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Original Article

Suppressive Effects of Transforming Growth Factor-β_1 Produced by Hepatocellular Carcinoma Cell Lines on Interferon-γ Production by Peripheral Blood Mononuclear Cells

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Transforming growth factor-β_1 (TGF-β_1) exerts potent immunosuppressive effects. In this study, we investigated the potential role of TGF-β_1 produced by hepatocellular carcinoma (HCC) cell lines in immunosuppression mechanisms. Using the Mv1Lu cell-growth inhibition assay and an enzyme-linked immunosorbent assay (ELISA), we detected optimal levels of TGF-β_1 in the culture supernatants conditioned by the HCC cell lines PLC/PRF/5, Hep3B, and HepG2. To determine the biological activity of TGF-β_1 in the supernatants, we examined the effects of the culture supernatants on the production of interferon (IFN)-γ induced during the culture of peripheral blood mononuclear cells (PBMCs) stimulated with interleukin (IL)-12. IFN-γ production of IL-12-stimulated PBMCs in the 1:1 dilution of the acid-activated conditioned medium of PLC/PRF/5, Hep3B, and HepG2 reduced to 14.7 ± 0.8, 17.3 ± 9.0, and 35.9 ± 14.6%, respectively, compared with the value in the culture with control medium (complete culture medium). These results suggest that HCC cells producing TGF-β_1 may reduce the generation or activation of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, and thus could enhance their ability to escape immune-mediated surveillance.

Key words: hepatocellular carcinoma, immunosuppression, transforming growth factor-β_1 (TGF-β_1), interleukin-12, interferon-γ (IFN-γ)

It is suggested that anti-tumor reactivity may be altered in cancer patients. Previously, the secretion of immunosuppressive factors from tumor cells appeared to be one of several immunosuppression mechanisms, by which tumors evolve to escape from the immune responses [1, 2]. Transforming growth factor-β_1(TGF-β_1), a member of a family of peptide factors regulating cell growth and differentiation [3], has also been shown to exert potent immunosuppressive effects [4]. The immunosuppressive properties of TGF-β_1 have been shown to modulate the immune response by affecting proliferation, the activation state, and differentiation of natural killer (NK) cells and cytotoxic T cells [5, 6].

TGF-β_1 is overexpressed in most common forms of cancer [7-10]. In addition, in patients with hepatocellular carcinoma (HCC), TGF-β_1 plasma levels have been found to be elevated [11] and expression in the HCC cells to be increased [12-14]. In contrast, in HCC patients, the NK cell activity, which is believed to play an

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important role in host anti-tumor defense mechanisms, appears to be significantly decreased. The reduced activity have been shown to be associated with the progression of HCC (15, 16). Taken together, it is possible that the TGF-β_1 produced by HCC cells may exert negative effects on anti-tumor immunity against HCC.

It has recently been reported that tumor cell-derived TGF-β_1 and interleukin (IL)-10 in conditioned media of pancreatic carcinoma cell lines inhibits both the proliferation and development of Th1-like responses in peripheral blood mononuclear cells (PBMCs) derived from normal donors [10]. However, the immunosuppressive effects of HCC-derived TGF-β_1 have not been fully studied. IL-12, a monocyte/macrophage-derived cytokine, promotes the cytolytic maturation and proliferation of T and NK cells, and the release of interferon (IFN)-γ from these effector cells [17, 18]. Furthermore, IL-12 has been shown to have potent anti-tumor activity in tumor models, including HCC [19, 20]. Thus, in the present study, the TGF-β_1 secreted from HCC cell lines was assessed for its inhibitory effects on the production of IFN-γ induced during the culture of PBMCs stimulated by IL-12, to investigate the potential role of HCC-derived TGF-β_1 in immunosuppression mechanisms.

Materials and Methods

Hepatocellular carcinoma cells. Human HCC cell lines PLC/PRF/5 [21], Hep3B [22], and HepG2 [23] were assessed for TGF-β_1 production. These HCC cells were grown in Minimal Essential Medium (MEM; Sigma Chemicals Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 ˚C in a humidified atmosphere of 5% CO_2 in 95% air.

