

Functional expression of a proliferation-related ligand in hepatocellular carcinoma and its implications for neovascularization

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Abstract

AIM: To detect the expression of a proliferation-related ligand on human hepatocellular carcinoma (HCC) cell lines (SK-Hep1, HLE and HepG2) and in culture medium.

METHODS: APRIL expression was analyzed by Western blotting in HCC cell lines. Effects of APRIL to cell count and angiogenesis were analyzed, too.

RESULTS: Recombinant human APRIL (rhAPRIL) increased cell viability of HepG2 cells and, in HUVEC, rhAPRIL provided slight tolerance to cell death from serum starvation. Soluble APRIL (sAPRIL) from HLE cells increased after serum starvation, but did not change in SK-Hep1 or HepG2 cells. These cells showed down-regulation of VEGF after incubation with anti-APRIL antibody. Furthermore, culture medium from the HCC cells treated with anti-APRIL antibody treatment inhibited tube formation of HUVECs.

CONCLUSION: Functional expression of APRIL might contribute to neovascularization via an upregulation of VEGF in HCC.

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Key words: A proliferation-inducing ligand; HCC; VEGF; Cell proliferation; Neovascularization

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INTRODUCTION

Cytokines regulate cellular proliferation and differentiation by binding to their specific receptors on target cells^[1]. Tumor necrosis factor (TNF) is the prototypic member of a family of cytokines playing an important role in immune regulation and cancer^[2,3].

A proliferation-inducing ligand (APRIL), a new member of the TNF family, is reported to stimulate tumor cell growth^[4-6], modulate tumor cell apoptosis^[7-9], or activate nuclear factor-kappa B (NF- κ B)^[10,11]. APRIL also is associated with regulation of humoral immunity^[6,12]. APRIL is a type II membrane protein, which is typical of the TNF ligand family member^[4]. The sequence of the extracellular domain of APRIL shows homology with FasL, TNF α , LT β , TRAIL, TWEAK, and TRANCE^[4]. In particular, APRIL shares significant homology with B-lymphocyte stimulator (BLys)^[13]. Though the TNF ligand family is synthesized into membrane-bound proteins, of which several are cleaved into a soluble form, APRIL is processed intracellularly and secreted from the cell surface^[14].

The expression of APRIL mRNA or protein has been detected in many tumor cells and tissues^[4,5,7], but is almost undetectable in normal tissues^[5]. Hence, APRIL may provide a significant growth advantage to malignant cells. Because soluble APRIL can stimulate tumor cell growth^[1], APRIL may provide its signal in an autocrine and/or paracrine mode^[5].

APRIL binds to two TNF receptor families, transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA) with relatively high affinity^[6,10,15]. It is clear that TACI and BCMA intracellular signaling is similar to that of other TNF receptor homologs without a death domain^[16,17]. However, the signaling transduction pathway triggered by APRIL that prolongs lymphocyte survival is still not characterized^[18]. A soluble form of BCMA inhibits APRIL activity and decreases tumor cell proliferation^[19]. NZB/WF1 mice, which develop chronic autoimmune disease, can inhibit development of proteinuria and prolongation of survival after being treated with soluble TACI-Ig fusion protein treatment^[20]. These observations imply the importance of APRIL via these receptors with tumor cell or lymphocyte survival. However, in Jurkat cells, APRIL induces cell death and binds to other TNF family receptors^[7]. An additional APRIL-specific receptor expressed on cell surfaces other than TACI or BCMA, is implied adenocarcinoma or fibroblast tumor cells lacking any detectable BCMA or TACI expression even though

these cells respond to growth stimulatory effects of APRIL^[5,19]. However, the function of APRIL in tumor cells is not well elucidated.

Therefore, we investigated the function of APRIL in HCC cell lines and human umbilical vein endothelial cells (HUVEC). We further investigated the expression of APRIL in HCC cells and evaluated the effects of APRIL on neovascularization.

MATERIALS AND METHODS

Cell lines and reagents

Human umbilical vein endothelial cells (HUVECs), and human HCC cell lines, HepG2 and SK-Hep1 cells were purchased from American Type Culture Collection (Rockville, MD, USA). Human HCC cell line, HLE was purchased from the Health Science Research Resource Bank (Osaka, Japan). HCC cell lines were cultured in Dulbecco's modified Eagle's medium (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) at 37 °C. All media were supplemented with 1% penicillin/streptomycin (GIBCO BRL, Grand Island, NY, USA) and 10% heat-inactivated fetal calf serum (GIBCO BRL). HUVECs were cultured in HuMedia-EG2 (KURABO, Osaka, Japan). Recombinant human APRIL (rhAPRIL) was purchased from ALEXIS Biochemicals (Switzerland). Anti-APRIL polyclonal antibody was purchased from Ψ ProSci Incorporated (Poway, CA, USA). Anti-vascular endothelial growth factor (VEGF) mAb and anti- α -tubulin mAb were purchased from Oncogene Research Products (Boston, MA, USA). Normal rabbit IgG was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Recombinant human VEGF (rhVEGF) was purchased from Peprotech EC Ltd. (London, UK).

