Hepatocellular Carcinoma Cells Induce Regulatory T Cells and Lead to Poor Prognosis via Production of Transforming Growth Factor-β1

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Key Words
Hepatocellular carcinoma • Foxp3 • Transforming growth factor-β1 • Regulatory T cell • Prognosis

Abstract
Background/Aims: Regulatory T cells (Tregs) are associated with a poor prognosis in hepatocellular carcinoma (HCC). The purpose of the study was to explore the mechanisms of Tregs accumulation in HCC. Methods: We analyzed the frequency of Tregs in HCC by flow cytometry and immunohistochemistry. We also established a transforming growth factor (TGF)-β1-knockdown cell line by lentivirus-mediated RNA interference. Mouse CD4+CD25− T cells were cultured in supernatants from various cell lines. Results: HCC patients had a high frequency of Tregs, and high numbers of Tregs correlated with a poor prognosis. Liver cancer cells induced Treg production by secreting TGF-β1. In vivo experiments indicated that knockdown of TGF-β1 reduced the numbers of Tregs and metastatic nodules in mice. Conclusions: These results indicate that cancer-secreted TGF-β1 may increase Tregs, and TGF-β1 knockdown might impair immunosuppression in the tumor microenvironment by decrease Tregs.

Introduction
Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide, especially in Asia, with an estimated 696,000 deaths from liver cancer in 2008 [1]. Despite improvements in survival rates associated with advances in diagnostic and surgical
techniques, frequent recurrence and metastasis remain the leading causes of deaths, even after HCC resection [2].

Regulatory T cells (Tregs) were discovered by Sakaguchi et al. in 1995 and are characterized by their immunosuppressive properties [3]. The transcription factor forkhead box P3 (Foxp3) provides a biomarker for Tregs' detection. In addition to Foxp3, GITR+, LAG3+ and CD127low are also used to detect Tregs. It has been shown that Tregs mediate immunosuppression by secreting TGF-β1 and IL-10. Decreased numbers or dysfunction of Tregs have been found in patients with autoimmune diseases [4-6], and increased numbers of Tregs in the peripheral blood and tumor stroma have recently been reported in several cancers [7-10]. Other studies have shown that a high frequency of Tregs is a frequent indicator of poor prognosis in tumor patients [11-13]. Several clinical trials have confirmed the efficacy of antibodies that attenuate Treg function [14]. However, the generation of Tregs in cancer patients remains poorly understood.

Overexpression of transforming growth factor (TGF)-β1 has been reported in several cancers [15-17], including HCC [18], indicating a relationship between this cytokine and malignant cell progression [19]. Several reviews have reported on the roles of TGF-β1 in cancer development, including in epithelial-mesenchymal transition, inducing angiogenesis, and suppressing anti-tumor immunity [20-22]. Recent studies on the tumor immune microenvironment have suggested that its status can affect the progression of established tumors [23, 24]. Tumor-derived TGF-β1 has been reported to affect tumor-infiltrating leukocytes (TILs) and modulate the microenvironment to avert immune surveillance [21]. Tregs form an important component of TILs in many solid tumors. Previous experiments confirmed an important role for TGF-β1 in the development of Tregs, but few studies have investigated the relationship between tumor-derived TGF-β1 and Tregs. We therefore hypothesized that HCC might influence the differentiation of naive T cells into Tregs by secreting TGF-β1.

In the current study, we examined the presence of Tregs in HCC patients. We also investigated the secretion of TGF-β1 by HCC cells, and its relationships with Treg production in mice and prognosis in patients with HCC. The results of this study suggest that TGF-β1 might provide a potential immunotherapeutic target for HCC treatment.

Materials and Methods

Cell lines

The mouse HCC cell line Hepa1-6 was purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (Hyclone, Tianjin, China) supplemented with 10% fetal bovine serum (Gibco, Victoria, Australia), 100 units/mL penicillin (Hyclone), and 100 μg/ml streptomycin (Hyclone). In some experiments, cancer cells were cultured in serum-free AIMV medium (Invitrogen, New York, USA) and the supernatant was collected for further investigations.

