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Functional expression of TWEAK in human hepatocellular carcinoma: possible implication in cell proliferation and tumor angiogenesis☆

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Abstract

TNF-like weak inducer of apoptosis (TWEAK) is a member of the TNF family whose transcripts are expressed in various human tissues. Since TWEAK has a variety of biological activities, we investigated TWEAK sensitivity, expression, and physiological role in human hepatocellular carcinomas (HCCs). Tweak receptor was detected in four kinds of HCC cells. TWEAK significantly promoted cell proliferation and induced nuclear factor-κB activation in all HCC cells. Surprisingly, we found that HCC cells constitutively express TWEAK. In addition, soluble TWEAK was detected in culture medium. We found that TWEAK also promotes cell proliferation and induces the secretion of IL-8 and MCP-1 in human umbilical vein endothelial cell. Finally, culture medium from Sh-Hep1 cells incubated with anti-TWEAK antibody significantly inhibited endothelial cell tube formation. In conclusion, these results indicate that TWEAK might play a critical role in HCC cellular proliferation using both autocrine and paracrine mechanisms, and modulate tumor-related angiogenesis.

The members of the tumor necrosis factor (TNF) family play pivotal roles in the regulation of host defense and immune system by inducing apoptosis, proliferation or differentiation of cells, and they are involved in the pathogenesis of various diseases [1–4].

TNF-like weak inducer of apoptosis (TWEAK) is a newly identified death-inducing ligand belonging to the TNF family. TWEAK was identified as a membrane-anchored protein, the gene encoding a 249-amino acid protein [5]. Like TNF, LTα, and Fas ligand (FasL), TWEAK is proteolytically processed into a soluble form released from the cells, suggesting a bystander function as well as local signaling. Tweak transcripts are abundant and found in many human tissues, with particularly highly expression in heart, brain, skeletal muscle, and pancreas [5]. These expression patterns differ from those of FasL, TNF, and CD40 ligand, and resemble the TNF-related apoptosis-inducing ligand (TRAIL) [6], suggesting that TWEAK has a more constitutive function.

TWEAK is expressed on human monocytes upon IFN-γ stimulation [7] and is involved in cytotoxicity of TWEAK-sensitive tumor cells, such as Kym-1 rhabdomyosarcoma cells [8] and IFN-γ-treated HT-29 colon carcinoma cells [9]. In addition, a recent report indicated that patients with systemic lupus erythematosus show an increase in expression of TRAIL, TWEAK, and FasL in T cells, which mediate autologous monocyte death [10].

☆ *Abbreviations:* TNF, tumor necrosis factor; TWEAK, TNF-like weak inducer of apoptosis; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; EC, endothelial cell; TweakR, TWEAK receptor; HUVEC, human umbilical vein endothelial cell; Fn14, fibroblast growth factor-inducible factor 14; HCC, hepatocellular carcinoma; ActD, actinomycin D; CHX, cycloheximide; IFNγ, interferon γ; NF-κB, nuclear factor-κB; MCP-1, monocyte chemotactic protein-1; TNFR, TNF receptor; VEGF, vascular endothelial growth factor.

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In contrast, TWEAK promotes cell proliferation in some cell types, such as human vascular endothelial cell (EC) and smooth muscle cell [11,12]. However, expression of TWEAK protein and its physiological role in cancer cells remain unknown.

TWEAK receptor (TweakR) was first identified using an expression cloning panning approach from a human umbilical vein endothelial cell (HUVEC) library [12,13]. DNA sequence analysis revealed that TweakR was identical to human fibroblast growth factor-inducible factor 14 (Fn14) [14,15]. TweakR/Fn14/Fn14 mRNA was detected in a variety of adult tissues, with relatively high levels found in heart, kidney, lung, and aorta and human vascular EC and SMC [12,14–16]. Furthermore, previous reports suggested that TWEAK–TweakR/Fn14 cross-linking may be implicated in angiogenesis [12,17,18]. These findings led to the notion that TWEAK signaling is involved in the development of malignant cells. In the present study, we therefore investigated the expression of TWEAK and TweakR/Fn14 and potential biological functions of TWEAK in HCC cells.

Materials and methods

Cell lines and HCC tissues. The human umbilical vein endothelial cells, HUVECs, the human HCC cell lines, HepG2 and SK-Hep1 cells were purchased from American Type Culture Collection. The HCC cell lines, Huh7 and HLE, were purchased from the Health Science Research Resources Bank (Osaka, Japan). HCC cell lines were cultured in Dulbecco's modified Eagle's medium at 37 °C. All media were supplemented with 1% penicillin/streptomycin (Gibco-BRL) and 10% heat-inactivated fetal calf serum (Gibco-BRL). Human HCC tissues, cirrhotic liver (5 cases), and chronic hepatitis (5 cases) were obtained from surgical resection or biopsy for immunohistochemical analysis. We obtained informed consent from all patients for subsequent use of their tissues.