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) centrifugation from the peripheral blood samples, which were obtained from 5 patients with unresectable huge HCC, or from 7 healthy volunteers. The patients with HCC were diagnosed based on ultrasound sonography, abdominal computed tomography, and angiography (Table 1). Seven healthy volunteers (5 males and 2 females, median age 36, range 33–40) with no known liver disease served as control subjects. Informed consent was obtained from all patients and healthy control subjects.

TGF-β_1 mRNA Expression in HCC cells. The expression of TGF-β_1 messenger ribonucleic acid (mRNA) in the HCC cell lines PLC/PRF/5, Hep3B, and HepG2 were assessed by reverse transcription-polymerase chain reaction (RT-PCR). The PCR was carried out with complementary deoxyribonucleic acid (cDNA) derived from 2 μg of RNA, 1 unit of Taq polymerase (Recombinant Taq DNA polymerase; Takara Shuzo Co., Ohtsu, Japan), and a reaction kit in a final volume of 7 μl. The PCR conditions included denaturation at 94 ˚C for 1 min, annealing at 55 ˚C for 1 min, and extension at 72 ˚C for 1 min. Thirty cycles of PCR were performed on each sample, after which the products were separated on 2% agarose gels.

The PCR primers used here for the amplification of TGF-β_1-, and β-actin-specific sequences were as follows; TGF-β_1: 5-'GCCCTGGACACCAACTATTGCT-3' (sense strand) and 5'-AGGCTCCAAATGGGGCAGG-3' (antisense strand), and β-actin: 5'-ATCTGGCACCA CACCTTCTACAATGAGCTGCG-3' (sense strand) and 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (antisense strand). The PCR using these primers yielded 161-bp and 838-bp products, respectively.

Measurement of TGF-β_1 in culture medium. Culture supernatants conditioned by HCC cell lines were tested for TGF-β_1. HCC cells (1×10^7 cells / 10 ml of culture medium) were cultured for 96 h at

Table I Clinical profiles of patients with hepatocellular carcinoma

	Patients with hepatocellular carcinoma
No. of cases	5
Gender (Male/Female)	4/1
Age (years)	50 (48–76)*
HBV/HCV	3/2
Liver disease (CH/LC)	4/1
Alb (3.9–4.9 mg/dl)	3.5 (2.79–3.96)
AST (11–32 IU/l)	86 (24–130)
ALT (6–39 IU/l)	42 (17–87)
AFP (< 7.0 IU/ml)	4.9 (1.3–57135)
PIVKA-II (< 28 mAU/ml)	629 (17–57500)
KICG	0.132 (0.085–0.198)
Size of main tumor (cm)	120 (27–145)

*, Values are expressed as Median (Minimum-Maximum). AFP, alpha-fetoprotein; Alb, albumine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus; LC, liver cirrhosis; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

37 ℃ in a humidified atmosphere of 5% CO_2 in 95% air. After the incubation, the culture supernatant conditioned by HCC cells was collected and used as conditioned medium for subsequent experiments.

TGF-β_1 is secreted as a latent form that can be converted into a biological active form by exposure to extremes of pH, heat or by treatment with proteases [24]. For subsequent experiments, conditioned medium samples were treated with 0.12 N HCl for 30 min on ice and neutralized with 0.1 M HEPES buffer containing 0.144 M NaOH (acid-treatment) [25]. The acid-treated conditioned medium samples of the HCC cells were assessed for TGF-β_1 by a biological growth-inhibition assay using Mv1Lu mink lung cells [26], the growth of which is inhibited by TGF-β_1 in a dose-dependent manner, and by a specific enzyme-linked immunosorbent assay (ELISA, Morinaga Institute of Biological Science, Yokohama, Japan).

To determine the TGF-β_1 levels in the culture medium of HCC cells by the Mv1Lu cell-growth inhibition assay, we incubated 5×10^4 cells of Mv1Lu mink lung cells with the acid-activated culture supernatant conditioned by the HCC cells diluted in the culture medium, or with the culture medium containing the recombinant human TGF-β_1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) ranging from 0.1 to 10 ng/ml in 96-well flat-bottom microtiter plates. The plates were incubated at 37 ℃ with 5% CO_2 for 72 h. After the 72-h incubation, the cell-growth in each well was determined in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Standard growth-inhibition curves were obtained by plotting the cell growth of the treated cells versus the recombinant human TGF-β_1 concentrations. TGF-β_1 levels in the culture medium samples were obtained by determining the concentrations from the standard curve.

