Reduced expression of the transporter associated with antigen processing 1 molecule in malignant glioma cells, and its restoration by interferon-\(\gamma\) and -\(\beta\)

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Object. It remains unclear whether malignant glioma cells can deliver tumor antigens efficiently to major histocompatibility complex (MHC) Class I molecules. To elucidate the mechanism of antigen presentation in malignant gliomas, the authors examined the expression of the transporter associated with antigen processing 1 (TAP1), which transports antigens to MHC Class I molecules, and low-molecular-mass polypeptide 2 (LMP2), which is a subunit of a proteasome. They also analyzed the effects of interferon (IFN)-\(\gamma\) and IFN-\(\beta\) on the expression of these molecules.

Methods. Five glioma cell lines expressed undetectable or very low levels of TAP1 protein and did not express TAP1 messenger (m)RNA. Normal brain tissue and glioma tissue specimens also showed undetectable levels of TAP1 protein and the same levels of LMP2 protein as lymphoblastoid B cells. Treatments of the tumor cells with IFN-\(\gamma\) or -\(\beta\) enhanced the expression of both TAP1 protein and mRNA as well as the expression of MHC Class I molecules. The expression of LMP2 protein was not altered by the IFN treatments. The authors analyzed structural alterations in the TAP1 promoter region in eight malignant glioma cell lines. Single nucleotide polymorphism was found in 446 bp upstream of the translation start site of the TAP1 gene, and a point mutation was found in 34 bp upstream of the TAP1 gene.

Conclusions. Malignant glioma cells may be less immunogenic due to low levels of TAP1 expression. Upregulated expression of TAP1 and MHC Class I molecules by IFN-\(\gamma\) and -\(\beta\) may enhance antigen presentation in malignant glioma cells.

KEY WORDS • malignant glioma • transporter associated with antigen processing 1 • low-molecular-mass polypeptide 2 • interferon-\(\gamma\) • interferon-\(\beta\)

Several immunotherapies using dendritic cells in patients with malignant gliomas have been reported.\(^{3,7}\) The immunotherapy elicits CTLs against malignant glioma cells.\(^{7,13}\) The CTL may recognize antigen peptides delivered to MHC Class I molecules on the tumor cell surface and kill the tumor cells. Whether malignant glioma cells deliver tumor antigen peptides efficiently to the MHC Class I molecule remains unclear, however. To date, gliomas have been shown to express MHC Class I molecules,\(^{11}\) but the expression of several other molecules that transport tumor antigen peptides to the MHC Class I molecule is unknown. The majority of peptides associated with MHC Class I molecules and presented to CTLs are derived from intracellular self-proteins or microorganisms in infected cells, which are processed into short peptides in several cellular compartments.\(^{31,32}\) Intracellular proteins are proteolytically degraded by a proteasome complex composed of the LMP, LMP2, and LMP7.\(^{43}\) The processed peptides are transferred from the cytoplasm to the endoplasmic reticulum by the TAP molecule\(^{29}\) in an adenosine triphosphate-dependent manner.\(^{19}\) In the endoplasmic reticulum peptides, MHC Class I and \(\beta_2\)-microglobulin assemble into a complex, which is then transported to the cell surface.

The TAP proteins consist of TAP1 and TAP2 and are encoded within the MHC Class II region.\(^{3,33}\) The mutant cells with a defect in the TAP genes resulted in a severe reduction in the levels of MHC Class I on the cell surface.\(^{22,28}\) Transgenic mice deficient in the TAP1 gene also lack MHC Class I expression on the cell surface and have a severe deficiency in the development of CTLs.\(^{34}\) It has been shown that the bidirectional promoters of TAP1 and LMP2 exist between these genes and regulate the expression of both TAP1 and LMP2 genes.\(^{36}\)

In the present study, we investigated TAP1 expression,
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![Graph depicting the results of flow cytometric analyses of the dose dependencies of IFN-γ and IFN-β treatment on TAP1 expression in U251 tumor cells. Values are expressed as the means ± SDs (three experiments).](image)

which plays an important role in the final step of antigen peptide transport to the MHC Class I molecules, and the effect of IFN-γ or -β treatment on TAP1, LMP2, and MHC Class I expression in malignant glioma cells, because it has been reported that IFN-γ upregulates TAP1,\textsuperscript{15,27} LMP2,\textsuperscript{27} and MHC Class I\textsuperscript{15,26} in endothelial and tumor cells. We also noted an SNP in the promoter region and then analyzed whether different types of SNP influence the induction of TAP1 through IFN treatments.