Detection of TWEAK. Total RNA of HCC cells was isolated by Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX) according to the manufacturer's protocol. Complementary DNA was synthesized by extension of $(dT)_{12-18}$ primers with 200 U of SuperScript II reverse transcriptase (Gibco-BRL) in a mixture containing 2 μg of total RNA for 60 min at 37 °C. PCR of the complementary DNA was performed in a final volume of 50 μL containing all four dNTPs (each at 200 μmol/L), 2.5 mmol/L $MgCl_2$, 2.5 U Ex Taq (Takara Shuzo, Kyoto, Japan), and each primer at 0.4 μmol/L. The amplification cycles were 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 2 min for 35 cycles. The PCR-amplified products were run on a 1% agarose gel containing ethidium bromide and visualized under ultraviolet light. The sequences for the relevant primers were as follows: TWEAK sense primer is 5′-CCCATGGCCGCC CGTCGGAG-3′ and TWEAK antisense primer is 5′-GGGCCAACA GCCCAGACACC-3′. β-actin sense primer is 5′-ATGGATGATGAT ATCGCCGCG-3′ and β-actin antisense primer is 5′-CTAGAAGCATT TGCGGTGGACGATGGAGGGGCC-3′.

Expression of TWEAK in HCC cell lines was also analyzed by Western blotting. Briefly, cells were harvested and lysed in lysis buffer (50 mmol/L Tris–HCl, pH 8, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% NP-40, and 1 mmol/L phenylmethylsulfonyl fluoride) on ice. After centrifugation, supernatants were collected, equal amounts of protein from each extract were separated by 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes (Toyo Roshi, Tokyo, Japan) using the Bio-Rad electrotransfer system (Bio-Rad Laboratories). Blots were probed overnight at 4 °C with rabbit anti-TWEAK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-TWEAK polyclonal antibody absorbed with amino acids 1–249 representing full-length TWEAK of human origin. The immunoblots were then probed with horseradish peroxidase-conjugated anti-rabbit immunoglobulin. After the final wash, signal was detected with an ECL kit (Amersham–Pharmacia Biotech, Buckinghamshire, UK).

Immunohistochemical staining for TWEAK. Briefly, deparaffinized sections were heated for 10 min at 120 °C in a pressure cooker to reactivate the antigen and treated with 0.3% H_2O_2 in methanol for 20 min to abolish endogenous peroxidase activity. Sections were blocked with normal goat serum or normal rabbit serum in phosphate-buffered saline, and incubated with a 1:40 dilution of rabbit anti-TWEAK polyclonal antibody overnight at 4 °C. The sections were incubated with a second biotinylated antibody, followed up by avidin–biotin–peroxidase complex. After washing, they were developed in a substrate solution of 0.01% 3,3′-diaminobenzidene-hydrogen peroxide and counterstained with hematoxylin.

Detection of proliferation. To assess the viability of HCC cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. The HCC cells were plated at a density of 1×10^4 cells/well in 96-well microtiter plates (Corning Glass Works, Corning, NY) and each plate was incubated for 24 h at 37 °C in 5% CO_2. Recombinant human TWEAK (BioVision, Mountain View, CA) (50 μL) was added in the absence or presence of 0.5 μg/ml actinomycin D (ActD) (Sigma), 2.0 μg/ml cycloheximide (CHX) (Sigma) or 20 ng/ml interferon γ (IFNγ) (Sigma), and the plates were incubated for 24 h.

NF-κB luciferase reporter gene assay. The pNF-κB-Luc Vector (Mercury Pathway Profiling System) was obtained from Clontech (San Diego, CA). Human HCC cells (2×10^5) were grown in six-well plates in triplicate the day before transfection. Cells were transfected using FuGENE 6 (Boehringer–Mannheim) and incubated for 18 h at 37 °C. Luciferase activity was determined from cell extracts by means of a luciferase assay system (Promega) and a luminometer (Berthold Analytical Instruments, Nashua, NH).

ELISA for TWEAK. The amount of secreted TWEAK in the culture medium of the 4 HCC cell lines was measured by ELISA. The culture supernatant was collected, centrifuged for 10 min, and the obtained supernatant was used for experiments. Anti-TWEAK polyclonal antibody (PEPRORECH EC, London, UK) was diluted with phosphate-buffered saline (PBS) to a concentration of 0.5 μg/ml and 100 μl was added to each ELISA microplate (Nunc Maxisorp) well for coating. Recombinant TWEAK was used as a reference standard and was serially diluted from 10 ng/ml to 0. 0.25 μg/ml biotinylated anti-TWEAK antibody was added to each well and incubated at room temperature for 2 h. After washing, bound antibody was detected with avidin peroxidase and 2,2′-AZINO-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma). Color development was measured at 405 nm with a microtiter plate reader (Bio-Rad Laboratories).

