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Elevated Levels of Plasma Transforming Growth Factor-β in Patients with Hepatocellular Carcinoma

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We measured the plasma transforming growth factor-β (TGF-β) concentration in 14 patients with human hepatocellular carcinoma (HCC) and 9 age-matched normal subjects using growth inhibition assay of mink lung epithelial cells. The calculated plasma TGF-β concentration in the patients with HCC was 28.6±27.9 ng/ml (mean±SE), showing significant elevation compared with that in 9 normal subjects (5.3±3.3 ng/ml, $P<0.01$). In three cases, we could measure plasma TGF-β levels before and after their treatment for HCC. The plasma TGF-β levels decreased from 59.0 to 18.2 ng/ml after hepatic resection in one case, and from 24.0 to 10.7 ng/ml and from 12.4 to 3.4 ng/ml after transhepatic arterial embolization in the other two cases. These data indicate that plasma TGF-β level is elevated in patients with HCC, probably due to release from HCC tissues.

Key words: Transforming growth factor-β — Plasma concentration — Bioassay — Hepatocellular carcinoma

Transforming growth factor-β (TGF-β) was first described as a polypeptide promoting transforming activity of non-tumorigenic cells.[1] Subsequent studies have demonstrated a variety of biological functions of TGF-β, for example, regulating cell growth[2-4] and differentiation,[5] stimulating extracellular matrix production[6,7] and suppressing immunoreactions.[8,9] This multifunctional polypeptide is widely distributed in normal tissues including platelets,[10] bone[11] and placenta.[12] However, overproduction of TGF-β in many malignant cell lines[13] and enhanced expression of TGF-β gene in some neoplastic tissues[14,15] have been reported. In our previous study, we demonstrated elevated levels of TGF-β1 mRNA and its polypeptide in human hepatocellular carcinoma (HCC) tissue.[16,17] On the basis of these facts, our interest was focused on the levels of plasma TGF-β in patients with malignant tumors. However, there has been no report on measurement of plasma TGF-β levels. In this paper, we measured plasma TGF-β concentration in normal subjects and patients with HCC to clarify whether the plasma level is increased in the patients.

Fourteen patients with HCC (11 males and 3 females, mean age 60±7.1 yr) and age-matched nine normal subjects (8 males and one female, mean age 56.7±8.8 yr) were included in this study. All patients were diagnosed as having HCC on the basis of their clinical features and images including ultrasonography, CT and angiography. Patients who had received any treatment for HCC before entry into this study, such as ethanol injection, transhepatic arterial empolization (TAE) and administration of anti-tumor agents, were excluded. All patients were negative for HBsAg but positive for HCV antibody. Underlying liver diseases were liver cirrhosis in 12 cases and chronic hepatitis in 2 cases.

Fasting blood samples were obtained from the medial cubital vein in EDTA-2Na-containing tubes and centrifuged at 2,500 rpm at 4°C for 20 min. Additional centrifugation of the supernatant at 10,000 rpm at 4°C for 20 min was done to eliminate platelets. The following acid/ethanol extraction of TGF-β from the supernatant was a minor modification of the method of Roberts *et al.*[18] Because acid/ethanol converts the latent form of TGF-β to the active form, TGF-β recovered from plasma by this method is regarded as being in the active form. Exactly 2.5 ml of plasma was mixed with 5 ml of a solution consisting of 375 ml of 95% ethanol and 7.5 ml of concentrated HCl, plus 33 mg of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and 1.9 mg of pepstatin (Sigma Chemical Co.) as protease inhibitors. After overnight extraction at 4°C, the mixture was centrifuged at 10,000 rpm for 60 min. Exactly 2.5 ml of the supernatant was kept in the acidic range by adjustment to pH 5.2 with concentrated ammonium hydroxide followed by the addition of 30 μl of 2 *M* ammonium acetate buffer, pH 5.3. Six volumes of cold ethanol/ether (2:1) were immediately added, after which the mixture was allowed to stand at −20°C for 48 h. The resulting precipitate was collected by centrifugation at 2,500 rpm

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for 10 min, and redissolved in 5 ml of 1 *M* acetic acid. After dialysis at 4°C for 48 h against 0.17 *M* acetic acid (Spectrapor tubing, molecular weight cut-off 3,500, Spectrum Medical Industries, Los Angeles, CA), the solution was lyophilized and stored at −20°C until assay. The lyophilized samples were dissolved in phosphate-buffered saline containing 5 m*M* HCl and 0.2% bovine serum albumin (solution A) before assay.

We employed growth inhibition assay of mink lung epithelial cells (Mv1Lu) for quantification of TGF-β content, since this bioassay is reproducible and sensitive[19] even at concentrations of 10^{-2} ng/ml order. Mv1Lu cells were seeded at the cell density of 3×10^3/well in 96-well plates and allowed to grow at 37°C for 24 h in RPMI supplemented with 10% fetal calf serum (FCS). Then, the medium was changed to fresh RPMI containing 2% FCS. Serially diluted recombinant human TGF-β1 (Takara Biochemicals, Kyoto) at a concentration from 0 to 0.5 ng/ml in solution A for the standard curve or serially diluted samples (×10, ×100 and ×1000) were added at 100 μl per well. After incubation for 24 h at 37°C, 10 μl (37 kBq) of [^{3}H]thymidine was added per well and incubation was continued for 5 h at 37°C. Cells were harvested and [^{3}H]thymidine incorporated into DNA was counted by using a liquid scintillation counter. The TGF-β content in the samples was determined from the standard curve by extrapolation of the values counted. Curves from serially diluted samples showed good parallelism with the standard curve, indicating reliability of this assay. That the growth inhibitory activity was due to TGF-β was verified by showing that it could be neutralized by an antibody against TGF-β, J069 (R&D Systems) (data not shown). The plasma TGF-β concentration was calculated from the extrapolated value.

