Abstract. The proliferative activity of hepatic carcinoma cells is directly associated with tumorigenesis, tumor development, metastasis and invasion. A variety of cytokines and peptides serve important roles in the development of hepatic carcinoma. The aim of the present study was to examine the effect of intermedin (IMD) on hepatic carcinoma cell proliferation and its mechanism of action. HepG2 hepatic carcinoma cell lines were treated with human recombinant IMD1-53 and its receptor antagonist IMD17-47. Cell proliferation was detected using a Cell Counting kit-8. The activation of the classical Wnt signaling pathway was demonstrated by the ratio of TOPflash:FOPflash luciferase activity. The expression of c-Myc and cyclin D1 downstream of the Wnt signaling pathway were detected using reverse transcription-quantitative polymerase chain reaction analysis. It was demonstrated that IMD may promote the proliferation of HepG2 cells in a time-dependent manner, and that the IMD receptor antagonist IMD17-47 could eliminate this promotion. IMD may activate classical Wnt signaling pathway transcriptional activity and the mRNA levels of certain downstream target genes. Furthermore, blocking of the Wnt signaling pathway may inhibit IMD-induced HepG2 cell proliferation to a certain extent. IMD may promote hepatic carcinoma cell proliferation by binding with receptor antagonist IMD17-47 and activating the Wnt signaling cascade, thus providing a novel avenue for the treatment of hepatic carcinoma.

Introduction

Primary hepatocellular carcinoma (HCC) is the most common type of malignant tumor worldwide, an incidence ranked third-highest of all types of cancer, a high degree of malignancy and a five-year survival rate of ~30% (1). To date, no effective treatments have been developed (2). HCC is commonly diagnosed numerous countries with a high incidence of viral hepatitis (3). HCC contributes 85-90% of all primary liver cancers, with 500,000 new cases and 250,000 HCC-associated mortalities occurring worldwide every year (4). The main reasons for the high mortality rate of HCC patients are ascribed to the lack of effective treatments and the increasing resistance to conventional radiotherapy and chemotherapy (5). The proliferation of hepatoma cells is closely associated with HCC occurrence, development, invasion and metastasis (1). Small molecule bioactive peptides have various unique biological characteristics, including simple structures, extensive tissue distribution and a range of biological effects, which serve important roles in the regulation of the human metabolism and physiological function (6). A number of cytokines and small peptides have essential roles in the development of liver cancer. Intermedin (IMD) belongs to the calcitonin gene-related peptide (CGRP) superfamily (7). It was initially identified in teleost fish in 2004, and has also been identified in cDNA clones of other species, such as rodents and primates (8). IMD is widely distributed in the body, and its expression levels in adrenocortical tumors (9) and colorectal cancer (10) are high compared with those in normal tissues. IMD may also promote angiogenesis (11), suggesting that it is associated with the occurrence and development of tumors. The present study explored the effect of IMD in HCC and its underlying molecular mechanisms.

Materials and methods

HepG2 cells were purchased from Shanghai Bogoo Biological Technology Co., Ltd. (Shanghai, China), recombinant human IMD1-53 and its receptor antagonist (IMD17-47) were purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The RNA extraction, reverse transcription (RT) kit and Luciferase reporter gene assay kit were purchased from...
Promega Corporation (Madison, WI, USA). The Taq enzyme was purchased from Tiangen Biotech Co., Ltd. (Beijing, China) and the fluorescent EvaGreen® dye was purchased from Biotium, Inc. (Fremont, CA, USA). The classical Wnt pathway inhibitor (IWR-1-endo) was purchased from Cayman Chemical (Ann Arbor, MI, USA), the TOPflash/FOPflash plasmids for Wnt signaling pathway activity detection were purchased from Merck KGaA (Darmstadt, Germany) and the jetPEI® transfection reagent was purchased from Polyplus-Transfection SA (Illkirch, France).

**Cell proliferation was detected using CCK-8.** HepG2 cells in the logarithmic growth phase were inoculated in flat-bottomed 96-well plates at a density of 1x10^4/100 cells/µl and cultured in 5% CO₂ incubator at 37°C. After 12 h, the medium was replaced with fresh culture medium (Shanghai Yuan Long Biology Technology Company, containing various concentrations (0, 1, 5, 10, 50 and 100 nM of IMD1-53, or with 100 nM IMD17-47 and 25 nM IWR-1-endo). The complete culture medium without IMD and RPMI-1640 was used as the control. A total of 48 h after inoculation, 10 µl CCK-8 was added into each well of the plate and placed in a 37°C incubator for 1-4 h. A microplate reader was then used to measure the absorbance value at a wavelength of 450 nm, as previously described (12).

