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Research paper

Anti-tumor effects of bemiparin in HepG2 and MIA PaCa-2 cells

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ABSTRACT

Recent researches have demonstrated improved survival in oncologic patients treated with low molecular weight heparins (LMWHs) which are anticoagulant drugs. We evaluated "second generation" LMWH bemiparin and its *in vitro* anti-tumor effects on HepG2 hepatocellular carcinoma and MIA PaCa-2 cancer cells. The aim of the study is to investigate anti-cancer mechanism of bemiparin in HepG2 and Mia-Paca-2 cancer cells. Cytotoxic effects of bemiparin were determined by XTT assay. IC_{50} dose of bemiparin was found to be 200 IU/mL in the 48th hour in the MiaPaCa-2 cell line and 50 IU/mL in the 48th hour in the HepG2 cell line. *CCND1* (*cyclin D1*), *CDK4*, *CDK6*, *p21*, *p16*, *p53*, *caspase-3*, *caspase-9*, *caspase-8*, *Bcl-2*, *BID*, *DR4*, *DR5*, *FADD*, *TRADD*, *Bax*, gene mRNA expressions were evaluated by Real-time PCR. Real-time PCR analysis showed that *CCND1* expression was reduced in HepG2 dose the group cells when compared with the control group cells and *p53*, *caspase-3*, *caspase p21*, *caspase-8* and expressions were increased in the dose group cells when compared with the control group cells. *CCND1*, *CDK4* and *CDK6* expressions were reduced in MIA PaCa-2 dose group cells when compared with the control group cells and *p53* expression was increased in the dose group cells when compared with the control group cells. Other expressions of genes were found statistically insignificant both of cell lines.
It was found that bemiparin in HepG2 and MIA PaCa-2 cells suppressed invasion, migration, and colony formation by using matrigel invasion chamber, and colony formation assay, respectively. In conclusion, it is thought that bemiparin indicates anti-tumor activity by affecting cell cycle arrest, apoptosis, invasion, migration, and colony formation on cancer cells.

1. Introduction

Bemiparin, which has mean molecular weight (3600 Da) and the longest half-life (5.3 h), called second-generation low-molecular-weight heparin (LMWH). Bemiparin is a member of LMWH which is new class of anticoagulants derived from commercial grade unfractioned heparin (UFH) (Martínez-González and Rodríguez, 2010; Planès, 2003). Although bemiparin demonstrates its anticoagulant effects *via* its anti-Xa activity, also other factors such as the release of tissue factor pathway inhibitors (TFPI) from endotelial cells can regulate this activity (Planès, 2003; Weitz, 1997; Hirsh et al., 2001). At the same time, heparin binds to tumoral cell proteins and macrophages with low affinity than other LMWHs or UFH (Morita et al., 2001; Gebska et al., 2002). This situation may be associated with decrease in anticoagulant potency and presumably dose–response (Planès, 2003).

Association between cancer and thrombosis is well defined for long years. It is reported that there is a two-way association in that presence of the tumor stimulates a hypercoagulable state in the host leading patients to thrombosis or in contrast to this situation, the induction of coagulation promotes the tumor progression. Focusing on these relation may have significant effect on survival of cancer patients (Falanga et al., 2003; Vignoli et al., 2011). It is also reported that venous thromboembolism (VTE) is responsible approximately 3.5% death of cancer patients (Khorana et al., 2007). LMWHs are frequently used for thromboprophylaxis in cancer patients with have high risk for both venous and arterial thrombosis (Khorana et al., 2009). The gold standard treatment for acute DVT patient is low-molecular weight heparins (LMWHs) and vitamin K antagonists (VKAs) together with compression stockings. The most important benefit of this treatment reduces mortality by preventing pulmonary embolism (PE) (Polat et al., 2015).