Assessment of count of HCC cells and HUVECs

To assess the viability of HCC cells and HUVECs, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. The cells were plated at a density of 5×10^3 cells/well in 96-well microtiter plates (Corning Glass Works, Corning, NY, USA) and each plate was incubated for 24 h at 37 °C in 50 mL/L CO₂. Reagents were added and the plates were incubated for the indicated time. The live-cell count was determined using a Cell Titer 96 assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The absorbance of each well was measured at 570 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting

Expression of APRIL was analyzed by Western blotting. HCC cells were harvested and lysed in lysis buffer (50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP-40, 1 mmol/L phenylmethyl-sulfonyl fluoride) on ice. Protein content was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories). 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). Blots were blocked by incubation in 5% non-fat dried milk in Tris buffered saline (TBS)

overnight at 4 °C and probed for 2 h at room temperature with primary antibody. The immunoblots were then probed with horseradish peroxidase-conjugated immunoglobulin (Ig) G (1:2 000 diluted with 5% non-fat dried milk in Tris-HCl; pH 7.5 and 0.05% Tween 20). Signal was detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Angiogenesis assay

Matrigel (BD Biosciences, MA) was placed in an 8-well Lab-tek II chamber slide (NUNC™ Brand Products, Denmark) (100 μ L/well) and allowed to set at 37 °C for 30 min. HUVECs were added to each well (4×10^4) and incubated in the culture medium of HCC cells with rabbit IgG or anti-APRIL polyclonal antibody at 37 \times for 6 h in a 50 mL/L CO₂ atmosphere. Tube formation was observed by microscopy (OLYMPUS, Tokyo, Japan).

RESULTS

APRIL expressions in human HCC cell lines

APRIL expression in 3 human HCC cell lines (SK-Hep1, HLE, HepG2) was investigated. Bands corresponding to the expression in 42 ku unprocessed form of APRIL were observed in all human HCC cell lines (Figure 1). SK-Hep1 or HepG2 cells, were similar, but weaker in HLE cells. In addition to the unprocessed form, a lower molecular weight form (17 ku) was observed in HCC cell lysates and supernatants (Figure 1). This was thought to be a soluble form of APRIL. The soluble form of APRIL was observed in the three HCC cell lysates and supernatants. The soluble form of APRIL was most strongly expressed in supernatant from SK-Hep1 cells.

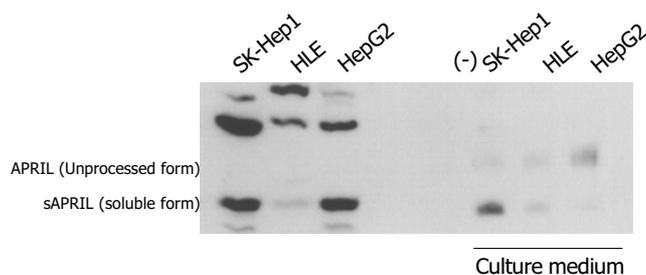


Figure 1 APRIL expression in human HCC cell lines and their supernatants by Western blotting. Closed arrowhead indicates the expression of unprocessed form of APRIL (42 ku) and open arrowhead indicates the soluble form of APRIL (17 ku).

Effect of recombinant human APRIL (rhAPRIL) in human HCC cell lines and HUVECs

We analyzed the effect of rhAPRIL on proliferation of human HCC cells. After 72-h incubation, the HCC cell counts slightly increased at the concentrations of 1, 10, or 100 ng/mL (Figure 2A), the greatest effects were observed at concentration of 1 ng/mL in HepG2 cells.

Since HCC is generally a hypervascular tumor^[21] and gains its hypervascularity during dedifferentiation and progression^[22], we investigated the effect of rhAPRIL stimulation on the proliferation of HUVECs.