**RNA extraction.** TRIzol® reagent (Promega Corporation) was used; the cells were aspirated from the culture medium and washed with PBS, following which 1 ml RNAtri reagent (Tideradar Beijing Technology Co., Ltd., Beijing, China) was added. The cells were triturated 30 times, transferred into an Eppendorf (EP) tube and placed at room temperature for 10 min. A total of 0.2 ml chloroform was added, and the samples were then incubated at room temperature for 5 min. The samples were centrifuged at 12,000 x g for 15 min at 4°C, and the resulting upper aqueous phase was carefully aspirated and transferred to a clean EP tube. Subsequently, an equal volume of isopropanol was added and mixed. The samples were allowed to precipitate for 2 h at -70°C, prior to being centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded, and the cells were precipitated and washed with 1 ml 0.75X ice-cold ethanol. They were then centrifuged again at 12,000 x g and 4°C for 10 min, following which the supernatant was discarded. When the whitish pattern appeared in the center, the RNA enzyme pure water 50-100 µl was added, and quantified with a spectrophotometer.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The reverse transcription reaction involved a 20 µl reaction system 5-(and-6)-carboxy-2,7'-dichlorofluorescein (pH 9.0; purchased from Beijing Jiamei Nuonuo Biological Technology Co., Ltd.), with 2 µg RNA (protector RNase inhibitor) starting, and used a reverse transcription kit according to the manufacturer's protocol (purchased from Promega Corporation) for first strand cDNA synthesis. RT-qPCR (Shanghai Solarbio Biological Technology Co., Ltd.) involved a 25 µl reaction system, and 1 µl reverse transcription reaction mixture was used as a template. Amplification conditions were as follows: Pre-degeneration for 5 min at 94°C, degeneration for 30 sec at 94°C, annealing for 30 sec at 58°C, extension for 30 sec at 72°C then extension for 5 min at 72°C, with a total of 40 cycles. Stratagene MX3000p software (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) was used for analysis. The 2^ΔΔCq method was used to quantify expression levels (13). Primer sequences were as follows: Human c-Myc upstream, 5'-TCTCAGATCCTGTCACAA-3' and downstream, 5'-GAGATGTCGCACCACCTG-3'; human c-Myc downstream, 5'-GTCCTCCTGCAGACTGTCACCTG-3'; human β-actin upstream, 5'-ATCTGGACCCACACCTTTC-3' and downstream, 5'-AGCCAGGTCCAGAGCA-3' (Shanghai, China Solarbio Biological Technology Co., Ltd.). The RT-qPCR protocol was repeated 5 times.

**Luciferase activity measurement.** HepG2 cells in the logarithmic growth phase were seeded on 12-well plates at 1x10^6 cells/well, then placed in a 5% CO₂ incubator at 37°C for 12 h, and each well was transfected with 0.5 µg various plasmids (using TOPFlash as the reporter plasmid, FOPFlash as a negative control and pRL-TK as an internal control) using jetPEI® reagent, and then incubated at 37°C for 24 h. The luciferase activity was measured with the Dual-Luciferase™ Reporter Assay System (Promega Corporation), according to the manufacturer's protocol (14).
Statistical analysis. All results are presented as the mean ± standard error of the mean. GraphPad Prism 5.0® software (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysis and creating the data graphs. A one-way analysis of variance was conducted for multi-group comparison, and the Student-Newman-Keuls method was used for post-hoc analysis and comparisons between the groups. Student’s t-test was used to compare the results between groups. P<0.05 was considered to indicate a significantly different difference.

Results

IMD induces HepG2 cell proliferation in a dose-dependent manner. The present study explored the direct effect of IMD on HepG2 cell proliferation. The results of the CCK-8 test demonstrated that the administration of 5-100 nM IMD for 48 h significantly promoted HepG2 cell proliferation (Fig. 1; P<0.05, compared with the control).

IMD promotes HepG2 cell proliferation in a dose-dependent manner. Additional analysis revealed that treatment with 100 nM IMD17-47 (competitive antagonist of the IMD receptor) significantly inhibited the promotion effect of 10 nM IMD on cell proliferation (P<0.05). Furthermore, IMD was observed to promote HepG2 cell proliferation in a dose-dependent manner, and exhibited a receptor-dependent manner (Fig. 2).

IMD activates the classical Wnt signaling pathway in HepG2 cells. The possible underlying mechanisms associated with IMD-promoted HepG2 cell proliferation were also investigated. The Wnt signaling pathway serves an important role in tumor cell proliferation. TOPflash was used as a reporter plasmid, FOPflash was used as the negative control, and a thymidine kinase promoter-Renilla luciferase reporter plasmid was used as the internal reference control. Subsequent to transfection for 6 h and treatment with 10 nM IMD for 24 h, a dual luciferase activity assay was conducted. The results demonstrated that the TOPflash/FOPflash ratio following IMD stimulation was significantly higher compared with in the control group (P<0.05; Fig. 3A). The mRNA levels of the downstream target genes c-Myc and cyclin D1 were identified to be significantly increased (P<0.05; Fig. 3B), suggesting that the classical Wnt signaling pathway was activated following IMD stimulation, and that specific transcriptional activity was significantly enhanced (P<0.05; Fig. 3).