**Materials and Methods**

*Cell Culture, Tissue Samples, and Reagents*

Three human GBM cell lines (A172, U251, and T98G) and a mixed glioma cell line (KG1C) were obtained from the Japanese Cancer Resources Bank (Tokyo, Japan). Several tumor lines were established in our laboratory: the YMG2 line was derived from anaplastic astrocytoma, and the YMG1, YMG4, and YMG5 lines from GBM. We have characterized the tumor cell lines previously.\textsuperscript{26} All of the tumor lines were positive for S100, and YMG1 and YMG5 were weakly positive and YMG2 and YMG4 were negative for glial fibrillary acidic protein on Western blot analysis. The Epstein–Barr virus–transformed B cell line was kindly provided by Dr. Jelena Levitskaya (Cancer Center Karolinska, Karolinska Institute, Stockholm, Sweden). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml kanamycin at 37˚ in 5% CO\textsubscript{2}. Three glioma tissues and three normal brain tissues were homogenized and lysed with the lysis buffer. Total RNA (10 μg) was separated in a 10 or 16% sodium dodecyl sulfate polyacrylamide gel (TEFCO, Tokyo, Japan) and electroblotted on a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was treated with blocking solution (Roche Diagnostics, Mannheim, Germany) overnight at 4˚C. After blocking, the membrane was incubated with rabbit anti–TAP1 antibody (StressGen, Victoria, BC, Canada) or rabbit anti–LMP2 antibody (Affiniti, Mamhead, United Kingdom) for 1 hour at 4˚C. After blocking, the membrane was incubated with rabbit anti–TAP1 antibody (StressGen, Victoria, BC, Canada) or rabbit anti–LMP2 antibody (Affiniti, Mamhead, United Kingdom) for 1 hour at room temperature. The membrane was subsequently incubated with alkaline phosphatase–conjugated swine anti–rabbit immunoglobulin (DAKO). After washing, the membrane was incubated with CDP-Star (Roche Diagnostics) substrate to induce expression. The immunoblot signals were visualized by chemiluminescence and recorded using a Fuji LAS1000 Lumino Image Analyzer (Fuji Film, Tokyo, Japan). The lymphoblastoid B cell line was used as the positive control for TAP1 and LMP2.

*Flow Cytometric Analysis*

The U251 GBM cells were cultured in six-well plates until they were subconfluent. Cells were then treated with 10, 50, 250, 500, or 1000 U/ml IFN-γ or -β for 12 hours to determine the effect of cytotoxic concentration on TAP1 expression. The cells were then treated with 500 U/ml IFN-γ or -β for 6, 12, 24, or 48 hours. After the treatments, cells were collected and washed twice with PBS. Cells were fixed and permeabilized with FIX & PERM reagents (Caltag Laboratories, Burlingame, CA) and incubated with rabbit polyclonal anti–TAP1 antibody (Rockland Immunochemicals, Gilbertsville, PA) or normal rabbit immunoglobulin. The tumor cells were washed twice with PBS and incubated with a secondary swine anti–rabbit antibody conjugated with fluorescein isothiocyanate (DAKO, Glostrup, Denmark). Using a flow cytometer (FACScaliber; Becton Dickinson, Mountain View, CA), the cells were analyzed.

We also examined the effect of IFN treatment on MHC Class I expression in malignant glioma cells. The glioma cell lines were treated with IFN-β or -γ (500 U/ml) for 24 hours. The cells were incubated with mouse anti–human leukocyte antigen-ABC antibody conjugated with phycoerythrin (DAKO) and analyzed using flow cytometry.

*Western Blotting*

Subconfluent tumor cells in 9-cm dishes were left untreated or treated with IFN-γ or -β (500 U/ml) for 12 hours and then lysed using a lysis buffer (Cellytic-MT with protease inhibitor cocktail; Sigma Chemical Co., St. Louis, MO). Three glioma tissues and three normal brain tissues were homogenized and lysed with the lysis buffer. Cell lysates (30 μg) were separated in a 10 or 16% sodium dodecyl sulfate polyacrylamide gel (TEFCO, Tokyo, Japan) and electroblotted on a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was treated with blocking solution (Roche Diagnostics, Mannheim, Germany) overnight at 4˚C. After blocking, the membrane was incubated with rabbit anti–TAP1 antibody (StressGen, Victoria, BC, Canada) or rabbit anti–LMP2 antibody (Affiniti, Mamhead, United Kingdom) for 1 hour at room temperature. The membrane was subsequently incubated with alkaline phosphatase–conjugated swine anti–rabbit immunoglobulin (DAKO). After washing, the membrane was incubated with CDP-Star (Roche Diagnostics) substrate to induce expression. The immunoblot signals were visualized by chemiluminescence and recorded using a Fuji LAS1000 Lumino Image Analyzer (Fuji Film, Tokyo, Japan). The lymphoblastoid B cell line was used as the positive control for TAP1 and LMP2.

