Abstract. In light of the poor prognosis for ovarian cancer patients, the research continues for innovative and efficacious treatment modalities. Along with surgical and chemotherapeutic regimens, gene therapy has emerged as one of the leading contenders in the treatment armamentarium. Hepsin is a type II transmembrane serine protease originally identified in the human liver as a cDNA clone. However, the biological function of Hepsin expression remains unclear. Apoptotic cell was increasingly common among higher proportions of Hepsin transfectants. We discovered that first-time stable transfectants of Hepsin significantly inhibited cell growth in the monolayer, anchorage-independent cell growth in the soft agar in vitro, and tumorigenicity in vivo in ovarian cancer cell lines. These results demonstrated that Hepsin has a potential therapeutic effect that inhibits through up-regulation of p53-dependent apoptosis and caspase-3, -6, and -7 activations. Our findings in this study identify Hepsin as a target for new approaches in ovarian cancer treatment.

Introduction

Despite its pervasive impact, the etiology of ovarian cancer and the factors that promote its progression are not yet well understood. Although chemotherapy remains a major treatment modality for human ovarian cancer, chemoresistance is a clinical problem that severely limits the success of the treatment. It is now widely accepted that the apoptotic capacity of ovarian cancer cells is pivotal in determining their responses to chemotherapeutic agents.

Hepsin is a type II transmembrane serine protease originally identified in the human liver as a cDNA clone (1,2). A 1.85-kb Hepsin mRNA is highly expressed in normal liver and kidney tissues and is poorly expressed in other tissues, including the normal prostate and lung. In normal ovarian tissue, Hepsin mRNA is not expressed, and Hepsin overexpression has been reported in ovarian, renal, and prostate carcinomas (3-5). However, the biological functions of Hepsin remain unknown.

The targets of p53-regulated genes or p53-interacting proteins have provided critical information to the understanding of the biochemical and biological functions of the p53 tumor suppressor gene. The purpose of this study is to examine the role of Hepsin in the wild-type (wt) p53-dependent apoptosis pathway and caspase family. We studied the p53 apoptotic-mediated factors and performed cell growth in the monolayer, anchorage-independent cell growth in the soft agar in vitro, and tumorigenicity in vivo. We provided new insights into the biological functions and gene therapeutic target in ovarian cancer.

Materials and methods

Cell culture and media. BG-1, OVCAR-3, CaOV-3 cell lines were derived from human epithelial ovarian carcinoma. These ovarian cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). The LNCaP cell line was derived from human prostate carcinoma. The prostate cancer cell lines were maintained in RPMI-1640 (Life Technologies, Inc., Rockville, MD) with 10% FBS. Cells were maintained at 37˚C in an atmosphere with 5% CO2 in the air.

Reverse transcription-PCR analyses. In reverse transcription-PCR (RT-PCR), we used primers 5'-TGTCCCGATGGCGAGTTT-3' (Hepsin-F) and 5'-CCTGTTGGCCCATAGTACTGC-3' (Hepsin-R), as described (3). The number of PCR cycles resulting in PCR products in the linear logarithmic phase of amplification curve was determined. PCR samples were electrophoresed on 2% agar gel and visualized by ethidium bromide.
Western blot analysis. Cell lysates were collected and estimated using a Protein Assay system (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. The protein from each cell line was subjected to SDS-PAGE and was transferred onto the nitrocellulose membrane. The polyclonal and monoclonal antibodies and working dilution ratios used for immunoblotting were as follows: Hepsin (Cayman Chemical, Ann Arbor, MI), p53, Puma-α, Puma-β/δ, Noxa, Bcl-2, Bak, p53AIP1, p53R2, caspase-3, -6, and -7 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma Chemical Co., St. Louis, MO). The membranes were then incubated with each second antibody. The expressions of antigen-antibody complexes were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Generation of transient transfected. A full-length Hepsin cDNA construct encoding the Hepsin protein was transiently transfected in the BG-1 cell lines using a TransFast transfection reagent (Promega, Madison, WI). BG-1 cell lines were treated either with or without Hepsin transient transfection for 24, 48 or 72 h.

Cell proliferation assay and FITC-conjugated Annexin V. In order to evaluate the growth effects of Hepsin, MTS assay (Promega) was performed. Cells were seeded into 96-well plates to obtain a density of 5x10⁴ cells/well and transfected for 24, 48 or 72 h. The absorbance was read at a wavelength of 490 nm using an ELISA plate-reader (Bio-Rad Systems). Moreover, apoptosis was measured by staining with FITC-conjugated Annexin V using a Mebcyto apoptosis kit (MBL, Nagoya, Japan) according to the manufacturer’s recommendations with flow cytometric analysis.