Flow cytometric analysis. Approximately 1×10^6 cells were washed PBS, harvested with 0.02% EDTA in PBS, and incubated for 30 min on ice with phycoerythrin (PE)-conjugated anti-human TWEAK (eBioscience, San Diego, CA), PE-conjugated anti-mouse and human TweakR/Fn14 (eBioscience) or isotype control PE-conjugated mouse IgG (eBioscience) at a concentration of 5 μg/ml. The cells were analyzed by flow cytometry using a FACScan cytometer and CellQuest software (Becton–Dickinson, Tokyo, Japan).

Angiogenesis assay. Matrigel (BD Biosciences, MA) was placed in eight-well Lab-tek II chamber slide (NUNC Brand Products, Denmark) and allowed to set at 37 °C for 30 min. Then 2×10^4 HUVECs were added to each well and incubated in the culture medium of SK-Hep1 with rabbit IgG or anti-TWEAK polyclonal antibody at 37 °C for 4 h under a 5% CO_2 atmosphere. Tube formation was observed by light microscopy (Olympus, Tokyo, Japan).

Results

Cell surface expression of TweakR/Fn14 in HCC cells

To investigate the biological function of TWEAK on HCC cells, we initially investigated the expression of TweakR/Fn14 antigen on the cell surface of HCC cells using flow cytometry (data not shown). All four cell lines examined were reactive with anti-TweakR/Fn14 antibody. The cell surface expression of TweakR/Fn14 was considerably higher in SK-Hep1 cells compared to the others.

TWEAK-induced cell proliferation in HCC cells

We investigated TWEAK-induced apoptosis in HCC cells because some transformed cell lines are sensitive to TWEAK-induced apoptosis. However, up to 100 ng/ml TWEAK failed to induce significant cytotoxicity in any of the HCC cells. With the exception of HLE cells, metabolic inhibitors, such as actinomycin D or cycloheximide, did not sensitize the HCC cells to TWEAK-induce apoptosis. In HLE cells, up to 40% TWEAK-induced cell death was observed in the presence of these metabolic inhibitors in a dose-dependent manner (Fig. 1). IFNγ had little effect on the viability of the HCC cells. In contrast, the proliferation of all HCC cell lines was augmented with TWEAK in a dose-dependent manner (Fig. 1).

TWEAK-induced NF-κB activation in HCC cells

Since all human HCC cell lines demonstrated resistance to TWEAK-mediated apoptosis, we investigated TWEAK pathways that may rely on NF-κB activation. As shown in Fig. 2, TWEAK-induced NF-κB activation was increased significantly in HLE and Huh7 cells in a dose-dependent manner. NF-κB activation in HepG2 and SK-Hep1 was weaker. These data demonstrate that TWEAK induces pathways leading to NF-κB activation in HCC cells. We failed to find a definitive relationship between the level of NF-κB induction and expression of TweakR/Fn14 (data not shown).

Expression of TWEAK in HCC cells

Next, expression of TWEAK transcripts in HCC cells was done using RT-PCR. Surprisingly, as shown in Fig. 3A, a 654-bp RT-PCR fragment of TWEAK was detected in all human HCC cell lines tested. Next expression of TWEAK at the protein level was analyzed using Western blotting. Bands corresponding to the expected size of TWEAK were observed in all HCC cells (Fig. 3B). Bands corresponding to the 30 kDa unprocessed form and the 18 kDa processed form of TWEAK were observed in all HCC cells. The larger 30 kDa form is thought to be the transmembrane form of TWEAK,