Inhibitory effects of recombinant human TGF-β_1 on IFN-γ production from IL-12-stimulated PBMCs. To test whether the inhibitory effects of TGF-β_1 involve a down-regulation of IFN-γ production from IL-12-stimulated PBMCs, we examined IFN-γ production in the presence of recombinant human TGF-β_1 added at the initiation of the PBMCs stimulation with IL-12. PBMCs (5×10^5 cells/100 μl/well) derived from 5 patients with HCC or from 7 normal donors were cultured with IL-12 (10 ng/ml) (R & D Systems, Minneapolis, MN, USA) in the presence or absence of recombinant human TGF-β_1 in 96-well flat-bottom microtiter plates. The plates were incubated at 37 ℃ in a 5 % CO_2 atmosphere for 48-h, and the supernatants were collected and assessed for IFN-γ production using specific ELISA (BioSource International, CA, USA).

Inhibitory effects of conditioned medium of HCC cell lines on IFN-γ production from IL-12-stimulated PBMCs. To show the biologic activity of TGF-β_1 in the supernatants conditioned by HCC cell lines, we examined the effects of the culture supernatants on the IFN-γ production induced in the culture of IL-12-stimulated PBMCs. Acid-activated conditioned supernatant samples diluted in the complete culture medium were added to the culture of PBMCs obtained from HCC patients in the presence of IL-12 (10 ng/ml) in 96-well flat-bottom microtiter plates. The supernatants were collected after the 48-h incubation and tested for IFN-γ. Results are presented as percentages of the control IFN-γ production (culture medium).

To confirm the specificity of the inhibition of IFN-γ production from IL-12-stimulated PBMCs, the supernatant samples conditioned by HCC cells were preabsorbed with anti-TGF-β_1 monoclonal antibodies (Wako Pure Chemical Industries, Ltd., Osaka, Japan) -conjugated Protein A sepharose (Sigma Chemicals Co. St. Louis, MO, USA). Normal (nonimmune) mouse monoclonal antibody was used as a negative control. Then, in a 1:1 dilution of preabsorbed conditioned supernatant, PBMCs were cultured with IL-12. IFN-γ production in the culture with preabsorbed supernatants was compared with that in the culture with nonabsorbed supernatants.

Statistical analysis. The changes in each of the variables were assessed by Wilcoxon's sign-rank test or the Student's t-test. Nonpaired data were compared by the analysis of the Mann-Whitney U-test. A P value less than 0.05 was considered to be statistically significant.

Results

Production of TGF-β_1 by HCC cells. The human HCC cell lines PLC/PRF/5, Hep3B, and HepG2 were assessed for TGF-β_1 production. To confirm the production of TGF-β_1, HCC cells were tested for the expression of TGF-β_1 mRNA by RT-PCR using a specific primer set of TGF-β_1. The expressions of TGF-β_1 mRNA were readily detected in these HCC cell lines (Fig. 1).

Using the Mv1Lu cell growth inhibition assay, we detected TGF-β_1 at a level of 13 ng/ml in the culture

supernatants conditioned for 4 days by HCC cell lines (Table 2). In addition, using an ELISA that recognized the biologically active form of TGF-β_1, we detect the optimal level of TGF-β_1 (2.3–7.3 ng/ml) in the culture supernatants conditioned by the three HCC cell lines (Table 2). These findings suggest that the HCC cell lines produced and secreted significant quantities of TGF-β_1 into the culture medium.

Inhibitory effects of recombinant human TGF-β_1 on IFN-γ production from IL-12-stimulated PBMCs. We examined the effects of recombinant human TGF-β_1 on IFN-γ production by IL-12-stimulated PBMCs, which were obtained from 5 HCC patients or from 7 normal donors. The mean IFN-γ production in the culture of IL-12-stimulated PBMCs derived from the HCC patients was 2043 ± 630 pg/ml (median 1766 pg/ml, range 1511–3022 pg/ml), which was not significantly different from that of the normal donors (1597 ± 400 pg/ml, median 1547 pg/ml, range 1170–2131 pg/ml) ($P = 0.223$). The results illustrated in Fig. 2 show that TGF-β_1 used at various concentrations from 0.1 to 10 ng/ml inhibited the production of IFN-γ by IL-12-stimulated PBMCs in a dose-dependent manner. The mean IFN-γ production by IL-12-stimulated PBMCs in the presence of 0.1, 1.0, or 10 ng/ml of recombinant human TGF-β_1 was 1572 ± 578, 947 ± 480, and 596 ± 290 pg/ml in the HCC patients, and 1277 ± 512, 607 ± 211, and 421 ± 219 pg/ml in the normal donors, respectively, suggesting that TGF-β_1 inhibited the IFN-γ production by IL-12-stimulated PBMCs derived from patients with HCC in a manner similar to those of healthy subjects.