*Northern Blotting*

Subconfluent tumor cells in 9-cm dishes were left untreated or treated with IFN-γ or -β (500 U/ml) for 8 hours. Total RNA was isolated from the cells through a one-step method by using ISOGEN (Nippongene, Toyama, Japan) according to the manufacturer’s protocol. Total RNA (10 μg) was electrophoresed with 1% agarose–formaldehyde gel and transferred to a nylon membrane (Zeta-Probe GT; BIO-RAD, Hercules, CA). Blots were hybridized with specific complementary DNA sequences containing the entire coding regions.
of TAP1 and glyceraldehyde-3-phosphate dehydrogenase labeled with digoxigenin. After hybridization, the membrane was washed and treated with blocking solution. The membrane was incubated with alkaline phosphatase–conjugated sheep anti-digoxigenin antibody (Roche Diagnostics) at room temperature for 30 minutes and developed using CDP-Star. Immunoblot signals were visualized through chemiluminescence and recorded using an image analyzer.

Sequence Analysis of the TAP1 Promoter Gene

Genomic DNA was extracted from cultured cells by using ISOGEN. Primers used for amplification of the TAP1 promoter included 5′-AAGTCCCCGGTTGGTCTCC-3′ (forward) and 5′-GCCTAG-AAGCCGACGCACAG-3′ (backward). The PCR amplification was performed using Taq polymerase (Takara, Otsu, Japan) and 10 μmol of each primer for 30 cycles in a DNA thermocycler (Takara), by denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 1 minute at 72°C. Amplified PCR products were analyzed in 1.5% agarose gels. For direct sequencing, the PCR products were extracted from agarose gels using the QiAquick gel extraction kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. The PCR products were directly subjected to DNA sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, CA) with the same primers as those used for PCR.

Results

Effect of IFN Treatment on TAP1 and MHC Class I Expression in U251 Tumor Cells

To investigate the dose dependency of TAP1 expression by IFN-γ or -β, U251 tumor cells were treated with various concentrations of IFN-γ or -β for 12 hours, and TAP1 expression was examined using flow cytometry. The TAP1 expression in the U251 tumor cells increased in a dose-dependent manner and reached a maximal level when 500 U/ml IFN-γ or -β was used (Fig. 1). To determine the time dependency of TAP1 expression by IFN-γ or -β, the U251 cells were treated with IFN-γ or -β (500 U/ml) for 0, 6, 12, 24, and 48 hours. Both IFN treatments increased TAP1 expression, and the expression level peaked at 24 hours (Fig. 2). Based on these data, we used 500 U/ml IFN-γ and -β to treat other cell lines. To determine how long the effect of IFN on TAP1 expression lasts, U251 cells were treated with IFN-β for 12 hours, and TAP1 expression was measured on flow cytometry 0, 12, 24, 36, and 60 hours after treatment. Before IFN-β treatment, the mean fluorescence intensity of TAP1 expression was 24.5 ± 0.6 (mean ± SD), and the values 0, 12, 24, 36, and 60 hours after treatment were 43.2 ± 0.5, 37.1 ± 1.7, 30.5 ± 1.8, 30.9 ± 4.1, and 23.7 ± 1.3, respectively. These results indicated that the enhancing effect of IFN-β on TAP1 expression lasted for 36 hours. Expression of MHC Class I molecules in U251 cells was greatly enhanced by IFN-γ and -β (Fig. 3). Results of flow cytometric analysis showed that mean values of fluorescence intensity of MHC Class I expression before and after the IFN-γ or -β treatments were 119.4 ± 66.9, 212.1 ± 80.3, and 178.8 ± 86.7, respectively, in the five malignant glioma cell lines. Basal expressions of MHC Class I molecules in the glioma cells were low compared with that (fluorescence intensity 202.8) in the lymphoblastoid B cells.

