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Short Communication

Identification of matrix metalloproteinases secreted by human hepatocarcinoma HepG2 cells

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ABSTRACT To date, the development of anticancer drugs has been conducted using two-dimensional (2D) cell culture systems. However, since cancer cells in the body are generated and developed in three-dimensional (3D) microenvironments, the use of 2D anticancer drug screening can make it difficult to accurately evaluate the anticancer effects of drug candidates. Therefore, as a step towards developing a cancer cell-friendly 3D microenvironment based on a combination of vinylsulfone-functionalized polyethylene glycol (PEG-VS) with dicysteine-containing crosslinker peptides with an intervening matrix metalloproteinase (MMP)-specific cleavage site, the types of MMPs secreted from human hepatocarcinoma HepG2 cells, a representative cancer cell, were analyzed transcriptionally and translationally. MMP3 was confirmed to be the most highly expressed protease secreted by HepG2 cells. This knowledge will be important in the design of a crosslinker necessary for the construction of PEG-based hydrogels customized for the 3D culture of HepG2 cells.

Keywords: hepatocarcinoma cells, hydrogel, matrix metalloproteinase, three-dimension culture

INTRODUCTION

Human body is organized three-dimensionally by a variety of cells (Cukierman et al., 2002; Abbott, 2003). Therefore, the occurrence, growth, and metastasis of cancers within a body occur in a three-dimensional (3D) microenvironment (Friedrich et al., 2007). Thus, *in vitro* maintenance of cancer cells in a 3D microenvironment is important for evaluating the cytological effects of specific substances. To date, the screening of anticancer drugs has generally been done in a two-dimensional (2D) microenvironment based on cell culture plates (Bhadriraju and Chen 2002; Arrondeau et al., 2010) due to a lack of techniques available to construct 3D cancer cell microenvironments. Thus, a platform that can implement a 3D microenvironment suitable for diverse types of cancer cells will be important for precise anticancer drug screening. As a step towards the development of human hepatocarcinoma HepG2 cell-friendly 3D microenvironments based on the conjugation of vinylsulfone-functionalized polyethylene glycol (PEG-VS) and dicysteine-containing peptides with an intervening matrix metalloproteinase (MMP)-specific cleavage site, we attempted to elucidate a peptide sequence cleaved specifically by MMPs secreted predominantly from HepG2 cells (Yun et al., 2013; Lim et al., 2016). For this, the transcription of different MMPs

and their activities in HepG2 cells were analyzed.

MATERIALS AND METHODS

Culture of human hepatocarcinoma cells

Immortalized human hepatocarcinoma (HepG2) cells were purchased from ATCC and cultured in the HepG2 culture medium consisting of low glucose Dulbecco's Modified Eagle Medium (LG-DMEM; Welgene, Gyeongsan, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) and 1% (v/v) penicillin-streptomycin solution (Welgene) at 37℃ under an atmosphere of 5% CO2 in air. The fresh medium was changed every second day. When the cultured HepG2 cells reached over 80% confluency, cells were dissociated with 0.25% trypsin-EDTA (Welgene) and then 5×10^5 HepG2 cells were replated into 60 mm culture dishes (SPL, Pocheon, Korea). Subculture was conducted at 6-day intervals.

Real-time polymerase chain reaction (RT-PCR)

According to the respective manufacturer's instructions, extraction of total mRNA from cells was conducted using the RNeasy™ mini kit (Qiagen, Hilden, Germany), and synthesis of cDNA from the prepared mRNA was performed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan). Subsequently, transcriptional levels of genes were quantified with Prime Q-Mastermix (Genetbio, Nonsan, Korea) using a qTOWER3 (Analytikjena, Jena, Germany). Melting curve data was analyzed for identifying PCR specificity. The mRNA levels are presented as $2^{-\Delta Ct}$ where Ct = threshold cycle for target amplification and $\Delta Ct = Ct_{\text{target gene}}$ (specific genes for each sample) – $Ct_{\text{internal reference}}$ (β-actin for each sample). Primers were designed using Primer 3 software (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA, USA) with the information of cDNA sequences obtained from GenBank. Table 1 shows general information and sequences of primers.

MMP antibody array

For preparing conditioned medium containing MMP proteins produced in HepG2 cells, cells with 80% confluency were cultured for 4 days in the fresh HepG2 culture medium and cell debris within the incubated culture medium was removed through centrifugation with the micro centrifuge Smart R17 plus (Hanil, Daejeon, Korea). In addition, HepG2 culture medium incubated for 4 days in HepG2 cell-free culture dishes was prepared as a negative control. Subsequently, the presence or absence of 7 MMP proteins (MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, and MMP13) in each medium was confirmed with Human MMP Antibody Array (ab-134004; Abcam®, Cambridge, UK) using with chemiluminescence imaging system Ez-Capture II (AE-9150; Atto, Tokyo, Japan), and ImageJ software (ver. 1.52a; NIH, Bethesda, MD, USA) was used for quantification of spots derived from each MMP protein.