One of the second generation LMWH, bemiparin has been recently utilized in clinical practice of either thromboprophylaxis or adjuvant therapy in oncological patients (Kakkar et al., 2010; Lecumberri et al.,

Abbreviations: XTT, [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]; CDK, cyclin dependent kinase; DMSO, dimethylsulfoxide; LMWH, low-molecular-weight heparin; UFH, unfractioned heparin; TFPI, tissue factor pathway inhibitors; VTE, venous thromboembolism; TFPI, tissue factor pathway inhibitors.

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2010). Studies on several cancer types showed that LMWHs have positive effects on patient survival (Gerotziafas et al., 2008). According to studies done for many years suggested that heparins have impact on growth and metastasis of primary tumors and inhibit the adhesion and migration capacity of cancer cells and influence tumor-induced neoangiogenesis by blocking the tissue factor/factor VIIa complex, factor Xa and thrombin (Falanga and Marchetti, 2007; Zacharski and Loynes, 2002). Recent *in vivo* and *in vitro* studies have demonstrated that heparins have effects on tumor angiogenesis and some results of these studies present that size of heparins may affect their antiangiogenic properties (Khorana et al., 2003; Norrby, 2006). Marchetti et al. showed that dalteparin and enoxaparin which is member of LMWH hinder the formation of microvascular endothelial cell capillary-like tubes activated through breast cancer and leukemia (Marchetti et al., 2008).

In this study, we examine the effects of bemiparin on apoptosis and cell cycle related gene expression, viability, colony formation and migration/invasion of the cultured MIA PaCa-2 pancreatic cancer cell and HepG2 hepatocellular carcinoma cells.

2. Material and methods

2.1. Cell Culture

MIA PaCa-2 human pancreatic cancer cells and HepG2 hepatocellular carcinoma cell (obtained from ATCC, USA) lines were used in this study. MIA PaCa-2 and HepG2 cells were grown in DMEM medium supplemented with 2 mM L-glutamine, penicillin (20 units/mL), streptomycin (20 μg/mL), and 10% (vol/vol) heat-inactivated fetal calf serum at 37 °C in a saturated humidity atmosphere containing 5% CO_2. MIA PaCa-2 and HepG2 cells were treated with 0.1 IU/mL, 0.5 IU/mL, 1 IU/mL, 10 IU/mL, 25 IU/mL, 50 IU/mL, 100 IU/mL, 200 IU/mL, 300 IU/mL bemiparin dissolved in medium up to 72 h, in a time and dose dependent manner.

2.2. Cell viability assay

Effects of bemiparin on cell viability and detecting of IC_{50} dose in MIA PaCa-2 and HepG2 cells were performed by using trypan blue dye exclusion test and XTT [2,3-bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay as indicated in manufacturers' instruction. MIA PaCa-2 and HepG2 cells were seeded in 96-well plates at a number of 3×10^4 cells/well and incubated for 24 h without reagent. The cells were treated with different concentrations of bemiparin and incubated for 24, 48 and 72 h. Then using XTT mixture as recommended by supplier assessed cell viability. Formazan formation was quantified spectrophotometrically at 450 nM (reference wavelength 630 nM) using a microplate reader. Viability was calculated using the background-corrected absorbance as follows:

$$\text{Cell Viability}(\%) = \text{A of experiment well}/\text{A of control well} \times 100.$$

2.3. RNA isolation and Real Time-PCR

Total RNA was isolated from the cells exposed to IC_{50} (inhibitory concentration where 50% of the cells die) doses of bemiparin using Trizol Reagent (İnvitrogen, USA) according to manufacturer instructions. cDNA synthesis from RNA template was performed *via* reverse transcription by using Transcriptor First-Strand cDNA Synthesis Kit (Roche, Germany) according to manufacturer protocol. *p21*, *p16*, *p53*, *caspase-3*, *caspase-9*, *caspase-8*, *FADD*, *TRADD*, *Bax*, *CCND1* (*cyclin D1*), *CDK4*, *CDK6*, *Bcl-2*, *BID*, *DR4*, *DR5* gene expression analysis was performed by Step One Plus Real Time RT-PCR (Applied Biosystems, USA) according to the SYBR Green qPCR Master Mix (Thermo Scientific, USA) protocol. RT-PCR assay was performed using gene-specific primers. The expression results were regulated to the *GAPDH* gene (housekeeping gene) expressions to calculate relative expression ratios. Primer sequences used in this study were given in Table 1.