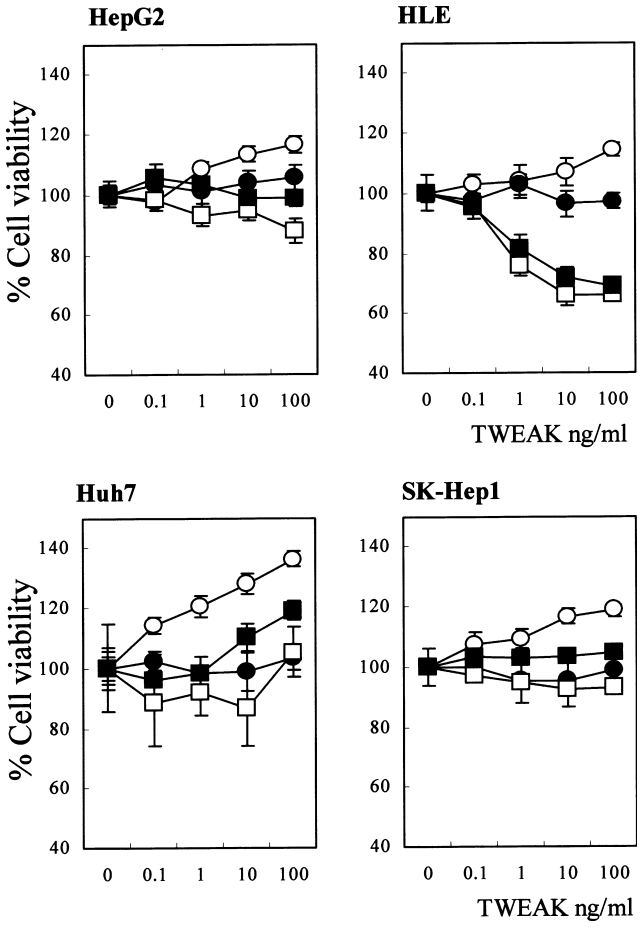

Fig. 1. Effects of rhTWEAK on cell viability of human HCC cells. HCC cells were incubated with the indicated concentrations of rhTWEAK alone (open circle) or in combination with 20 ng/ml interferon γ (closed circle), 0.5 μg/ml actinomycin D (open square) or 2.0 μg/ml cycloheximide (closed square) for 24 h. The cell count was analyzed by MTT assay. The data shown are means ± SD of eight independent experiments.

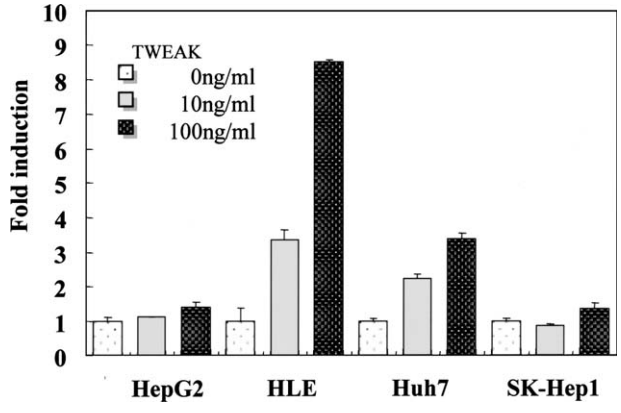

Fig. 2. TWEAK-induced nuclear factor-κB (NF-κB) activation in HCC cells. Cells were transfected with the pNF-κB-κLuc vector. NF-κB activation was analyzed using an NF-κB-luciferase reporter gene assay. The results are presented as fold induction of the luciferase activity observed in cells incubated with the indicated concentration of TWEAK for 24 h compared with that of cells without TWEAK. The data shown are means ± SD of three independent experiments.

