Double-stranded RNA-induced TLR3 activation inhibits angiogenesis and triggers apoptosis of human hepatocellular carcinoma cells

ZHONGYING GUO[1*], LI CHEN[1*], YUANYUAN ZHU[2], YIXIN ZHANG[3], SONG HE[3], JIN QIN[1], XIAOJUN TANG[2], JIAMING ZHOU[1] and YINGZE WEI[1]

[1]Department of Pathology, Nantong University School of Medicine, Nantong 226001; [2]The Affiliated Tumor Hospital, Nantong University, Nantong 226361; [3]Biomics Biotechnologies Co., Ltd., Nantong 226010, P.R. China

Abstract. Toll-like receptor 3 (TLR3) is a member of the Toll-like receptors which recognize pathogen-associated molecular patterns leading to the activation of the innate immune response. Recent reports have strongly indicated that they play important roles in cancer cells. Since TLR3 has been recently suggested as a possible therapeutic target in certain types of cancers, in the present study, TLR3 expression and its function were explored in hepatocellular carcinoma (HCC) and human umbilical vein endothelial cells (HUVECs). The expression of TLR3 in various HCC cell lines and HUVECs was detected using quantitative real-time PCR (qRT-PCR) and immunocytochemistry. TLR3 activity was determined by Luciferase reporter assays. The effects of TLR3 double-stranded RNA (dsRNA) agonists on angiogenesis were tested by aortic ring assay and HUVEC tube formation experiments. After dsRNA treatment, cell apoptosis was assessed by Annexin V and PI staining through FACS, and the migration ability was measured by a migration assay. The results showed that TLR3 was expressed in HCC cell lines and HUVECs at the mRNA and protein level. Luciferase reporter assays demonstrated that TLR3 was activated by the dsRNA analog BM-06 or poly(I:C). Rat aortic ring outgrowth and endothelial cell tube formation were suppressed after treatment with dsRNA. In addition, dsRNA triggered apoptosis in MHCC97H, SMMC-7721 and HUVEC cell lines and inhibited cell migration. In conclusion, TLR3 agonists not only affect tumor microenvironment by suppressing angiogenesis but also directly induce tumor cell apoptosis and inhibit tumor cell migration. TLR3 may be a new target for HCC therapy.

Correspondence to: Dr Li Chen, Department of Pathology, Nantong University School of Medicine, Nantong 226001, P.R. China
E-mail: chenlipro@163.com

*Contributed equally

Introduction

Mammalian Toll-like receptor 3 (TLR3), a member of the interleukin-1 (IL-1)/Toll receptor superfamily, recognizes double-stranded RNA (dsRNA) (1), such as that found in viral genomes or replication intermediates, which through dimerization (2-4) and phosphorylation (5) initiate a signaling cascade that can ultimately result in inhibition of angiogenesis (6-8) and apoptosis of cancer cells and blood endothelial cells (BECs) (9,10). Many researchers have demonstrated that the growth of solid neoplasms is always accompanied by angiogenesis (11,12). The population of tumor cells and the population of capillary endothelial cells within a neoplasm may constitute a highly integrated ecosystem. The two cell populations depend upon each other. Tumor cells are capable of stimulating endothelial cells to form new capillary sprouts which support tumor growth, while nonvascularized tumors are held in a dormant state, unable to grow beyond a size larger than 2-3 mm^3 (13). Similarly, endothelial cells may have an indirect effect on the rate of tumor growth, metastasis, invasion, progression, dormancy and apoptosis. Tumor growth inhibitors are considered the most promising approach to cancer therapy; thus there are a myriad of anti-angiogenic strategies, such as antibodies, soluble receptors and receptor antagonists, which target various growth factors and cytokines involved in abnormal neovascularization. Among the various proposed strategies, small interfering RNA (siRNA) has attracted much attention as a new therapeutic platform for achieving target-specific gene silencing. However, 'naked' siRNA (whether chemically modified or not) cannot be uptaken by mammalian cells without cell-permeating entities (14-16), and unintended 'off-target' effects remain formidable (17).

Recently, Kleinman *et al* found that 21-nt or longer siRNA suppressed hemangiogenesis in mouse models of choroidal and dermal neovascularization not via RNAi but by activating cell surface TLR3 on BECs in a sequence- and target-independent manner (7). At present, TLR agonists and antagonists are being developed for the treatment of cancer and as adjuvants for potential new vaccines to prevent or treat cancers (8,9,18). Hepatocellular carcinoma (HCC) is a common malignancy and is chemoresistant to currently available chemotherapeutic

agents. It is the leading cause of cancer-related deaths in the world, with increasing incidence in many countries (19). Recently, researchers have confirmed that TLR3 expression exists not only in immune cells, but also in other cells, such as prostate, human colon, pharyngeal and human breast cancer cells, and human melanoma cells (9,20-22). Thus, we investigated whether TLR3 is expressed in HCC cells and explored the role of the receptor in HCC cells. In this study, BM-06, a synthetic dsRNA analog, was used as a TLR3 agonist, which was designed and synthesized by Biomics Biotechnologies Co., Ltd. according to the highly conserved domain of entero-virus type 70 (EV70) virus genome sequences. Biomics first designed several specific small nucleotides, which can efficiently activate TLR3, then through mouse age-related macular degeneration (AMD) models to screen a small nucleotide sequence which can significantly inhibit choroid neovascularization (CNV) named BM-06. Polyinosinic-polycytidilic acid [poly(I:C)], a dsRNA mimic, which has been proven to active TLR3 was used as the positive control.