Generation of stable transfected. Hepsin stable transfected in the BG-1 cell lines was generated using a TransFast transfection reagent. Zeocin (0.8 mg/ml) (Invitrogen, Carlsbad, CA)-resistant colonies were selected after 4 weeks. Drug
200 μl of sterile PBS were injected subcutaneously above the A cell suspension (1x10^4 cells/well) in monolayer cultures of BG-1/Hepsin-24 and BG-1/Hepsin-16 cells. However, the empty vector type weakly expressed the mRNA levels of Hepsin. Furthermore, BG-1/Hepsin-24 cells were significantly over-expressed compared to BG-1/Hepsin-16 cells (Fig. 2).

**Results**

The transient transfection of Hepsin increased the progress of apoptosis. The mRNA of Hepsin was determined using quantitative RT-PCR (3). Among the established ovarian cancer lines, Hepsin expression was detected in the CaOV-3, OVCAR-3, and BG-1 cell lines. As a positive control, we used the LNCaP prostate cancer cell line (Fig. 1A). Among these three, the BG-1 cell line exhibited the strongest transformation on anchorage-independent cell growth. For this reason, the BG-1 transfectant was chosen for further experiments. Several previous reports also showed that Hepsin acts during apoptosis and in the G2 phase of the cell cycle in prostate cancer (8). We examined apoptosis expression transient transfection of Hepsin treatment in the BG-1 ovarian cancer cell line. Protein expression of Hepsin transient transfectant was detected using Western blotting. Hepsin expression was significantly detected only after 72-h treatment (Fig. 1B). Proliferation was significantly suppressed in 72-h transfectant in accordance with the pattern of protein expression (p<0.05) (Fig. 1C).

Representative flow cytometric data revealed that transient transfection of Hepsin for 72 h showed increased FITC-positive and propidium iodide (PI)-positive signals (Fig. 1D). Therefore, Hepsin was likely to regulate the apoptosis induced by transient transfection of Hepsin.

**Evaluation of Hepsin expression stably transfected BG-1/Hepsin cells.** mRNA from stably transfected cells was measured by real-time quantitative RT-PCR and was adjusted to the GAPDH levels. As a positive control, we used the LNCaP prostate cancer cell line. Both the mRNA and protein levels of Hepsin were similarly and highly expressed by BG-1/Hepsin-24 and BG-1/Hepsin-16 cells. However, the empty vector type weakly expressed the mRNA levels of Hepsin. Furthermore, BG-1/Hepsin-24 cells were significantly over-expressed compared to BG-1/Hepsin-16 cells (Fig. 2).

**Inhibition of cell growth in monolayer culture.** The effect of Hepsin expression on cell proliferation was analyzed in BG-1/Hepsin-16 and BG-1/Hepsin-24. We found a significant inhibitory effect of Hepsin expression on cell growth in the monolayer cultures of BG-1/Hepsin-16 and BG-1/Hepsin-24 (~70% inhibition) (p<0.05) compared to the empty vector type of BG-1 (Fig. 3).
Increased accumulation of p53-dependent apoptosis and caspases in stably transfected BG-1/Hepsin cells. To determine whether the p53 tumor suppressor gene regulates Hepsin expression in ovarian cancer cells, p53 mRNA and protein expression were analyzed in BG-1/Hepsin-16 and BG-1/Hepsin-24 cells. The mRNA and protein levels of BG-1/Hepsin-24 and BG-1/Hepsin-16 cell showed overexpression of p53 compared with the BG-1 empty vector. BG-1/Hepsin-24 cells showed the highest overexpression levels compared with BG-1/Hepsin-16 cells (Fig. 4A and B).

Accordingly, we experimented on the promoters of apoptotic cell death, which are pro-apoptotic Bcl-2 protein (Bax-, Bak- and BH-3-only proteins such as Noxa, Puma-α, Puma-β/δ), and p53-regulated apoptosis-inducing proteins (p53AIP1), the repair of damaged DNA proteins (p53R2), and anti-apoptotic Bcl-2 protein (Bcl-2). In BG-1/Hepsin-24, Noxa, Puma-α, Puma-β/δ, Bax, Bak, p53AIP1, and p53R2 proteins were up-regulated. Subsequently, down-regulation of Bcl-2 resulted in p53-dependent apoptosis. The p53-related apoptosis factors were highly up-regulated in BG-1/Hepsin-24, unlike BG-1/Hepsin-16 and the empty vector type (Fig. 4B).

To investigate the specific apoptotic regulated by Hepsin, we examined the effect of Hepsin on the caspase family by Western blotting. The protein levels of BG-1/Hepsin-24 and BG-1/Hepsin-16 showed significant overexpression of caspase-3, -6, and -7 (Fig. 4C), but not caspase-1, -2, -8 and -9 (data not shown), compared with the BG-1 empty vector. Moreover, caspase-3, -6, and -7 of BG-1/Hepsin-24 cells showed the highest overexpression levels compared with BG-1/Hepsin-16 cells.