The mean of plasma TGF-β concentration in nine normal subjects was 5.3±3.3 ng/ml (mean±SE, range from 1.1 to 10.1, Fig. 1). On the other hand, the mean of plasma TGF-β levels in fourteen patients with HCC was 28.6±27.9 ng/ml (range from 7.5 to 116.4, Fig. 1). This elevation of plasma TGF-β concentration in the patients with HCC compared with that in 9 normal subjects was statistically significant ($P<0.01$). In 11 of the 14 patients (78.6%) with HCC, the plasma TGF-β level exceeded

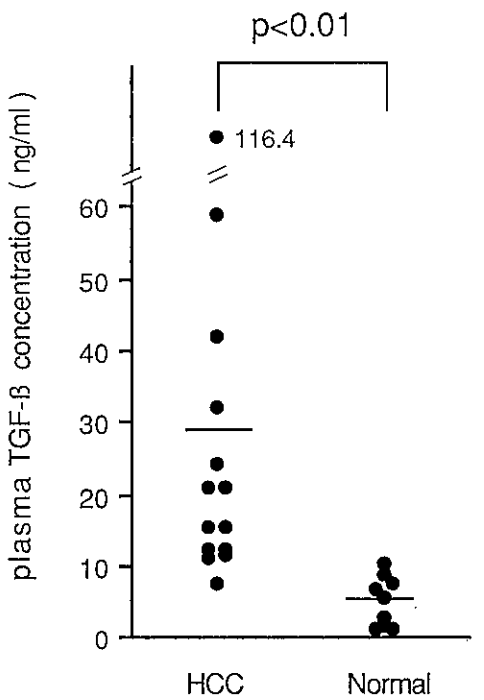

Fig. 1. Comparison of plasma TGF-β concentration in patients with HCC and normal subjects. Plasma TGF-β was acid/ethanol extracted and its concentration was determined by growth inhibition assay using Mv1Lu cells as described in the text. The Wilcoxon rank sum test was used for statistical analysis.

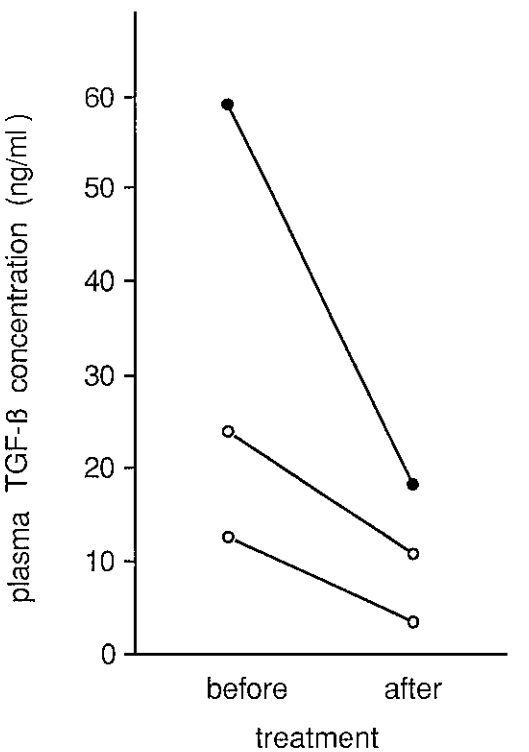

Fig. 2. Effect of treatment for HCC on the levels of plasma TGF-β concentration in three patients. Plasma TGF-β levels decreased in the three cases who were treated with hepatic resection (●) or TAE (○).

11.9 ng/ml (mean+2 SE of normal subjects). This implies that the presence of HCC is related to the high levels of plasma TGF-β. To elucidate whether the presence of HCC increases the plasma levels of TGF-β, we observed the changes in plasma TGF-β levels before and after treatment for HCC in three patients, one of whom underwent hepatic resection and the remaining two, TAE (Fig. 2). The post-treatment plasma samples were obtained one month after the therapy to avoid direct influence of the procedures on the levels of TGF-β. The plasma TGF-β levels decreased from 59.0 ng/ml to 18.2 in the patient given hepatic resection, and from 24.0 ng/ml to 10.7 and 12.4 ng/ml to 3.4 in the two treated with TAE. The marked reduction of the plasma levels strongly suggested that the presence of HCC caused the elevation of plasma TGF-β.

In the previous study, we demonstrated high levels of TGF-β1 mRNA and TGF-β protein expression in human HCC tissues. Taken together, the data suggest that the bulk of the elevated plasma TGF-β in patients with HCC is likely to be derived from their HCC tissues. However, it is necessary to take into consideration the presence of underlying liver diseases such as chronic hepatitis and liver cirrhosis. Since TGF-β content is known to increase in hepatic tissues with inflammation and fibrosis,[20-23] the increased plasma TGF-β in the patients with HCC may be derived in part from underlying liver diseases.

In conclusion, we determined the plasma TGF-β concentration for the first time in patients with HCC and normal subjects. The plasma TGF-β level in the patients with HCC was significantly elevated compared with that in the normal subjects. The elevated levels of plasma TGF-β were decreased by the treatment for HCC in three patients, indicating that most of the elevated TGF-β is derived from HCC tissue.

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