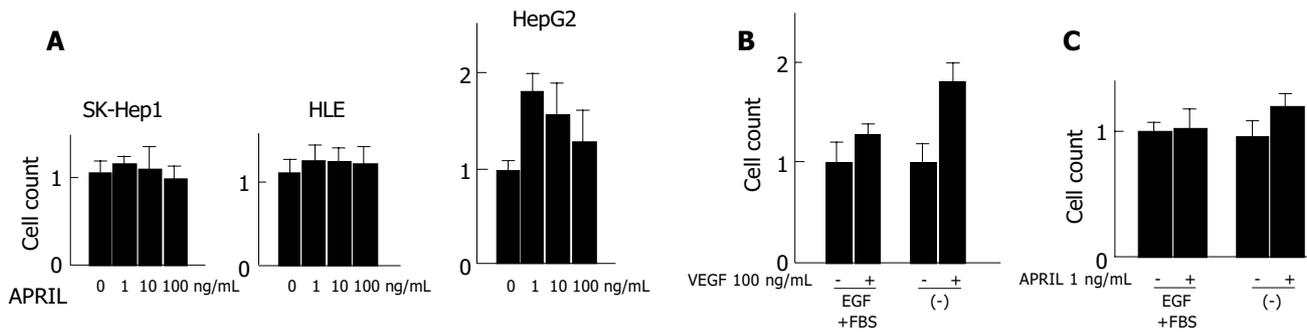


Figure 2 Effects of rhAPRIL stimulation on viability of human HCC cells (A) and

HUVECs (B and C) incubated at different time periods.

An increase in proliferation at the concentration of 100 ng/mL rhVEGF was observed in the presence or absence of FBS and EGF (Figure 2B). In particular, rhVEGF effectively inhibited cell death induced by cell starvation. The cell viability of HUVECs slightly increased with the stimulation of 1 ng/mL rhAPRIL and the same concentration of rhAPRIL inhibited cell death with starvation (Figure 2C). From these results, we confirmed that APRIL could induce cell proliferation and cell death inhibition in HCC cells and HUVECs, but we also observed that the effect of APRIL on HCC cell lines was not so potent.

APRIL could mediate regulation of cell growth, induce XIAP expression and decrease caspase activity^[23]. We examined the expression of apoptosis-related proteins, including FLICE/caspase-8 inhibitory protein (FLIP), X-chromosome-linked inhibitor of apoptosis (XIAP), BclL, Apaf-1, and smac in HCC cell lines and HUVECs after APRIL stimulation. However, these apoptosis-related proteins did not show changes in expression levels after APRIL stimulation (data not shown).

Regulation of APRIL in human HCC cell lines

We examined the expression of APRIL after serum starvation in HCC cells and HUVECs (Figure 3). Serum starvation increased the expression of both the unprocessed and soluble forms of APRIL in HLE. In SK-Hep1 and HepG2 cells, serum starvation did not induce upregulation of APRIL, however its expression was maintained. sAPRIL eventually disappeared after 24 h of serum starvation in SK-Hep1 cells, whereas HepG2 cells maintained sAPRIL expression.

APRIL was associated with VEGF expression in HCC cell lines

We observed APRIL expression and regulation by serum starvation in human HCC cell lines. In particular, the serum starvation induced APRIL expression in HLE cells. VEGF might be associated with angiogenesis or tumor progression^[24-31] and VEGF expression could be induced by stress, for example hypoxia or ischemia, in HCC cells^[24,25,28,29]. All HCC cell lines showed expression of VEGF with serum starvation, although the modulation of VEGF expression differed among three cell lines (Figure 4A). In SK-Hep1 and HepG2 cells, VEGF expression was stable for 12 h. In contrast, VEGF expression in HLE cells was not observed until 48 h and was less than that in SK-Hep1 or HepG2 cells.

Because APRIL was expressed constitutively in SK-Hep1 and HepG2 and upregulated in HLE cells after starvation,

we considered that APRIL might be associated with VEGF expression in HCC cells. So, we used anti-APRIL antibody to bind to sAPRIL in culture medium and to inhibit APRIL effects mediated by an autocrine or paracrine system. As shown in Figure 4B, blockade of APRIL by anti-APRIL antibody induced downregulation of VEGF expression in SK-Hep1 and HepG2 cells, compared to controls with normal IgG.

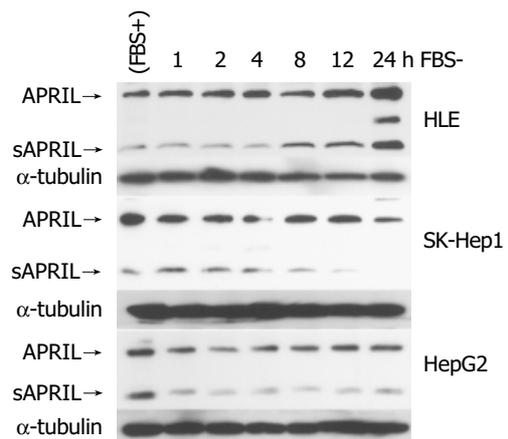


Figure 3 Regulation of APRIL expression in HCC cells. Closed arrowhead indicates the expression of unprocessed form of APRIL. Open arrowhead indicates soluble form of APRIL.