Patients and specimens

A total of 184 patients with HCC, 29 with liver cirrhosis, and 30 healthy controls were enrolled in the current study. Immunohistochemical analysis was carried out on 151 tumor specimens derived from HCC patients who had undergone surgical resection at Zhongshan Hospital, Fudan University, between 2006 and 2008. Venous blood was obtained from 33 HCC patients, 29 cirrhosis patients, and 30 healthy controls. Plasma was separated and stored at −20˚C for subsequent enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells were isolated from donor blood using Ficoll density-gradient centrifugation and checked by flow cytometry. The current study was approved by the Ethical Committee of Zhongshan Hospital and informed consent was obtained from all patients.

Animals

SPF female C57BL/6 mice aged 4 – 8 weeks were purchased from the SLAC Laboratory Animal Company (Shanghai, China). All animal experiments were approved by the University Committee for the Care and Use of Laboratory Animals, Fudan University.
Short hairpin RNA construction and establishment of stable TGF-β1-knockdown cell line

Short hairpin RNAs (shRNAs) targeting TGF-β1 (5′-CCG GCG GCA GCT GTA CAT TGA CTT TCT CGA GAA AGT CAA TGT ACA GCT GCC GCT GCA GGG AGG GCG ACT TAA CCT TAG GTT TTGG-3′) and scrambled shRNA (5′-CCG GCC TAA GGT TAA GTC GCC CTC GCT CGA GGG AGG GCG ACT TAA CCT TAG GTT TTGG-3′) were used in the current study. Lentivirus was produced in 293T cells, using 2 μg/ml puromycin to select infected cells.

RNA extraction and real-time PCR

Total RNA was extracted from the cultured cells as described previously [25]. The relative expression levels of TGF-β1 and Foxp3 mRNA were detected by real-time PCR (ABI7500 Fast system, UAS) using a SYBR Prime Script kit (TaKaRa, Dalian, China). The results were analyzed using the 2^ΔΔCt method. The following primers were used: TGF-β1 forward: 5′–CAC CTG CAC AGC TCA CGGCA -3′, reverse: 5′- AAG CGC CCG GGT TGT GTTGG-3′; Foxp3 forward: 5′-TTG AGG GTG GGT GTC AGGAG-3′, reverse: 5′-AGG GTT GGG CAT TGG GTTCT-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5′- CAT CAA GAA GGT GGT GAAGC-3′, reverse: 5′-CCT GTT GCT GTA GCC GTATT-3′.

Lymphocyte isolation

Mice were sacrificed and spleens and lymph nodes were collected. CD4^+CD25^- T cells were isolated according to the manufacturer’s introductions (Miltenyi Biotec, Germany). The purity (> 90%) was analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

Lymphocyte culture in different media

CD4^+CD25^- T cells were cultured in different media. Hepa1-6, scramble, and shRNA-TGF-β1-Hepa1-6 cells (2 × 10^6) were plated into T25 flasks with complete medium for 2 days, and the supernatants were then replaced with fresh AIMV medium. After a further 3 days, the supernatants were collected and diluted with fresh AIMV medium (1:3) and were called Hepa1-6, scramble, and shRNA-TGF-β1-Hepa1-6 supernatants, respectively. These supernatants were added to 30 U/ml recombinant mouse interleukin (IL)-2 (R&D Systems, Minnesota, USA), 10 mM HEPES buffer (Hyclone), and 0.05 mM 2-ME (Gibco). Isolated CD4^+CD25^- T cells (5 × 10^5/well) in 48-well plates were cultured in either complete T cell medium (AIMV, 30 U/ml recombinant mouse IL-2, 10 mM HEPES buffer, 0.05 mM 2-ME), or Hepa1-6, scramble, or shRNA-TGF-β1-Hepa1-6 supernatant for 3 days in the presence of anti-CD3/CD28-coated beads (5:1, Invitrogen).