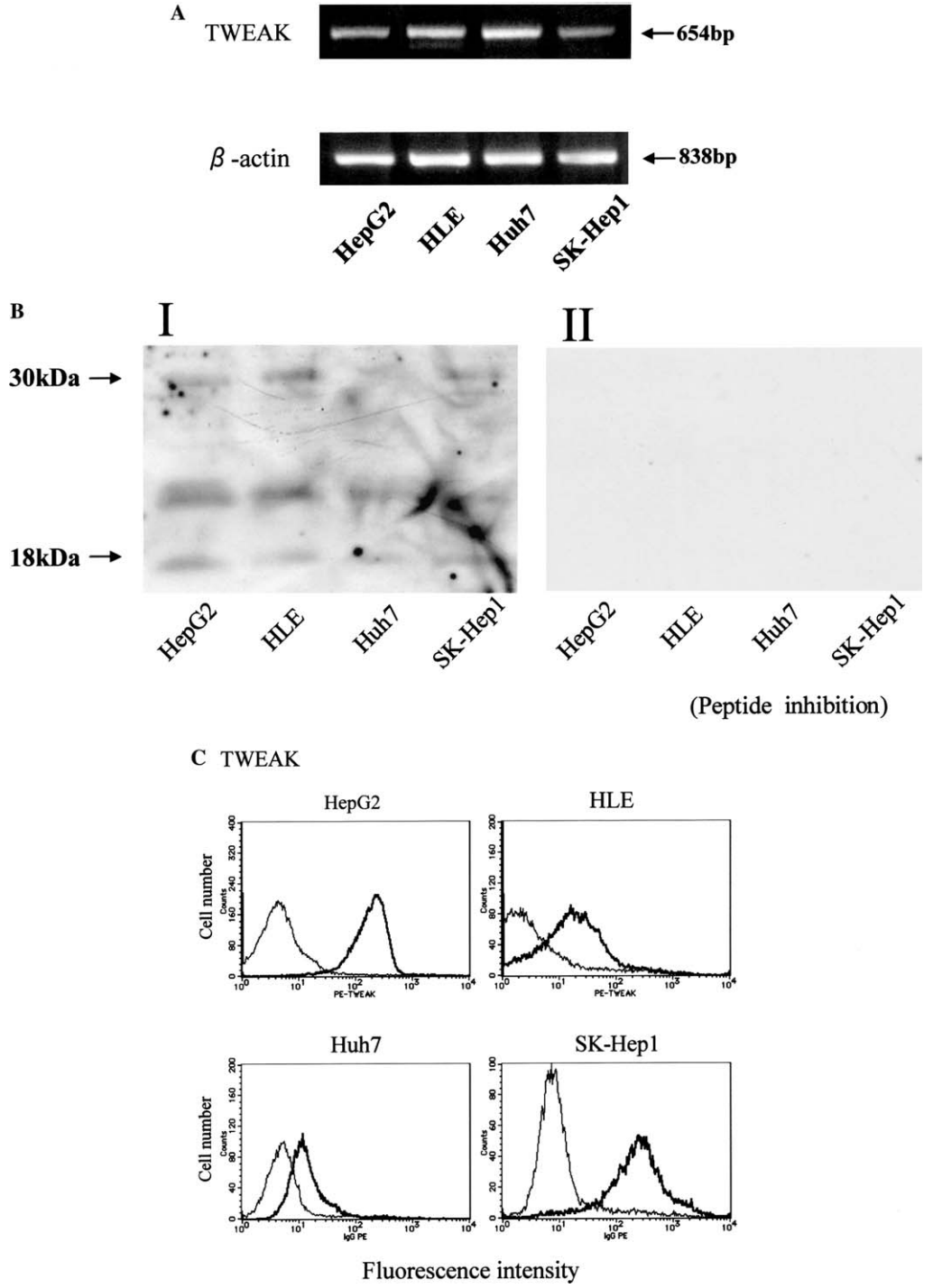

Fig. 3. (A) Detection of TWEAK transcripts in human HCC cell lines by RT-PCR. A 413 bp human TWEAK sequence and an 838-bp β-actin sequence were amplified from total RNA, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining. (B) TWEAK expression in human HCC cell lines by Western blotting with (I) anti-TWEAK antibody, (II) anti-TWEAK antibody absorbed with the TWEAK peptide (control) (original magnification 200×). Upper arrowhead indicates the expression of transmembrane form of TWEAK (30 kDa) and lower arrowhead indicates the soluble form of TWEAK (18 kDa). (C) Cell surface expression of TWEAK on HCC cell lines. The cells were stained with PE-anti-human TWEAK (thick line). Autofluorescence was determined with cells incubated with PE-control mouse IgG (thin lines). Similar results were obtained in four independent experiments.

and the smaller 18 kDa may be the soluble form of TWEAK. Another band of intermediate cleavage products was observed between these bands.

Expression of TWEAK antigen on the cell surface of HCC cells was investigated using flow cytometry (Fig. 3C). All four cell lines examined were reactive with

Table 1
Summary of immunohistochemical study for TWEAK

Tissues		Positive staining (%)
HCC ($n = 64$)	21	32.8*
Liver ($n = 51$)	15	29.4
Well-diff HCC ($n = 14$)	4	28.6
Mod-diff HCC ($n = 27$)	9	33.3
Poorly diff HCC ($n = 9$)	2	22.2
Metastasis ($n = 13$)	6	46.1
Cirrhosis ($n = 5$)	0	0
Chronic hepatitis ($n = 5$)	0	0

* $P < 0.05$ vs. cirrhosis and chronic hepatitis.

anti-TWEAK antibody. The highest surface expression of TWEAK was observed on HepG2 and SK-Hep1 cells.

Then, 64 HCC tissues, five samples of cirrhotic liver, and five liver tissues from patients with chronic hepatitis were examined for TWEAK by immunohistochemistry. The results are summarized in Table 1. In HCC tissues, 21 of 64 cases (32.8%) revealed staining for TWEAK. Expression of TWEAK was located mainly in the cytoplasm and membrane (Fig. 4). The positivity for TWEAK in metastatic HCC was relatively high comparing with that in HCC. In contrast, all the cirrhotic or hepatitis tissues were negative or only weakly stained for TWEAK expression, except for lymphocytes in the portal areas (Fig. 4).

Expression of soluble TWEAK in culture medium

Detection of soluble TWEAK from HCC cells was done on conditioned media from the cells using a newly established ELISA. As shown in Fig. 5A, the expression of soluble TWEAK was observed in all culture medium of HCC cells for 72 h.

Effect of TWEAK on human umbilical vein endothelial cell

We analyzed cell proliferation effects of TWEAK on HUVECs because TweakR/Fn14 is known to be highly expressed in endothelial cells, smooth muscle cells, and aorta. As shown in Fig. 5B, the HUVEC cell count increased in a dose-dependent manner after 72 h incuba-