Statistical analysis

All numerical data in each parameter was analyzed using the Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC, USA). Comparisons among groups were conducted using a generalized linear model (PROC-GLM) in the SAS package. A p value of less than 0.05 was determined as a significant difference.

Table 1. Oligonucleotide primers and PCR cycling conditions

Genes	GenBank number	Primer sequence		Size (bp)	Temp (℃)
		Sense (5' > 3')	Anti-sense (5' > 3')		
ACTB	NM_001101.5	GAGCGAGCATCCCCCAAAGT	TTGGGAGAGGACTGGGCCAT	166	60
MMP1	NM_002421	GAGCAGATGTGGACCATGCCA	CCTGGGCCTGGTTGAAAAGC	199	60
MMP2	NM_004530	TGAGGACTACGACCGCGACAA	CATCTTTCCGTCACTGCGGC	168	60
MMP3	BC074815	CGAGCTGGATACCCAAGAGGC	TTGGGAAAGCCTGGCTCCAT	175	60
MMP8	NM_002424	CCATTCTTTGGGGCTCGCTC	CAGGGTTTGGGTGTGCTTGG	186	60
MMP9	NM_004994	GTTTGGAAACGCAGATGGCG	GCATTGCCGTCCTGGGTGTA	195	60
MMP10	NM_002425	GGCTCTTTCACTCAGCCAAC	CTCAGATCCCGAAGGAACAG	182	60
MMP13	NM_002427	GGCGACTTCTACCCATTTGA	GGTCCTTGGAGTGGTCAAGA	190	60

ACTB, actin beta; MMP, matrix metalloproteinases; Temp, temperature.

RESULTS AND DISCUSSION

In a 3D cancer cell culture system using hydrogels based on PEG functionalized by vinylsulfone, interconnection between PEG-VS arms and the biodegradability of PEG-based hydrogels are essential for the successful formation of PEG-based hydrogels and construction of the microspace required for the proliferation of cancer cells inside the hydrogel. Therefore, a crosslinker should contain an amino acid sequence cleaved specifically by MMPs secreted by cancer cells, flanked by a cysteine at both ends.

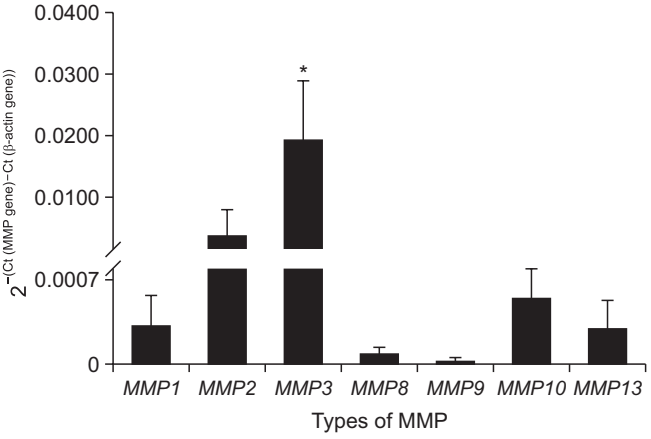

Fig. 1. Transcription of genes encoding matrix metalloproteinases (MMPs) in HepG2 cells. HepG2 cells were cultured for 6 days in HepG2 cell culture medium and the mRNA levels of MMPs (MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, and MMP13) were quantitatively monitored using real-time PCR. MMP3 exhibited the highest mRNA level; the expression of the remaining MMP genes decreased in the following order: MMP2 > MMP10 > MMP1 > MMP13 > MMP8 > MMP9. Data are shown as the mean ± standard deviation (SD) of four independent experiments. *$p < 0.05$.

As a step towards inserting MMP-specific cleavage peptide sequences into a crosslinker required for the construction of a 3D PEG-based hydrogel customized to HepG2 cells, we analyzed the types of MMPs released from HepG2 cells. Among seven MMP genes, MMP3 showed the highest level of transcription; decreased levels of transcription of the remaining genes were observed in the following order: MMP2 > MMP10 > MMP1 > MMP13 > MMP8 > MMP9 (Fig. 1). In addition, a quantitative analysis of MMP proteins released from HepG2 cells demonstrated that protein expression of MMP3 was the highest; decreased levels of protein expression of the remaining MMP proteins were observed in the following order: MMP13 > MMP10 > MMP9 > MMP1. Expression of MMP2 and MMP8 was not detected in HepG2 cells (Fig. 2). Thus, we confirmed that MMP3 is an active protease released from HepG2 cells, indicating that amino acid sequences cleaved specifically by MMP3 will be useful in the development of a crosslinker for a HepG2 cell 3D culture system.