Recombinant human TGF-β1 (1 ng/ml, R&D Systems) and TGF-β1 receptor kinase inhibitor LY-364947 (3 μM, Merck, Germany) were added to some cultures and T cell proliferation was measured using the carboxyfluorescein succinimidyl ester (CFSE) method, according to the manufacturer’s instructions (Invitrogen).

Flow cytometry

Human Tregs were detected using a Treg-staining kit (eBioscience, California, USA), as described previously [25]. Mouse Tregs were detected using peridinin chlorophyll protein complex-conjugated anti-mouse CD4 antibody (BD Bioscience, California, USA) and phycoerythrin-conjugated anti-mouse Foxp3 Ab (BD).

Western blotting

Rabbit anti-mouse Foxp3 primary antibody (Abcam, Massachusetts, USA) and rabbit anti-mouse GAPDH primary antibody (Abgent, California, USA) were used to detect Foxp3. Immunoblotting experiments were performed as described previously [25].

ELISA

Cell lines were plated at a density of 5 × 10^5/well in a 24-well plates and cultured for 48 h in serum-free medium to exclude the possibility of TGF-β1 in the serum. Supernatant and serum TGF-β1 concentrations were measured by ELISA, according to the manufacturer’s instructions (eBioscience).

Immunohistochemistry and double-immunofluorescence staining

Immunohistochemical analysis was performed using mouse anti-human TGF-β1 primary antibody (Santa Cruz, Texas, USA), mouse anti-human Foxp3 primary antibody (236A/E7, Abcam), and rabbit anti-
mouse Foxp3 primary antibody (Abcam), as described previously [25]. The cut-off value for Treg expression was the median value. Co-expression of TGF-β1 and Foxp3 was determined by double-immunofluorescence staining. Alexa-fluor 488 and Alexa-fluor 555 conjugated second antibodies were used.

**Evaluation of immunohistochemical staining**

Immunohistochemical staining for TGF-β1 was assessed by two pathologists and the mean value was taken to represent TGF-β1 expression. The following criteria were used: strong staining (++), appreciable staining in >50% tumor cell cytoplasm; weak staining (+), less staining in tumor cell cytoplasm; absent (0), no staining in tumor cells [26]. Foxp3 staining was determined in five independent microscopic fields (×40) for each sample, and the mean was considered as the frequency of Tregs in one sample [27].

**Animal models**

Subcutaneous tumor models were generated by injecting $6 \times 10^6$ cells into the right flank of each C57BL/6 mouse. Lung metastasis models were generated by injecting $2 \times 10^6$ cells suspended in 150 μL phosphate-buffered saline via the tail vein.

**Evaluation of lung metastasis**

Mice were sacrificed and the lungs were excised, perfused with 15% India ink (15% India ink, 85% water, and 3 drops of NH$_2$OH per 100 mL), and fixed in Fekete’s solution (100 mL 70% ethanol, 10 mL 37% formaldehyde, 1.5 mL glacial acetic acid) [28].

**Statistical analysis**

Statistical analyses were conducted using SPSS 17.0 for Windows. Differences between groups were analyzed using Student’s $t$-tests and correlations were analyzed by linear regression. Cumulative survival time was calculated by Kaplan–Meier analysis and analyzed with log-rank tests. $P < 0.05$ was considered statistically significant.

**Results**

**Increased CD4+Foxp3+ cells in peripheral blood**

We analyzed the frequency of CD4+Foxp3+ T cells in peripheral blood using flow cytometry. Dot plots were used to show the prevalences of CD4+Foxp3+ T cells in different groups (Fig. 1A). The frequencies of CD4+Foxp3+ T cells were higher in HCC (9.61 ± 2.55%) and cirrhosis patients (9.62 ± 2.88%) compared with healthy controls (7.42 ± 1.68%) ($P < 0.05$) (Fig. 1B). TGF-β1 concentrations were significantly higher in HCC patients (11,549 ± 5204 pg/mL) compared with healthy controls (8448 ± 5094 pg/mL). TGF-β1 serum levels were also higher in HCC patients compared with cirrhotic patients (9491 ± 4323 pg/mL), but the difference was not significant ($P > 0.05$). A similar trend was observed between cirrhotic patients and healthy controls (Fig. 1C).

