakly increased the KMO mRNA level.
but the effect of IFN-γ treatment on KYNase and QPRT expression was not clear. Regardless of IFN-γ treatment, 3HAO was not detected. The neuroblastoma (SY5Y) cells also expressed IDO mRNA, and the IDO mRNA level was increased after IFN-γ treatment, but the extent of the increase was lower than in glioma cells. Expression of QPRT mRNA was also found, but there was no evidence of expression of any other enzymes of interest in SY5Y cells, even after IFN-γ treatment. Independent of IFN-γ treatment, HepG2 and HEK did not express IDO or KMO mRNA, whereas strong expression of 3HAO and QPRT mRNA was detected (Fig. 2).

Using LC/MS/MS, Trp and its KP metabolites, Kyn, 3-hydroxykynurenine (3HK), and 3-hydroxyanthranilic acid (3HA), were measured in culture medium with or without IFN-γ treatment and with different FCS concentrations. No detectable decrease in Trp or increase in Kyn was noted in the culture medium of glioma cells without IFN-γ treatment, while a drastic decrease in Trp concomitant with an increase in Kyn was observed with IFN-γ treatment. These changes observed in the culture medium of the glioma cells exposed to IFN-γ increased with higher FCS concentrations. In HepG2 cells, which lack IDO induction, no changes in the amount of Trp or Kyn were noted with IFN-γ treatment (Fig. 3). Among the metabolites downstream of Kyn, 3HK and 3HA were detected after a longer incubation period (Fig. 4).

These results indicate that all 4 human malignant glioma cell lines that were examined have IFN-γ-inducible IDO, which used up Trp and resulted in metabolite accumulation on exposure to IFN-γ. This suggests that malignant glioma cells can produce a local environment that is depleted of Trp and rich in Kyn, which results in the inactivation of the T cells that are part of the patients’ immune defense against the glioma.

**Effect of 1MT on Trp Deprivation and Kyn Production**

Next, we assessed whether 1MT, a commercially available inhibitor of IDO that has been most often used for IDO inhibition in previous reports, inhibits IDO and restores Trp levels in culture medium. The Trp concentration was restored to 34, 45, and 52 μM with 200, 500, and 900 μM 1MT, respectively. Concomitantly, Kyn production was reduced to 5.8 μM with the addition of 500 μM 1MT. The addition of 1MT did not stimulate the proliferation of glioma cells (Fig. 5).

**Combination of 1MT With Anticancer Drugs**

We also investigated the effect of 1MT on the inhibition of glioma cell growth by anticancer drugs used therapeutically in treating patients with malignant glioma. First, we confirmed that 100 IU/ml IFN-γ exerted no effect on cell viability under our experimental conditions (data not shown). Next, IFN-γ–stimulated LN229 cells were incubated with temozolomide, BCNU, etoposide, or CDDP. These chemotherapeutic agents reduced cell growth to 55–73% (Fig. 6). Due to this decrease in cell number, these anticancer drugs by themselves prevented Trp degradation and Kyn accumulation. The addition of 1MT did not interfere with the cytotoxic effects of the

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**Fig. 4.** Graph showing the concentration of Trp and its metabolites over time. Glioma (LN229) cells (5 x 10^3) were incubated in 0.1 ml of medium containing 100 IU/ml IFN-γ for the times indicated. The amounts of Trp and its metabolites in the culture media were determined using LC/MS/MS. Data in all graphs are expressed as means of 3 independent experiments performed in triplicate. Error bars indicate SDs. *p < 0.05; **p < 0.01, compared with control (0 hours).

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**Fig. 5.** Graph showing the effect of 1MT on Trp metabolism and cell viability. The LN229 cells (5 x 10^3) seeded onto 96-well culture plates were incubated in 0.1 ml of the medium containing 100 IU/ml IFN-γ supplemented with the indicated concentrations of 1MT for 48 hours. The amounts of Trp and Kyn in the culture media were determined using LC/MS/MS. Cell viability was determined using the WST-1 assay. n.s. = no significant difference. *p < 0.05; ***p < 0.001, compared with control (without 1MT).
Inducible immunosuppressive IDO and its inhibitor’s effect

In this paper, we have demonstrated that IDO and KP enzymes except 3HAO were present in cultured human malignant glioma cells, and that IDO expression was greatly enhanced with IFN-γ stimulation, which resulted in Trp depletion and Kyn accumulation in the culture medium. We have also shown that the IDO inhibitor 1MT effectively prevents Trp depletion. The combination of the IDO inhibitor and anticancer drugs augmented the inhibitory effect of these agents on cell growth and Trp degradation. These results suggest that IDO expression induced by IFN-γ secreted from T cells could lead to the immune resistance of gliomas through local Trp depletion and KP metabolite accumulation, and that IDO inhibitors could preserve Trp levels for T cells. Finally, IDO inhibitors could be combined with anticancer drugs as immune adjuvant therapy to obtain better therapeutic effects in patients with malignant glioma.