Inhibition of cell growth in soft agar. The inhibitory effect in colony formation was approximately 80% higher than the empty vector type. Furthermore, BG-1/Hepsin-24 cells produced a smaller number of colonies compared to BG-1/Hepsin-16 cells and the empty vector type (p<0.05 or p<0.01) (Table I).

Inhibition of tumor growth in vivo. We examined the tumorigenicity of BG-1/Hepsin-24 or the empty vector type in nude mice. The inhibitory effect in tumor growth was approximately 80% higher than the empty vector type. Furthermore, BG-1/Hepsin-24 cells produced smaller tumors compared to BG-1/Hepsin-16 cells and the empty vector type (p<0.05 or p<0.01) (Table I).
mice. Characteristically, there was a noticeable suppression of tumor growth in a statistically significant manner by BG-1/Hepsin-24. The extent of reduction in the tumor volume compared to the empty vector type was more than 95% (p<0.01) (Fig. 5).

**In vivo expression of p53-dependent apoptosis protein.** To confirm the p53-dependent apoptosis protein overexpression by immunohistochemistry staining, we used an empty vector and Hepsin transfectants (BG-1/Hepsin-24). The representative staining of the p53-dependent apoptosis factors (Bax, Bak, Puma-α, Puma-β/δ, Noxa, p53AIP1, and p53R2) were also densely stained in BG-1/Hepsin-24 tissue. Furthermore, p53AIP1 was more densely stained than the other p53-dependent apoptosis factors (Fig. 6).

**Discussion**

In light of the poor prognosis for ovarian cancer, the research continues for innovative and efficacious treatment modalities. Along with surgical and chemotherapeutic regimens, gene therapy has emerged as one of the leading contenders in the treatment armamentarium.

Data from the present study show that Hepsin is essential for the functional activity of ovarian cancer. Previous reports have documented overexpression of Hepsin in ovarian cancer; however, its biological functions remain to be understood. In the current study, we demonstrated that the overexpression of Hepsin inhibited cell proliferation, colony formation in vitro,
and tumorigenicity in vivo. Prostate cancer studies showed that Hepsin acts during apoptosis and in the G2 phase of the cell cycle (8).

DNA damage and oncogene expression lead to the stabilization and activation of the p53 tumor suppressor protein. The wild-type p53 (wt p53) protein exerts inhibitory effects toward the growth of abnormal cells, and has been considered as a guardian of the genome by preventing cancer development (9). To our knowledge, the present study is the first report of Hepsin-induced activation of the p53-dependent pathway. Further research is needed to define the mechanism involved in the upstream activation of p53 by Hepsin.

Depending on the cellular context, p53 activation can cause cell cycle arrest or apoptosis, and both responses contribute to tumor suppression (10). In this study, we discovered that Hepsin induced activation of p53 leading to a p53-dependent apoptosis.

Several promoters of apoptotic cell death, such as promoters of pro-apoptotic Bcl-2 protein: Puma (11,12), Noxa (13,14), Bax (15), Bak (16), and other promoters such as p53AIP1 (17), and p53R2 (18), were considered to regulate p53-dependent apoptosis. In this study, we used protein analysis to study the p53-dependent apoptosis pathway. Our results showed that Hepsin-induced expression up-regulated the Puma, Noxa, Bak, Bax, p53AIP1, and p53R2 proteins. This confirms the fact that Bcl-2, which is a death antagonist and has the capacity to suppress caspase-dependent pathways of apoptosis, plays a potentially important role in p53-dependent apoptosis (13-19).

To date, there is no evidence for a regulatory role for Hepsin in apoptosis-mediated caspase signaling complex activity. Our data indicates that p53-dependent apoptosis, which mediated the activation of caspase-3, -6, and -7, was enhanced by Hepsin expression.

In the present study, we discovered that the inhibitory effect of Hepsin overexpression isn’t only present in experimental cell growth in the monolayer but also in anchorage-independent cell growth in vitro. In the present study, we first reported the vigorous inhibitory effect of tumorigenesis expression in vivo. In vivo studies showed that the extent of reduction in the tumor volume compared to the empty vector type was more than 95% (Fig. 5). Combining these results, it is suggested that Hepsin has a potentially therapeutic effect through growth inhibition of the ovarian cancer cell line.

In conclusion, we propose that Hepsin induces activation of apoptosis and inhibits cell growth in vitro as well as tumorigenicity in vivo. Our findings identify Hepsin as a potential therapeutic target gene for new approaches to treating ovarian cancer.

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