**Immunohistochemical characteristics and prognosis**

We investigated the presence of Tregs in HCC samples by immunohistochemical staining. Representative images of Foxp3 staining showed abundant Foxp3+ cells in the tumor margin, and slight Foxp3+ cells in the tumor center and paracancerous tissues. Foxp3+ cells were abundant around micrometastases (Fig. 1D). Kaplan–Meier analysis showed that patients with low numbers of Tregs in the tumor stroma had a better prognosis (Fig. 1, E and F).

Two-, 3- and 4-year overall survival (OS) and disease free survival (DFS) rates were significantly higher in patients with low Tregs compared with those with high Tregs (77% and 47% vs. 51% and 31%; 68% and 44% vs. 44% and 31%; 66% and 38% vs. 38% and 25%, respectively) (Fig. 1E, F). Patients with low intratumoral Tregs had longer OS (median, 44.7 months) and DFS (median, 28 months) than those with high Tregs (OS median 34.5 months; DFS median 13 months).
Correlation between Tregs and TGF-β1 in tumor stroma

The relationship between TGF-β1 and Tregs in the tumor microenvironment was explored by immunohistochemical double-staining of formalin-fixed surgical specimens for Foxp3 and TGF-β1. Foxp3 staining corresponded to the region of high TGF-β1 expression (Fig. 2).

Fig. 1. Tregs were increased in HCC patients and a high frequency of Tregs in the tumor microenvironment predicted a poor prognosis. (A) Dot plots gated on CD4+ T cells showed CD4+Foxp3+ Tregs. Results are expressed as the proportion of CD4+Foxp3+ Tregs among CD4+ T cells. (B) Histogram representing the mean frequencies of CD4+Foxp3+ T cells in the peripheral blood of HCC (n = 33) and cirrhotic patients (n = 29), and healthy controls (n = 30). (C) Histogram showing the mean concentrations of TGF-β1 in different donor sera. (D) Immunohistochemical detection of Foxp3 in the tumor microenvironment. (E and F) Kaplan–Meier analysis of OS and DFS for tumor-infiltrating Tregs. Data are presented as mean ± SEM.
Induction of Foxp3 expression in vitro

shRNA-TGF-β1-Hepa1-6 cells were established to evaluate the correlation between tumor-derived TGF-β1 and Tregs. Real-time PCR and ELISA confirmed that > 80% of TGF-β1 mRNA was silenced in shRNA-TGF-β1 cells (Fig. 3A). Dot plots were used to represent the effects of the different media on Foxp3 induction in CD4+CD25− T cells (Fig. 3B). Hepa-1-6 (4.66 ± 0.16%) and scramble media (4.01 ± 0.69%) significantly increased Foxp3 expression compared with control medium (1.69 ± 0.46%), while LY-364947 (2.81 ± 0.37%) and shTGF-β1 media (2.69 ± 0.67%) had the opposite effect. The addition of recombinant human TGF-β1 to shTGF-β1 medium restored decreased Foxp3 expression (36.23 ± 2.33%), while LY-364947 inhibited recombinant TGF-β1-induced Foxp3 expression (12.88 ± 1.4%) (Fig. 3B). Hepa-1-6 medium and recombinant TGF-β1 induced Foxp3 mRNA and protein expression levels more than shTGF-β1 and control medium, as shown by real-time PCR and western blotting (Fig. 3C, D).

In mice, most Foxp3+ cells were CD4+CD25+ cells, with a few CD4+CD25− Foxp3+ lymphocytes. Based on cell proliferation assays, CD4+CD25− cells showed higher proliferative capabilities than CD4+CD25+ cells, producing six and four generations, respectively (Fig. 3E). Foxp3 was induced in a time-dependent manner, with CD4+Foxp3+ cells reaching a peak at 72 h in Hepa-1-6 medium, followed by a decrease (Fig. 3F).