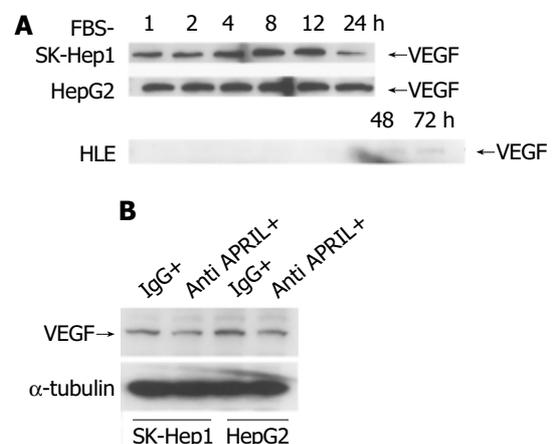


Figure 4 Regulation of VEGF expression with starvation in HCC cells incubated with FBS-negative culture (A) and 10 µg/mL anti-APRIL polyclonal antibody or normal rabbit IgG (B).

Finally, we observed that culture medium from the HCC cells incubated with anti-APRIL polyclonal antibody inhibited endothelial cell tube formation in matrigel (Figure 5).

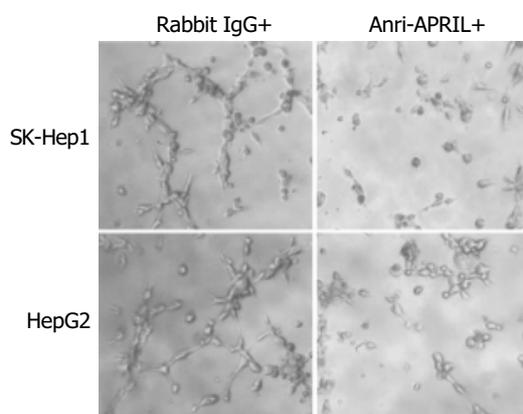


Figure 5 Effect of APRIL on *in vitro* endothelial cell tube formation.

DISCUSSION

Though HCC is the most common primary cancer of the liver and preceded by chronic hepatitis and cirrhosis, the mechanism of hepatocarcinogenesis or tumor progression is complex and not well understood. Cell killing and stimulation of mitosis leading to cell transformation or chromosomal instability induced by recombinogenic proteins during chronic hepatitis may cause carcinogenesis of HCC^[32].

HCC is resistant to conservative chemotherapy and difficult to induce apoptosis with TNF family members^[33]. On the other hand, TNF families, including TNF, TRAIL or Fas, can induce activation of NF- κ B^[33-41] and the tumor itself expresses these ligands^[42,43]. These phenomena suggest that TNF families contribute to tumor progression by autocrine or paracrine systems. In this study, we demonstrated that APRIL, a TNF family member, expressed in HCC cell lines, including SK-Hep1, HLE and HepG2, and sAPRIL was detected in culture medium of these HCC cell lines. However, addition of rhAPRIL did not induce any significant effect on viability of HCC cell lines, except for HepG2 cells stimulated with 1 ng/mL rhAPRIL. Because APRIL can induce both cell proliferation^[4,5,19] and death^[7] of malignant tumors, the result of present study may reflect that APRIL does not induce cell death so much as proliferation in HCC cells. In contrast to other TNF members, which are processed from the cell surface, APRIL is processed intracellularly and sAPRIL is purified^[14]. sAPRIL is used to stimulate tumor cells and induce cell proliferation in previous studies^[4,14]. The unprocessed form of APRIL may have less ability to induce tumor cell proliferation than sAPRIL.

APRIL enhances the growth of several tumor lines, however, the effect is more prominent at the point of tumor implantation than established tumor^[23]. Because expanding solid tumors, including HCC, are advanced with insufficient nutrition or oxygen, they have to get new vascular support to grow. In this study, HUVECs did not show significant cell proliferation or apoptosis inhibition after APRIL

stimulation. But HLE cells showed upregulation of APRIL after 24 h starvation, and further prolonged starvation induced upregulation of VEGF. In SK-Hep1 and HepG2 cells, starvation did not significantly induce the expression of APRIL, however, starvation promptly induced upregulation of VEGF. These results suggest that APRIL may be associated with expression of VEGF and induction of neovascularization in HCC. Further more, blockade of APRIL activity by anti-APRIL antibody induced downregulation of VEGF and inhibited tube formation of HUVECs. These results support the notion that neovascularization and progression of HCC may be induced by APRIL via VEGF expression.

Until now, some factors including VEGF have been identified as mediators of angiogenesis associated with tumors. With regard to HCC, recent studies indicate that angiopoietin^[44,45] is a target of angiogenesis. APRIL may not be critical for HCC progression as a direct effector, but plays an important role in neovascularization of HCC.

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