Inhibitory effects of culture supernatants conditioned by HCC cells on IFN-γ production from IL-12-stimulated PBMCs. We found that

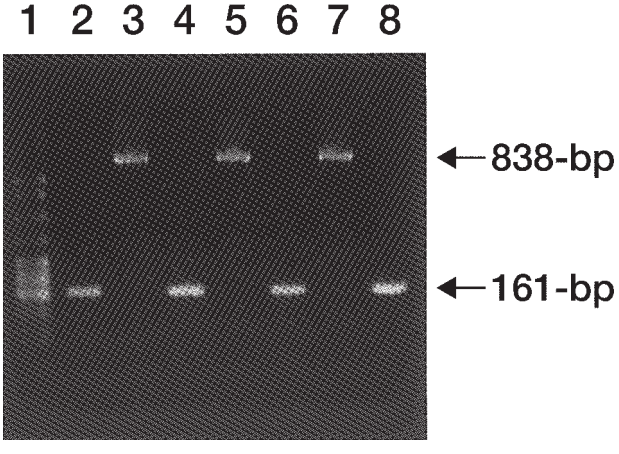

Fig. 1 Analysis of transforming growth factor (TGF)-β_1 mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was prepared from human the hepatocellular carcinoma cell lines PLC/PRF/5 (lane 2 and 3), Hep3B (lane 4 and 5), and HepG2 (lane 6 and 7). Total RNA was analyzed by RT-PCR for the expression of TGF-β_1 mRNA (lane 2, 4, and 6; 161 bp) and β-actin mRNA (lane 3, 5, and 7; 838 bp). Lane 1, molecular size-marker; lane 8, positive control cDNA fragment.

Table 2 TGF-β_1 concentration in the culture supernatant of HCC cell lines

HCC cell line	Bioassay (ng/ml)	ELISA (ng/ml)
PLC/PRF/5	13.6	2.3
Hep3B	13.6	7.3
HepG2	13.5	3.4

ELISA, enzyme-linked immunosorbent assay.

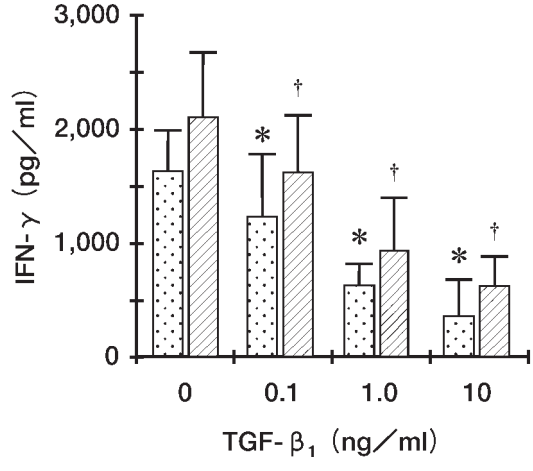

Fig. 2 Suppressive effects of transforming growth factor (TGF)-β_1 on interferon (IFN)-γ production in the culture of peripheral blood mononuclear cells (PBMCs) stimulated with interleukin (IL)-12. PBMCs (5×10^5 cells/100 μl/well) derived from 7 healthy subjects (▨) and from 5 patients with HCC (▨) were cultured with IL-12 (10 ng/ml) in the presence or absence of human recombinant TGF-β_1 added at the beginning of the culture period at the indicated concentrations. Culture supernatants were collected after the culture for 48-h, and tested for the IFN-γ production. IFN-γ production is presented as the mean $\pm$ SD of the 7 normal subjects and the 5 HCC patients, respectively. Asterisks indicate statistically significant differences ($P < 0.05$ in Wilcoxon's sign-rank test) between data sets when compared to controls in the absence of TGF-β_1 in healthy subjects (*) and in HCC patients (†), respectively.