Table 1
Primer sequences of the genes used in this study.

Name	Primer sequence
BAX	F: AGAGGATGATTGCCGCCGT
	R: CAACCACCCTGGTCTTGGATC
CASPASE-3	F: GCAGCAAACCTCAGGGAAAC
	R: TGTCGGCATACTGTTTCAGCA
BCL-2	F: TTGGCCCCCGTTGCTT
	R:CGGTTATCGTACCCCGTTCTC
GAPDH	F: TTCTATAAATTGAGCCCGCAGCC
	R: CCGTTGACTCCGACCTTCAC
CASPASE-9	F: GGCTGTCTACGGCACAGATGGA
	R: CTGGCTCGGGGTTACTGCCAG
P21	F: TGGAGACTCTCAGGGTCGAAA
	R: GGCGTTGGAGTGGTAGAAATC
P53	F: ATCTACAAGCAGTCACAGCACAT
	R: GTGGTACAGTCAGAGCCAACC
BID	F: CCTACCCTAGAGACATGGAGAAG
	R: TTTCTGGCTAAGCTCCTCACG
DR4 (*TNFRSF10A*)	F: GCGGGGAGGATTGAACCAC
	R: CGACGACAAACTTGAAGGTCTT
DR5 (*TNFRSF10B*)	F: ACAGTTGCAGCCGTAGTCTTG
	R: CCAGGTCGTTGTGAGCTTCT
FADD	F: GCTGGCTCGTCAGCTCAAA
	R: ACTGTTGCGTTCTCCTTCTCT
TRADD	F: GCTGTTTGAGTTGCATCCTAGC
	R: CCGCACTTCAGATTTCGCA
CCND1	F: AGCTCCTGTGCTGCGAAGTGGAAAC
	R: AGTGTTCAATGAAATCGTGCGGGGT
CDK4	F: ATGTTGTCCGGCTGATGGA
	R: CACCAGCGTTACCTTGATCTCCC
CDK6	F: AGACCCAAGAAGCAGTGTGG
	R: AAGGAGCAAGAGCATTCAGC
P16	F: CAGTAACCATGCCCGCATAGA
	R: AAGTTTCCCGAGGTTTCTCAGA
CASPASE-8	F: TCTGGAGCATCTGCTGTCTG
	R: CCTGCCTGGTGTCTGAAGTT

2.4. Determination of cell invasion and migration effect of bemiparin

Effects of bemiparin on invasion activities of HepG2 and MIA PaCa-2 cells were determined *via* the Bio Coat Matrigel Invasion Chamber guide (BD Biosciences). HepG2 and MIA PaCa-2 cells with serum-free DMEM-medium were seeded at a density of 2×10^5 cells/well onto the upper chambers of Matrigel-coated filter inserts and serum-containing DMEM medium (500 μL) was added to the lower chambers. The control and dose group cells were then incubated at 37 °C for 24 h. After the incubation, filter inserts were removed from the wells. The cells exist on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with crystal violet. The cells that invaded the lower surface of the filter were counted using a light microscope. Each experiment was performed in triplicate. Percentage of invasion was calculated as follows:

$$\text{Invasion}\ (\%) = \frac{\text{The number of cells in matrigel matrix basement membrane}}{\text{The number of cells in control membrane}} \times 100.$$

2.5. Colony assay

To define colony formation effects of bemiparin in HepG2 and MIA PaCa-2, the cells were seeded in six-well plates at a density of 10^3 cells per well. The medium was renewed every 3 days during three weeks until visible colonies formed. Colonies were fixed in methanol for 10 min and stained with crystal violet to make them visible. Thus, colony numbers of control and bemiparin treated cells were compared.