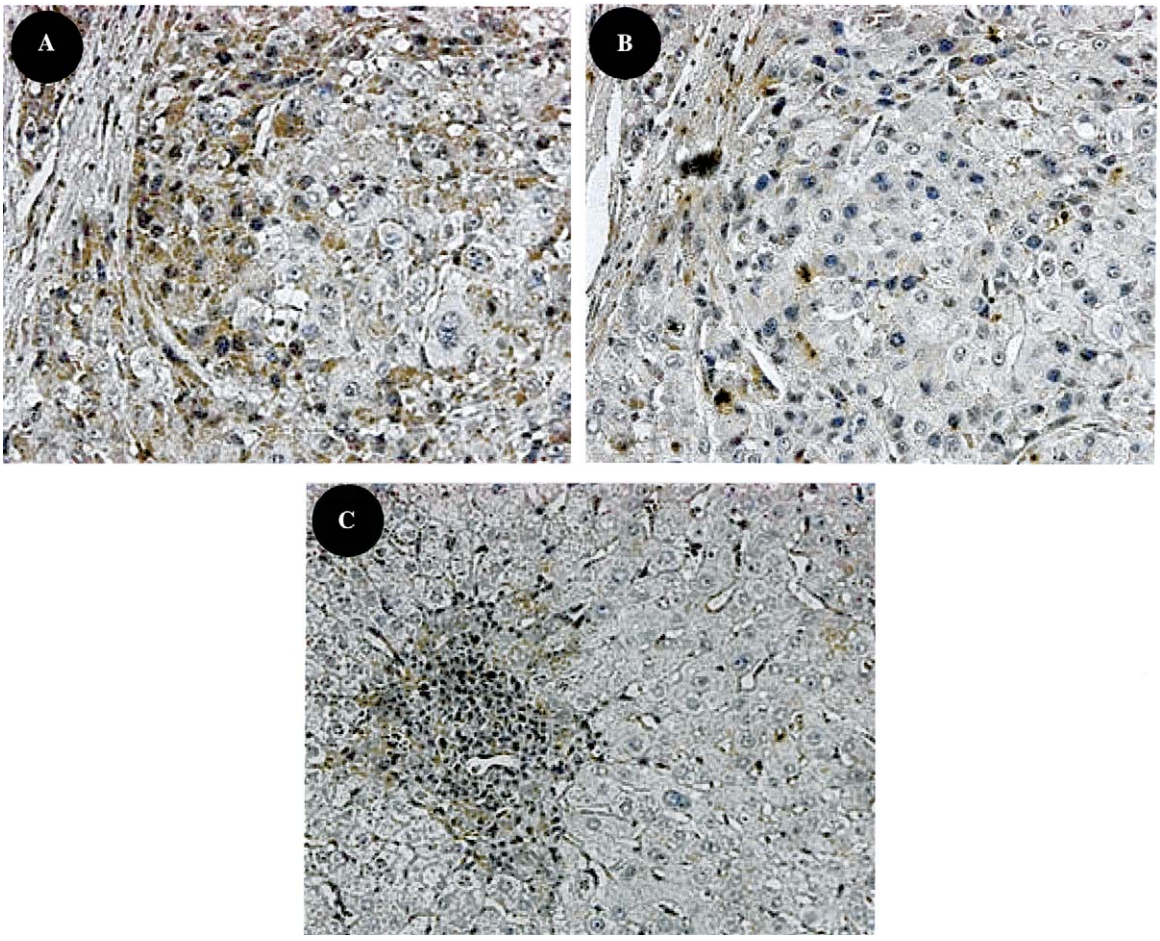

Fig. 4. Immunohistochemical staining of human HCC tissues with (A) anti-TWEAK antibody, (B) anti-TWEAK antibody absorbed with the TWEAK peptide (control) (original magnification 200×). Note that TWEAK staining was found mainly in the cytoplasm and membrane. In contrast, (C) non-tumor tissues were negative for TWEAK staining.