HCC cells secreted TGF-β_1 into the culture medium in a biologically inert form that was unmasked by the acid-treatment. We next investigated whether TGF-β_1 in the culture supernatants was effective in suppressing the IFN-γ production in the culture of PBMCs stimulated with IL-12. As shown in Fig. 3, IFN-γ production of IL-12-stimulated PBMCs in the 1:1 dilution of the acid-activated conditioned medium of PLC/PRF/5, Hep3B, and HepG2 was reduced to 14.7 ± 0.8, 17.3 ± 9.0, and $35.9 \pm 14.6\%$, respectively, compared with the value in the culture with control medium (2370 ± 880 pg/ml, complete culture medium). IFN-γ production of IL-12-stimulated PBMCs in the 1:4 dilution of the conditioned medium of PLC/PRF/5, Hep3B, and HepG2 were 54.1 ± 48.6, 53.3 ± 14.6, and 46.0 ± 17.9 %, respectively. These results suggest that the acid-activated supernatants suppressed in a dose-dependent manner the IFN-γ production that was induced in the culture of PBMCs stimulated with IL-12.

To confirm that the biological activities of the supernatants were indeed due to TGF-β_1, the supernatants were preabsorbed with anti-TGF-β_1 monoclonal antibody-conjugated Protein A-Sepharose. As shown in Fig. 3, the inhibitory effects of the 1:1 dilution of conditioned supernatants on the IFN-γ production from IL-12-stimulated PBMCs were completely overcomed by the preabsorption (114.6 ± 12.6, 125.1 ± 34.0, $132.5 \pm 27.0\%$, respectively), indicating that the suppressive effects on IFN-γ production were due to the TGF-β_1 in the supernatants conditioned by HCC cells.

Discussion

Certain tumors have been shown to produce large amounts of TGF-β_1 [7–10]. TGF-β_1 is also produced from hepatocellular carcinoma cells [12–14]. In the present study, we determined that TGF-β_1 is present in culture supernatants conditioned with the human HCC cell lines PLC/PRF/5, Hep3B, and HepG2 in a biologically inert form that can be unmasked by acid treatment. In addition, the expressions of TGF-β_1 mRNA were detected in these HCC cell lines. These results suggest that the HCC cell lines continue to synthesize TGF-β_1 in a latent but potentially activated form.

TGF-β_1 is a cytokine involved in the regulation of cell growth and differentiation [4]. It has also been shown to modulate immune responses by affecting the proliferation, activation, and differentiation of immune cells within the