The CNS has been thought to be an immune-privileged site; however, recent studies have shown that T cells play important roles in antitumor response in the brain. Thus, the feasibility of immunotherapy against brain tumors, including gliomas, has been actively pursued.

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**Discussion**

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multiple human malignant glioma cell lines are shown to have a strong potential for decreasing Trp, on stimulation with IFN-γ, with resulting accumulation of downstream metabolites and possible suppression of T-cell function. When stimulated by IFN-γ, one of the major antitumor cytokines that may be produced by activated T cells, glioma cells increase IDO expression, which converts Trp to Kyn in the culture medium. Munn et al. showed that a low Trp concentration halted cell cycle progression in T cells at a mid-G1 arrest point and suggested that one of the stress response kinases, GCN2, which is activated by Trp-uncharged tRNA, is a molecular sensor in T cells that stops immune cell activation and proliferation. Metabolites of KP may also play a role in T-cell regulation. Terness et al. reported that Kyn or another metabolite of KP inhibits T-cell proliferation in an additive manner. To further elucidate the contribution of enhanced IDO expression in the glioma cell suppression of T-cell function and to clarify the mechanism by which T cells exhibit differential sensitivity to Trp depletion and metabolite accumulation, coculture experiments dealing with the effects of glioma cells on T-cell function and proliferation are required.

The feasibility of IDO involvement in the immune tolerance of gliomas should be assessed in the clinical context: determination of CSF and serum Trp/Kyn concentrations in patients with malignant glioma, histological examination of IDO expression in glioma cells and T-cell infiltration in tumor tissues, as well as determination of their correlations with tumor invasion/metastasis and clinical prognosis. Recently, low serum Trp levels and increased IDO expression in tumor tissues have been reported in patients with malignant diseases, and these features have been shown to be correlated with a poor prognosis. For example, in colorectal cancer, reduced serum Trp is correlated with a deterioration in quality of life, and high IDO expression is associated with an increased frequency of liver metastasis. Thus, IDO expression in tumor tissues, as well as CSF or serum Trp/Kyn concentrations, in patients with malignant glioma may predict prognosis.

Under the present experimental conditions, no unfavorable effects were noted with the combined use of 1MT and chemotherapeutic drugs that are clinically used for the treatment of malignant gliomas. In IFN-γ–unstimulated LN229 cells, overexpression of IDO by transient transfection of the expression plasmid restored cell viability to levels similar to those of untransfected cells in an IDO-dose dependent manner; on the other hand, vector-transfected cells had decreased viability (unpublished observation). Thus, IDO itself may exert some protective effect on survival/growth of glioma cells under stressful conditions. The addition of 1MT to anticancer drug therapy could prevent T-cell suppression by glioma cells through IDO inhibition, augmenting rather than impairing the cytotoxic effect of the chemotherapeutic drugs. In fact, in a mouse breast cancer model, 1MT has been shown to successfully reduce tumor volumes when given in combination with chemotherapeutic agents. By giving 5 mg/ml 1MT in drinking water, a serum 1MT level of at least 205 μM could be achieved in mice without serious toxicity. In addition, systemic administration of 1MT has been shown not only to reach mouse brain but also to inhibit IDO activity, leading to the exacerbation of Th1 cell–induced experimental autoimmune encephalomyelitis, an experimental model of multiple sclerosis. These observations suggest the possibility of IDO targeting by 1MT and other IDO inhibitors in malignant glioma therapy. In vivo experiments using intracranial tumor models with implanted human malignant glioma cells are required to evaluate the efficacy of IDO inhibitors given in combination with chemotherapeutic agents.

Conclusions

The present study demonstrated the following: 1) the KP features common to human malignant gliomas, 2) highly inducible IDO in multiple human glioma cell lines and drastic Trp depletion with accumulation of its metabolites in these cells on stimulation with a major antitumor cytokine IFN-γ, and 3) effective prevention of the depletion of Trp with an IDO inhibitor 1MT without adverse effects on the cytotoxicity of anticancer drugs against the glioma cells. The results suggest a possible mechanism for the immune escape of malignant gliomas and provide candidate variables that could be used as clinical prognostic indicators for patients with malignant glioma. The results also provide the basic information required to develop a new immunoadjuvant therapy targeting IDO in patients with these devastating brain tumors.

Disclosure

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References

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