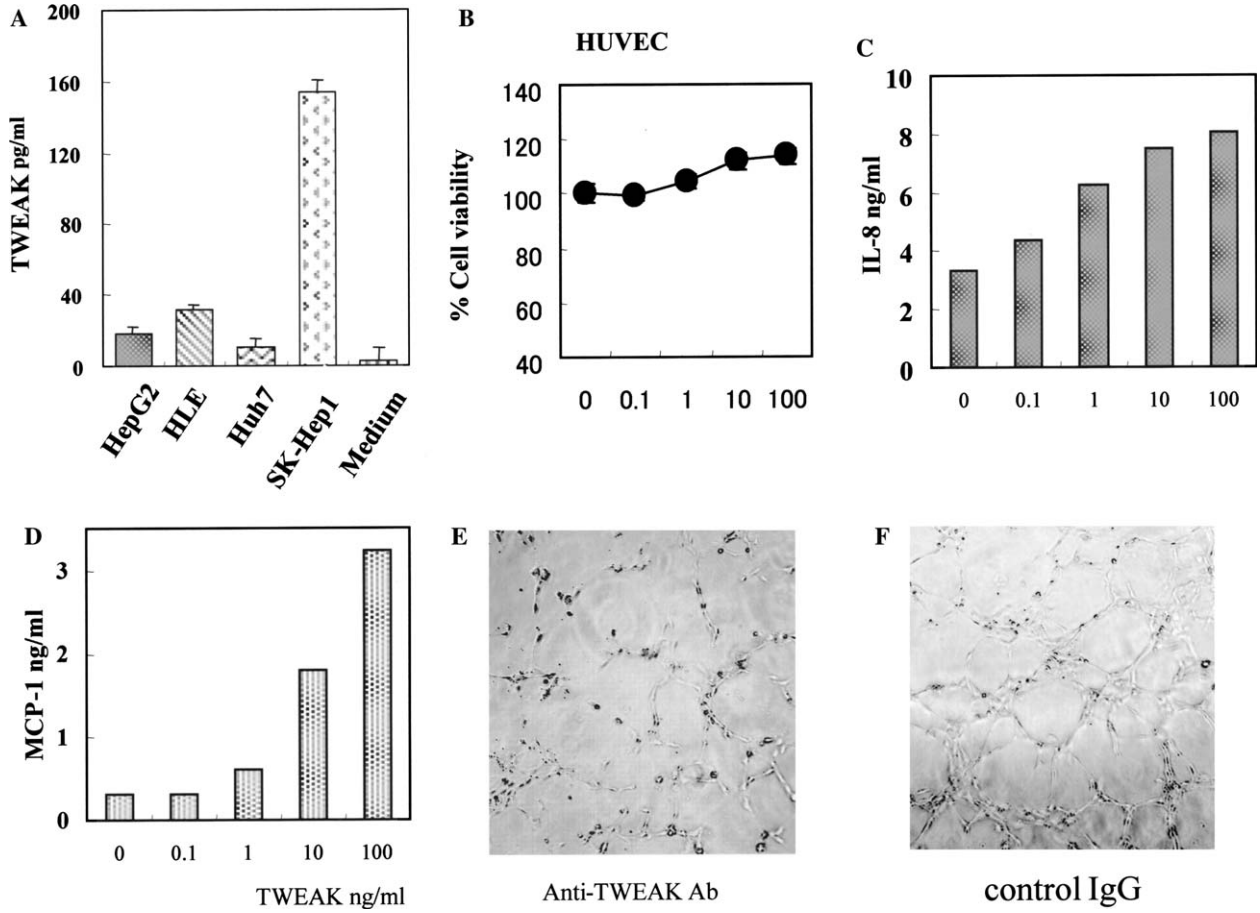

Fig. 5. The level of soluble TWEAK in the supernatants of HCC cells for 72 h was measured by ELISA. Soluble TWEAK was detected in all culture medium. The data shown are means ± SD of three independent experiments. (A) Effects of rhTWEAK on HUVEC viability, cytokine production, and tube formation. HUVECs were incubated with the indicated concentrations of rhTWEAK for 72 h. (B) Cell viability was determined by MTT assay. The data shown are means ± SD of eight independent experiments. The levels of IL-8 (C) and MCP-1 (D) in the supernatants were measured by ELISA. Endothelial cell tube formation on Matrigel is shown in the culture medium of SK-Hep1 incubated with (E) anti-TWEAK polyclonal antibody or (F) rabbit IgG for 4 h.

tion (Figs. 5C and D). TWEAK also induced secretion of IL-8 and monocyte chemotactic protein-1 (MCP-1). Finally, we investigated the effect of irrelevant rabbit IgG or anti-TWEAK polyclonal antibody and HCC conditioned media on HUVEC cell tube formation. Culture medium from SH-Hep1 cells incubated with anti-TWEAK polyclonal antibody significantly inhibited endothelial cell tube formation in Matrigel (Figs. 5E and F).

Discussion

TNF family members, such as FasL or TRAIL, induce apoptosis in a variety of human transformed cells and play an important role in the host's defense against tumor cells via T lymphocytes or macrophages [3,4, 19,20]. Most HCC cells show strong resistance to Fas and TRAIL receptor-mediated apoptosis, and have several mechanisms, such as down-regulation of death signaling component [21,22] or expression of anti-apoptotic proteins [23,24]. However, the resistance of HCC cells to FasL and TRAIL-induced apoptosis can be overcome by a subtoxic level of ActD, accompanied by decreased expression of caspase inhibitors [25].

The present study demonstrated that exogenous TWEAK could not efficiently induce apoptosis in HCC cells even in the presence of CHX or ActD. These results suggested that HCC cells are generally resistant to TWEAK and this is probably not due to the presence of anti-apoptotic proteins that may be sensitive to the metabolic inhibitors. One report indicated that TWEAK-induced apoptosis is indirect and is mediated by the interaction of endogenous TNF and TNF receptor (TNFR), since neutralizing TNFR efficiently antagonized cell death induction [8]. However, other reports demonstrated that TWEAK could directly induce apoptosis via caspase activation in HSC cells and IFNγ-treated HT-29 cells [9]. Our findings, together with previous findings, indicate that TWEAK has

cytotoxic activity toward some human tumor cells, but this function requires co-incubation with sensitizing agents in most cases, suggesting that TWEAK-induced cell death depends on multiple pathways and is cell-type dependent.