Polymorphism and Mutation in the TAP1 Promoter in Malignant Glioma Cells

Eight human malignant glioma cell lines were analyzed for structural alterations in the TAP1 promoter region. An SNP was present in the TAP1 promoter at 446 bp upstream from the translation initiation site of the TAP1 gene. Three types of SNPs were found. Four glioma cell lines (A172, KG1C, YMG2, and YMG4) showed T and T in alleles, two cell lines (T98G and YM5G) G and T (Fig. 4), and two cell lines (U251 and YMG1) G and G. One glioma cell line (U251) showed a point mutation where a nucleotide G was substituted for A at 34 bp upstream of the TAP1 gene.

Protein Expression in Malignant Glioma Cells

We examined TAP1 expression through Western blotting in glioma cell lines carrying different SNP types and in U251 cells having a mutation in the TAP1 promoter. Levels of TAP1 expression in all five glioma cell lines, glioma tissues, and normal brain tissues were very low compared...
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To examine the effect of IFN-γ and -β treatments on the induction of TAP1 expression, five glioma cell lines (U251, KG1C, YMG1, YMG2, and YMG5) were treated with IFN-γ or -β (500 U/ml) for 12 hours. Both IFN treatments induced the expression of TAP1 protein in all glioma cell lines (Fig. 6). Interferon-mediated upregulation of the TAP1 protein was independent of SNP types in the TAP1 promoter. We also analyzed LMP2 expression in glioma cell lines, glioma tissues, and normal brain tissues. Basal expression levels of LMP2 in U251 and KG1C cells were similar to that in the lymphoblastoid B cell line and lower in YMG1, YMG2, and YMG5 cells (Fig. 7). One glioma tissue sample showed an LMP2 expression level comparable to that in B cells, whereas the other two glioma samples showed lower expression levels. Three normal brain tissue samples showed lower levels of LMP2 expression compared with that in the B cells. Interferon treatment did not change the LMP2 expression levels in the glioma cell lines (Fig. 8).

Expression of TAP1 mRNA in Malignant Glioma Cells

To investigate the effects of IFN treatments on the expression of TAP1 mRNA, five glioma cell lines (KG1C, U251, YMG1, YMG2, and YMG5) were treated with IFN-γ or -β (500 U/ml) for 8 hours. Northern blotting was performed to detect TAP1 mRNA in these cell lines. The TAP1 mRNA was undetectable in all of these glioma cell lines, whereas the IFN-γ or -β treatment increased TAP1 mRNA in all glioma cell lines (Fig. 9). The SNP types of the TAP1 promoter did not affect the induction of TAP1 mRNA by the IFN treatments.

Discussion

Expression of TAP1 in Malignant Glioma Cells

Results in the present study showed that TAP1 protein expression in malignant glioma cell lines, glioma tissue specimens, and normal brain tissue samples was undetectable or remained at very low levels compared with that in lymphoblastoid B cells and that the expression of TAP1 mRNA was not detected in malignant glioma cell lines on Northern blotting. These findings suggest that the characteristics of downregulation of TAP1 expression in normal brain tissue was taken over by the malignant glioma cells and that if tumor antigens are present and processed in the cytoplasm of the glioma cells, the tumor antigen peptides

Fig. 3. Graphs demonstrating the results of a flow cytometric analysis of MHC Class I expression in U251 tumor and lymphoblastoid B cells. The U251 cells were treated with IFN-γ or -β (500 U/ml) for 24 hours. Left: Interferon-γ treatment of U251 cells. Center: Interferon-β treatment of U251 cells. Right: Expression of MHC Class I in lymphoblastoid B cells.

Fig. 4. Results of an SNP analysis in the TAP1 promoter region in malignant glioma cells. Representative SNPs were presented. Upper: Arrow indicates SNP at 446 bp upstream of the translation start site of the TAP1 gene in YMG2 cells. Center: Arrow indicates SNP in YMG5 cells. Lower: Arrow indicates SNP in YMG1 cells.
cannot be loaded efficiently to the MHC Class I molecules via the TAP1 molecule, which may in turn allow glioma cells to escape an attack by the CTL. Reduced expression of TAP1 protein has been reported in various types of carcinomas. Results of several immunohistochemical analyses showed that TAP1 downregulation or loss was found in breast carcinoma, cervical carcinoma, colorectal cancer, lung cancer, melanoma, and renal cell carcinoma. Vitale, et al., demonstrated an association of MHC Class I and TAP downregulation with tumor progression in breast carcinoma. Although TAP1, TAP2, and MHC Class I were strongly stained in low-grade breast carcinoma, downregulation of these molecules was observed in 22% and complete absence in 8% of high-grade lesions. Furthermore, TAP1 downregulation in malignant melanoma has been shown to be associated with metastases, time to progression, and survival. These data suggest that downregulation of TAP molecules in some malignant tumors worsens the clinical course by providing tumor cells with a mechanism of escape from an attack by CTLs during tumor progression. Malignant glioma cells may have the same mechanism of escape from the immune system.