Decreased Tregs in model mice

In a 4-week lung metastasis model, flow cytometry demonstrated that cancer-derived TGF-β1 caused an increase in Tregs in the spleen, which effect was reversed by TGF-β1 knockdown (Fig. 4A, B). TGF-β1 concentrations measured by ELISA were slightly higher in the Hepa-1-6 and scramble groups compared with the shTGF-β1 group, but the differences were not significant (Fig. 4C) (P > 0.05). Animal experiments indicated that Tregs were significantly reduced in subcutaneous tumors with selective TGF-β1 knockdown (Fig. 4D, E).

HCC cells could secret TGF-β1 and predict a poor prognosis

TGF-β1 expression was detected in 105 HCC patients by immunohistochemistry (Fig. 5A). Among 105 neoplastic tissues, TGF-β1 was strongly stained in 29 cases, weakly stained...
in 47, and absent in 29. Among 101 non-neoplastic tissues, TGF-β1 was strongly stained in 29, weakly stained in 66, and absent in six. Kaplan–Meier analysis indicated that HCC
patients overexpressing TGF-β1 in neoplastic tissues usually had significantly shorter OS and a higher recurrence rate than patients with lower expression, after surgery (Fig. 5B).

**Tumorigenicity of cancer cells in model mice**

TGF-β1 interference significantly suppressed metastasis and prolonged survival time in a lung metastasis model (Fig. 6A, B). After 60 days of feeding, nine mice in the Hepa1-6 group had died, compared with none in the shRNA-TGF-β1 group. In addition, TGF-β1 silencing was
associated with reduced tumorigenicity of cancer cells in a murine in situ hepatoma model (Fig. 6C).

**Discussion**

Tregs are known to have a negative immunomodulatory effect in vivo, and a high frequency of Tregs can indicate a poor prognosis in HCC patients [27, 29]. Tregs act as a prognostic marker for both OS and DFS in HCC patients after surgery. The results of the current study were consistent with previous reports, indicated by the presence of abundant Foxp3+ cells in tumor and peripheral blood. We found that most Foxp3+ cells were at the tumor edge, surrounding the mass and protecting it from the anti-tumor immune response. Although recruitment can partly explain the increase in Foxp3+ cells in the tumor stroma [30], the mechanisms responsible for the increase in Tregs in patient blood remain unclear, and a better understanding of the means whereby cancer cells induce the production of Tregs is urgently needed.

Many patients with cancer have shown high serum concentrations of TGF-β1 [15-17, 31]. In the current study, immunohistochemistry and ELISA confirmed that HCC tumors
secreted TGF-β1, suggesting that the tumor itself may be one of the most important sources of TGF-β1 in HCC patients. Previous studies identified TGF-β1 as a major factor in the induction of the regulatory phenotype and the maintenance of the biological functions of Tregs [32, 33]. Immunofluorescence analysis revealed that Foxp3⁺ cells corresponded to the region of TGF-β1 expression. We therefore hypothesized that cancer cells could secrete TGF-β1 to induce Treg production from CD4⁺CD25⁻ T cells in HCC patients.

In vitro experiments demonstrated that mouse Hepa1-6 cell supernatant significantly increased Foxp3 gene expression in mouse CD4⁺CD25⁻ T cells. However, this increased expression was reduced by inhibiting TGF-β1 downstream signaling or by supernatant from shRNA-TGF-β1-Hepa1-6 cells. Furthermore, recombinant human TGF-β1 significantly increased Foxp3 expression, and this increase was effectively inhibited by TGF-β receptor I inhibition. TGF-β1