The current study showed that TWEAK promoted cell proliferation in a dose-dependent manner in all HCC cells examined. Other TNF family members such as FasL [26], TRAIL [25], and CD40 [27] ligand do not have this proliferation effect. This particular function of TWEAK was rather weak in colon cancer cells (K. Shiraki et al., unpublished data). Our data provide novel evidence that TWEAK function includes promotion of HCC cell proliferation, although the mechanism for this effect is not elucidated.

Only a few studies have investigated TweakR/Fn14 intracellular signal transduction pathways [12,16,28]. Our study revealed that TWEAK stimulation significantly induced NF-κB activation in two HCC cell lines, consistent with similar previous findings in human embryonic kidney 293 cells or NIH 3T3 fibroblast cells [16]. In HCC cells, we found that this activation did not correlate with the level of TWEAK-induced cell proliferation. Therefore, TweakR/Fn14 signaling pathways may be complex and cell-type dependent.

We further found that TweakR/Fn14 is strongly and constitutively expressed on the surface of HCC cells using flow cytometry. TweakR/Fn14 mRNA is expressed in a variety of adult tissues [12,14–16]. Although TweakR/Fn14 is expressed at a relatively low level in normal liver tissue, it is expressed at a high level in HCC specimens [14]. Furthermore, the murine TweakR/Fn14 gene is induced rapidly during liver regeneration in vivo [14]. These findings suggest that TweakR/Fn14 signaling may play an important role in hepatocyte growth control, differentiation, and liver neoplasia. Our observation that TweakR/Fn14 is functionally expressed on HCC cell surfaces and that TWEAK promotes cell proliferation supports these possible roles initiated by TWEAK–TweakR/Fn14 interaction.

We found TWEAK mRNA expressed in all HCC cells examined and protein expression was confirmed by Western blotting, flow cytometry, and ELISA, indicating that TWEAK was constitutively and abundantly expressed in HCC cells. We also found TWEAK expression in human HCC tissue sections. Our Western blotting analysis revealed both the full-length form and cleaved form, as well as other intermediate cleavage products, which was consistent with previous report [5]. Each HCC cell line demonstrated different expression patterns of these forms, although TWEAK is primarily a secreted cytokine-like TNF and LTα [5].

TWEAK is expressed in a variety of tissues including most organs and immune system, however, its expression in malignant cells has not been well characterized. FasL and TRAIL are also expressed on the surface of tumor cells by chemotherapeutic drugs and the cells subsequently induce apoptosis of co-cultured-Jurkat cells [24,29]. These results raised the possibility that tumor cells can induce peripheral deletion of tumor-reactive T cell clones via a Fas and TRAIL receptor signaling. TWEAK characteristics differ somewhat in that it is expressed at relatively high levels without drug or cytokine stimulation. Further study is to be needed to evaluate whether TWEAK expression on HCC cells might influence the tumor immunological environment.

TWEAK and TweakR/Fn14 appear to be co-expressed in HCC cells, indicating that cell surface TWEAK and released TWEAK from these cells may function in an autocrine and/or paracrine manner to influence cell proliferation. The fact that TweakR/Fn14 is highly expressed in human aorta, vascular ECs and SMC [12] supports the notion that the TWEAK-TweakR/Fn14 interaction contributes to angiogenesis. We also observed TWEAK-induced cell proliferation of HUVECs. In addition, tubular formation of HUVECs by culture media of HCC cells was inhibited by anti-TWEAK antibody, indicating that the soluble TWEAK released from the HCC cells was functional. These findings are consistent with previous reports that TWEAK induces proliferation and migration of human vascular EC cells and corneal angiogenesis in vivo [11,13]. TNF [30] and Fas ligand [31] have also previously been implicated in angiogenesis. However, these effects are mediated indirectly by the up-regulation of endothelial growth factor. In contrast, TWEAK may have a more direct effect on angiogenesis and endothelial cell proliferation independent of vascular endothelial growth factor [12,32,33]. In the present study, TWEAK also increased pro-inflammatory cytokines IL-8 and monocyte chemotactic protein-1 in HUVECs, suggesting that TWEAK may influence its immediate immunologic and angiogenic environment.

In conclusion, our study reveals that TWEAK is constitutively expressed on human HCC cells and HCC cells secrete a soluble, functional form of TWEAK. TWEAK in HCC cells may play an important role in cell proliferation and angiogenesis. Since HCC cells usually grow rapidly with hypervascular characteristics, these functions of TWEAK may be critically important for the tumor development. Further evaluation of these possible roles of TWEAK and the regulation of TWEAK expression in malignant cells is important for development of new strategies to control the growth of malignant cells accompanied by aggressive tumor-induced angiogenesis.

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