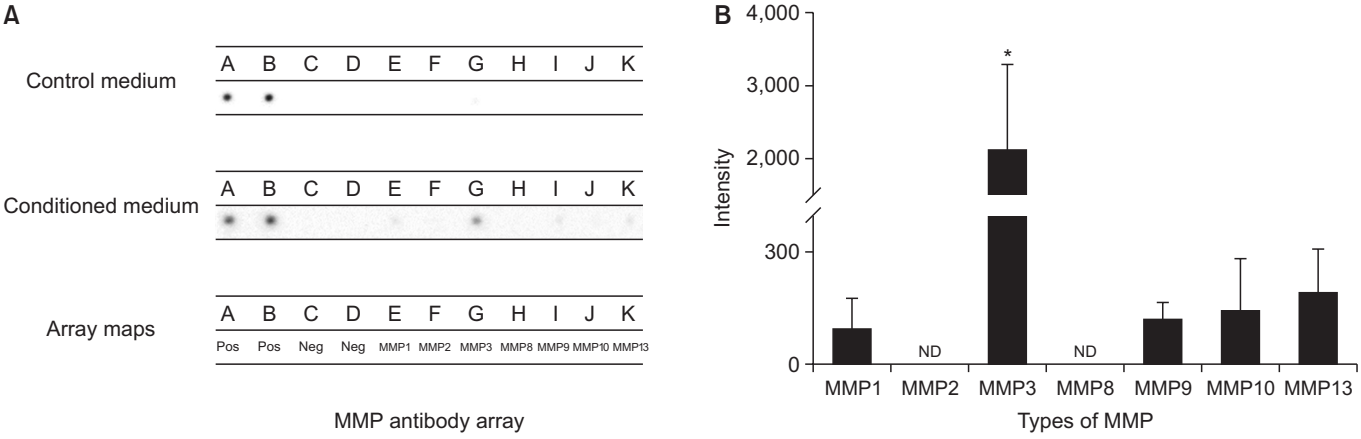

Fig. 2. Identification of MMP proteins secreted by HepG2 cells. To prepare a conditioned medium including the MMP proteins released from HepG2 cells, fresh HepG2 culture medium was added to HepG2 cells at 80% confluency and incubated for 4 days (Conditioned medium). For the control, fresh HepG2 culture medium was incubated for 4 days in a HepG2 cell-free culture dish (Control medium). The different MMPs present in the conditioned medium were identified using a chemiluminescence immunoassay and quantified using ImageJ software. Five MMP proteins (MMP1, MMP3, MMP9, MMP10, and MMP13) were observed in the conditioned medium; two (MMP2 and MMP8) were not expressed. No MMP proteins were observed in the control condition. The protein level of MMP3 was the highest among the MMPs detected. The expression of the remaining MMPs decreased in the following order: MMP13 > MMP10 > MMP9 > MMP1. Data are shown as the mean ± SD of three independent experiments. *$p < 0.05$. ND, not detected; Pos, positive control; Neg, negative control.

MMPs are generally synthesized and secreted according to cell type (Elkington et al., 2009; Tandara and Mustoe 2011); MMPs also play a major role in the regulation of cell behaviors such as proliferation, migration, differentiation, angiogenesis, apoptosis, and host defenses (Sternlicht et al., 2000; Snoek-van Beurden and Von den Hoff 2005; Winer et al., 2018). In the case of hepatocellular carcinoma, MMP3 is specifically produced (Bodey et al., 2000; Naim et al., 2017) and it plays a pivotal role in the induction of tumor invasion, metastasis, and angiogenesis (Sternlicht et al., 2000; Monvoisin et al., 2002). Previous studies support the high level of secretion of MMP3 by HepG2 cells immortalized from a liver hepatocellular carcinoma.

CONCLUSION

MMP3 was shown to be the most highly expressed protease in HepG2 cells. This result will be important in the design of a crosslinker for a PEG-based hydrogel customized for the 3D culture of HepG2 cells.

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Ethical Approval: This study was approved by the Kangwon National University Institutional Review Board (IRB) according to the guidelines of the Kangwon National University IRB committee (IRB approval no. KWNU-IRB-2022-01-010).

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Consent to Publish: Not applicable.

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