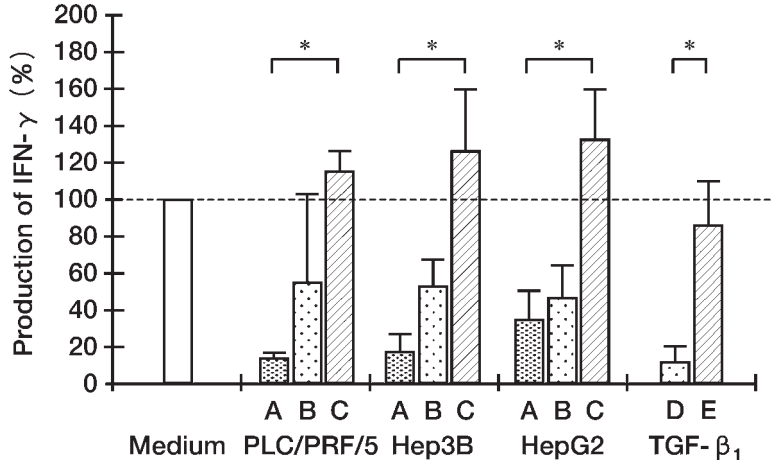

Fig. 3 Effects of the culture supernatants conditioned by hepatocellular carcinoma (HCC) cell lines PLC/PRF/5, Hep3B, and HepG2 on the production of interferon (IFN)-γ induced in the culture of peripheral blood mononuclear cells (PBMCs) stimulated with interleukin (IL)-12. PBMCs (5×10^5 cells/100 μl/well) derived from 3 HCC patients were cultured with IL-12 (10 ng/ml) in (**A**) a 1:1 dilution of the conditioned supernatant; (**B**) a 1:4 dilution of the conditioned supernatants; (**C**) a 1:1 dilution of the conditioned supernatants, which were preabsorbed with anti-TGF-β_1 monoclonal antibodies conjugated Protein A-Sepharose; (**D**) the culture medium containing human recombinant TGF-β_1 (5 ng/ml); or (**E**) the culture medium containing human recombinant TGF-β_1, which was preabsorbed with anti-TGF-β_1 monoclonal antibody-conjugated Protein A-Sepharose. Culture supernatants were collected after the incubation for 48-h and tested for IFN-γ. Results are presented as percentages of the control IFN-γ production (culture medium). Values are presented as the means $\pm$ SD of the 3 HCC patients. Asterisks indicate a statistically significant difference ($P < 0.05$ in Student's t-test) between 2 mean values.

framework of the immune-cell network [5, 6]. Therefore, it is reasonable to consider that the TGF-β_1 produced by HCC cells could suppress the immune cells surrounding HCC cells, subsequently enhancing the ability of HCC to escape immune surveillance. However, with regard to the TGF-β_1 produced by HCC cells, there have been few studies of the immunosuppressive effects on the cell-mediated immune response.

The findings of the present study clearly show that recombinant human TGF-β_1 inhibits the IFN-γ production induced in the culture of PBMCs stimulated with IL-12, as reported previously [27–29]. These results suggest that TGF-β_1 inhibits the responsiveness of T and NK cells to IL-12. IL-12 acts on T and NK cells by inducing the proliferation and production of cytokines, and by enhancing the activity of CTL and NK cells [17, 18]. Therefore, the TGF-β_1-dependent inhibition of biological activities of IL-12 may contribute to the inhibition of cell-mediated immune responses and the subsequent suppression of anti-tumor immunities. It has recently been reported that TGF-β_1 antagonizes the ability of IL-12 to stimulate the IFN-γ production of lymphocytes by inhibiting IL-12-inducing signaling molecules [27] or by interfering with the normal expression of IL-12 receptors (IL-12R) [28].

The present results demonstrate that the TGF-β_1 secreted by HCC cell lines into a culture medium also inhibits the IFN-γ production by IL-12-stimulated PBMCs obtained from HCC patients. These findings indicate that TGF-β_1-producing HCC cells may reduce the generation or activation of CTL and NK cells, and thus could enhance their ability to escape immun-mediated surveillance. Bellone G *et al.*, also have reported that TGF-β_1 and IL-10 secreted into the conditioned medium of pancreatic carcinoma cell lines significantly reduced the production of IFN-γ by PBMCs obtained from three normal donors upon activation by anti-CD3 antibody. From these results, they suggested that production of either TGF-β_1 or a combination of IL-10 and TGF-β_1 by pancreatic carcinoma cells contributes to the inhibition of Th1-like responses in naïve PBMCs.

On the other hand, the escape from immune destruction, which is partially acquired by secreting immunosuppressive factors, might be circumvented by inhibiting the expression of these factors. Fakhrai *et al.* [29] have recently reported that subcutaneous immunization of glioma-bearing rats with 9L glioma cells genetically modified to inhibit TGF-β_1 expression with an antisense plasmid vector results in a significantly higher number of animals surviving for 12 weeks compared with immunizations with control vector-modified 9L cells. They also found that *in vitro* tumor cytotoxicity assays with lymphnode effector cells results in a 3- to 4 fold increase in lytic activity in animals immunized with TGF-β_1 antisense-modified tumor cells compared with immunization with control vector. These results suggest that inhibition of TGF-β_1 expression significantly enhances tumor-cell immunogenecity and supports the view that immunization with tumor cells genetically modified to suppress TGF-β_1 may be efficacious against established tumors expressing TGF-β_1.

HCC is liable to occur even after the curative therapy. Therefore, the development of new therapies such as immunotherapy is essential to prevent recurrence. The present findings may have important implications for the development of immunotherapies for HCC. Strategies to inhibit the expression of TGF-β_1 in HCC cells may provide clues to develop active immunotherapies for HCC.

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