**Fig. 6.** TGF-β1 silencing impaired invasion and metastasis of Hepa1-6 cells *in vivo*. (A) Hepa1-6, scramble, and shRNA-TGF-β1-Hepa1-6 cells (2×10⁶) were injected into C57BL/6 mice via the tail vein (n = 5). Mice were sacrificed after 4 weeks of feeding. The lungs were removed and perfused with 15% India ink, and fixed in Fekete’s solution. All mice in the Hepa1-6 and scramble groups had lung metastases, but no nodules were found in the shRNA-TGF-β1-Hepa1-6 group. Representative images are shown. (B) Mice (n = 11) were injected with cells via the tail vein to induce lung metastasis, as described previously, and observed daily for up to 60 days. TGF-β1 knockdown prolonged survival in this lung metastasis mouse model. (C) Mice were sacrificed on day 28. shRNA-TGF-β1-Hepa1-6 cells showed low tumorigenicity after implantation in an *in situ* liver cancer model (n = 4). The experiments were repeated twice.
gene interference in vivo significantly reduced the expression of Tregs in the subcutaneous region and the spleen. These results suggest that cancer-cell-secreted TGF-β1 may be largely responsible for the high frequency of Tregs in HCC patients.

The potential of Tregs as a therapeutic target has been discussed previously [34]. However, no successful clinical trials have been reported, suggesting that depletion of Tregs alone might not be sufficient to prevent tumor-mediated immunosuppression. TGF-β1 may provide an alternative approach. Previous studies demonstrated that TGF-β1 inhibited lymphocyte proliferation and suppressed immune surveillance [35], supporting cancer-cell-secreted TGF-β1 as an important factor mediating immunosuppression in HCC patients. To verify the mechanisms, we repeated the animal experiments and showed that lung metastatic nodules were significantly reduced by TGF-β1 gene silencing. Moreover, TGF-β1 knockdown prolonged survival in a mouse lung metastasis model and decreased the tumorigenicity of cancer cells in situ in a hepatoma model. TGF-β1 knockdown may thus impair cancer cell proliferation in vivo, while TGF-β1-mediated immunosuppression is confirmed to play a major role in cancer development.

TGF-β1 plays a complex role in cancer progression [36, 37]. In the current study, cancer-derived TGF-β1 modulated the tumor microenvironment by inducing the production of Tregs. Cancer-derived TGF-β1 and Tregs may cooperatively contribute to HCC growth, invasion, and metastasis. Anti-TGF-β1 antibodies, anti-receptor antibodies, and small-molecule inhibitors targeting TGF-β receptor kinases have all been involved in preclinical trials [38, 39]. However, TGF-β1 is widely present in blood and tumor tissues and is thus difficult to neutralize completely. We also used a TGF-β receptor I inhibitor in a mouse model, but this failed to reduce metastatic nodules (data not show). These results indicated that blocking the downstream pathway did not effectively restore TGF-β1 immunosuppression. It is possible that the upstream regulatory signals of TGF-β1 in cancer cells may represent a more effective way of impairing TGF-β1-mediated immunosuppression. Further studies are planned to investigate this possibility.

In conclusion, Tregs may reflect internal immunosuppression in HCC patients and may act as prognostic factors for both OS and DFS. Cancer-cell-secreted TGF-β1 modulates the tumor microenvironment by increasing Tregs, and silencing TGF-β1 prolonged survival in a mouse model. These findings suggest that TGF-β1 might be a potential immunotherapeutic target for the treatment of HCC, and support the need for further studies.

**Abbreviations**

- CFSE (Carboxyfluorescein succinimidyl ester); DFS (Disease free survival); ELISA (Enzyme-linked immunosorbent assay); Foxp3 (Transcription factor forkhead box P3); HCC (Hepatocellular carcinoma cells); IL-2 (Interleukin-2); OS (Overall survival); TGF-β1 (Transforming growth factor-β1); TILs (Tumor-infiltrating leukocytes); Tregs (Regulatory T cells); 2-ME (2-Mercaptoethanol).

**Acknowledgments**

The authors would like to thank the team members of Prof. Shen, Xizhong’s laboratory for their helpful discussion and critical reading of the manuscript.

Grant sponsor: Shanghai Science and Technology Commission; Grant numbers: 10410709400; Grant sponsor: National Nature Science Foundation of China; Grant numbers: No. 81272388.

**Disclosure Statement**

The authors declare that there are no conflicts of interest.
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