**Enhancement of TAP1 and MHC Class I Expression by IFN-γ and -β**

We showed that levels of TAP1 mRNA and protein in malignant glioma cells were undetectable or very low and enhanced by treatment with IFN-γ or -β, and that MHC Class I expression was also increased by the IFN-γ and -β treatments. Previous reports demonstrated that several molecules participating in antigen processing were upregulated by some cytokines. Interferon-γ treatment in vitro enhances expression of MHC Class I molecules, TAP1, TAP2, LMP2, and LMP7 in small cell lung carcinoma and increases antigen presentation capacity in tumor cells. Similar effects of IFN-γ on TAP1 induction have been reported in embryonal carcinomas and renal cell carcinoma. Interferon-β also induces TAP1, LMP2, and LMP7 in respiratory syncytial virus–infected pulmonary epithelial cells, and tumor necrosis factor–α treatment elevates TAP1 expression in ex vivo human tumor samples. These findings agree with those in the present study. Note, however, that LMP2 expression in malignant glioma cells was not altered by IFN-γ and -β treatments. This phenomenon suggests that IFN-γ and -β regulate only TAP1 expression in malignant glioma cells, whereas the bidirectional promoters for TAP1 and LMP2 regulate the expression of both TAP1 and LMP2. Interferon-β has been used in the treatment of malignant gliomas following resection and may upregulate TAP1 molecules in malignant glioma tissues and enhance the ability to present antigens to immunocompetent cells. Accordingly, IFN-β may be effective as an adjuvant drug for several immunotherapies that induce CTLs against malignant glioma cells.

**Single Nucleotide Polymorphism in the TAP1 Promoter**

Recently, the mechanism of transcriptional regulation for the TAP1 gene has been identified. Human TAP1 and LMP2 genes are transcribed in a divergent orientation with only 593 bp separating the two ATG translation initiation codons, and the expression of both genes is linked. Some regulatory elements within the TAP1/LMP2 promoters and the transcription factors that bind these elements have been defined. Interferon-γ functions through Stat1α/Stat1α homodimers binding to the GAS within TAP1/LMP2 promoters and IFN-α/β act through Stat1/Stat2/p48 binding on the ISRE in HelA cells. Tumor necrosis factor–α induces
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TAP1 and LMP2 gene expression by acting through p50/p65 and p52/p65 NF-κB heterodimers binding to the NF-κB site. The 200 bp proximal to the TAP1 ATG translation start codon is necessary for TAP1 transcription.36 The GAS, ISRE, and NF-κB site exist within 200 bp proximal to the TAP1 gene, and the experimentally altered mutations at cytokine-activating sites block the induction of TAP1 gene expression.17,36 In the present study, we found an SNP at 446 bp upstream of the TAP1 gene, and a point mutation gene in normal human samples. This finding agrees with our results and suggests that the site of an SNP in the TAP1 promoter is preserved in malignant glioma cells.

Expression of LMP2 in Malignant Glioma Cells

In the present study, expressions of the LMP2 protein in malignant glioma cell lines, glioma tissues, and normal brain tissues followed almost the same pattern, and their levels were lower than that in the lymphoblastoid B cell line. Furthermore, LMP2 expression was not affected by IFN-γ or -β treatment. The LMP2 gene promoter region coordinates with that of the TAP1 gene. Expression of LMP2 genes has been shown to be induced by IFN-γ treatment in various tumor cells.16,23,27 The reason why IFN treatments did not upregulate the expression of LMP2 protein in malignant glioma cells is not clear. It is generally accepted that components of the multicatalytic proteasome complex, LMP2 and LMP7, are involved in the generation of an-
Conclusions

We investigated the expression of TAP1, LMP2, and MHC Class I in malignant glialoma cells. The TAP1 expression was very low or undetectable, whereas the level of LMP2 expression was comparable to lymphoblastoid B cells. Interferon-γ and -β treatments upregulated TAP1 expression as well as MHC Class I molecules. These results suggest that IFNs can be used as an adjuvant agent to enhance the capacity to present tumor antigen to immunocompetent cells.

References

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