IWR-1-endo is able to partially inhibit IMD-induced HepG2 cell proliferation. Pre-treatment with 25 µM IWR-1-endo for 1 h significantly inhibited 10 nM IMD-induced HepG2 cell proliferation (P<0.05, compared with the control). Therefore, IMD may promote HepG2 cell proliferation via the Wnt signaling pathway (Fig. 4).

Discussion

The results of the present study have indicated that IMD is able promote HepG2 cell proliferation via the classical Wnt signaling pathway, according to the data that suggest the following: IMD treatment may directly induce HepG2 cell proliferation; IMD17-47 may block HepG2 cell proliferation; IMD may activate the classical Wnt signaling pathway transcriptional activity and affect downstream target gene mRNA expression levels; blocking the Wnt signaling pathway may inhibit IMD-induced HepG2 cell proliferation.

IMD is a member of the CGRP superfamily, which exhibits ~30% similarity with the amino acid structure of adrenomedullin (AM), and is therefore also known as...
AM2 (7). Its precursor consists of 148 amino acid residues with multiple protease cleavage sites, and may be cut into three active fragments: IMD 1-47; IMD 8-47; IMDI-53 (15). CGRP may serve its role through binding with calcitonin receptor like receptors/receptor activity modifying protein receptor complexes (RAMPs) (16). CGRP primarily acts on RAMP1, AM principally acts on RAMP2/3 and IMD non-selectively acts on RAMP1/2/3; therefore, IMD may have a wider range of biological effects (8). A number of studies have investigated the effect of IMD on homeostasis regulation, hypertension, myocardial ischemia, heart failure and renal failure (15,17,18). Although previous studies have revealed that the expression of IMD in pancreatic cancer and breast cancer was higher than the control group (19,20), the effect of IMD in tumors, particularly in the occurrence and development of liver cancer, remains unclear. The results of the present study demonstrated that IMD may promote HepG2 cell proliferation (Fig. 1), and this effect is achieved through receptor-dependence (Fig. 2). The autocrine hypothesis proposed that numerous tumor cells may react with self-secreted growth factors, such as basic fibroblast growth factor, and thereby escape from growth inhibition (21,22). The present study also observed that the IMD receptor blocker IMD17-47 can still inhibit the proliferation of HepG2 cells without exogenous IMD stimulation (Fig. 2), indicating that HepG2 cell may express and secrete IMD. This result is concordant with the data obtained by Guo et al. (23).

The Wnt signaling pathway has an important role in the proliferation of tumor cells. Activation of the classical Wnt signaling pathway is dependent on the abnormal accumulation of β-catenin in the cytoplasm, which then enters the nucleus and binds with TCF/LEF to form transcription factor complexes, these complexes then regulate the expression of various downstream genes, including cyclin D1 and c-Myc (24,25). Previous studies have demonstrated that 30-40% of liver cancer cases also exhibit a disorder of the Wnt signaling pathway or a β-catenin gene mutation; β-catenin expression may be observed in the cytoplasm and nucleus of 62-70% of liver cancer cells, and has been observed to be marked in poorly differentiated HCC cell nuclei (26-28). c-Myc is the first discovered downstream target gene of the β-catenin/TCF complex, and c-Myc serves an important role in various biological processes, including cell apoptosis, proliferation, metabolism, DNA repair and angiogenesis (29). The cyclin D1 gene is an additional direct target gene of the β-catenin/TCF pathway, sustained high expression levels of cyclin D1 may cause cells to remain in the S phase of growth, resulting in excessive proliferation and eventual malignant transformation (30). In the present study, the results of the TOPflash/FOPflash luciferase assay indicated that IMD may enhance the transcriptional activity of the β-catenin/TCF complex; the expression of the Wnt downstream genes cyclin D1 and c-Myc were also significantly upregulated, suggesting that IMD may activate the Wnt signaling pathway, thus promoting HepG2 cell proliferation.

In conclusion, the present study demonstrated that, as a member of the CGRP superfamily, IMD binds with receptor, and then activates the Wnt signaling pathway, thus, it serves a role in promoting the proliferation of liver cancer cells, which presents a novel strategy for the treatment of liver cancer. In addition, IMD antagonists may be used as a potential anti-liver cancer agent.

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Competing interests

The authors declare that they have no competing interests.

References