HCC cell lines (MHCC97H, SMMC-7721, BEL-7402, HepG2) were found to express TLR3. After activation of TLR3 in endothelial and HCC cells by BM-06, BM-06 was found to inhibit angiogenesis affecting the tumor microenvironment and driving HCC and endothelial cells to apoptosis.

Materials and methods

Cell culture. HCC cells MHCC97 and HepG2, were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. HCC cell lines SMMC7721, BEL-7402 and human umbilical vein endothelial cells (HUVECs) were cultured in DMEM medium supplemented with 10% calf serum (CS) (Invitrogen), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

HUVECs were used between the 3rd and 8th passages. All of the cell lines were purchased from the Shanghai Cellular Institute of the Chinese Scientific Academy, and cell cultures were maintained at 37°C in a humidified incubator in 5% CO_2.

Reagents. Poly(I:C) was purchased from Invivogen (San Diego, CA), Matrigel was purchased from BD Biosciences (Bedford, MA) and the goat anti-human TLR3 primary and CD31 primary antibodies were obtained from Santa Cruz Biotechnology. The pNFκB-TA-luc reporter plasmid was obtained from Beyotime Institute of Biotechnology, and the Renilla luciferase plasmid and Dual Luciferase reporter assay system were obtained from Promega Corp. (Madison, WI). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA), and BM-06 (25 nt) was kindly provided by Biomics Biotechnologies Co., Ltd. (Nantong, Jiangsu, China).

RNA isolation and one-step quantitative real-time PCR (qRT-PCR). Total RNAs were isolated from the four HCC cell lines and HUVECs using TRIzol reagent (Invitrogen). Reactions were performed in a 25-μl volume with 12.5 μl of 2X master mix, 1 μl of each primer mix (10 μM/ml), 0.5 μl of 50X SYBR-Green I and 4 μl RNA using the One-Step Quantace kit (Quantace, Australia). The PCR mixtures were first subjected to 30 min at 42°C for reverse transcription and initially denatured at 94°C for 10 min and then 40 cycles of amplifi-cation at 94°C for 20 sec, 56°C for 30 sec and 72°C for 30 sec. All samples were run in triplicate. Amplification of the house-keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was conducted for each sample as an endo-genous control. In the experiment, the following sequences for the forward and reverse primers were used: for TLR3, 5'-CCT GGTTTGTTAATTGGATTAACGA-3' (forward) and 5'-TGA GGTGGAGTGTTGCAAAGG-3' (reverse); for GAPDH, 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAG ATGGTGATGGGATTTC-3' (reverse).

Detection of TLR3 by immunocytochemistry. A total of $2x10^4$ cells were cultured on a small round slide in a 24-well plate overnight. Then cells were fixed for 30 min with 4% para-formaldehyde in PBS and permeabilized for 5 min with 0.2% Triton X-100 in PBS before blocking for 2 h with 1% bovine serum albumin (BSA). Subsequently, cells were incubated overnight at 4°C with the TLR3 primary antibody (1:100 dilution), and then with TRITC-conjugated secondary antibodies (1:100 dilution, Sigma-Aldrich, St. Louis, MO) for 2 h. The nuclei were counterstained with Hoechst 33258 (5 μg/ml) (Invitrogen) for 10 min, and the stained cells were observed with immunofluorescence microscopy.

Luciferase reporter assays. MHCC97H, SMMC7721 and HUVECs were seeded in 24-well plates at a density of $5x10^5$/ ml overnight. The following day, cells were transfected with 667 ng of pNFκB-TA-luc reporter plasmid (for NF-κB activation) and 133 ng Renilla luciferase-expressing plasmid as an internal control using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, the cells were left untreated or stimulated with BM-06 (10 μg/ml) or poly(I:C) (10 μg/ml) for 6 h before harvesting. Firefly luciferase and Renilla luciferase activities were analyzed by using the Dual luciferase reporter assay system. Firefly luciferase activities were normalized to Renilla luciferase activities. Data are expressed as the mean relative stimulation ± SD.

Endothelial cell tube formation assay. Matrigel was plated at 100 μl/well in 48-well plates and allowed to reach the solid phase after 30 min in a 37°C incubator. HUVECs were then suspended in BM-06 (10 μg/ml) or poly(I:C) (10 μg/ml) or vehicle buffer (PBS) and plated on top of the Matrigel at a density of $3x10^4$ cells/well. After a 16-h incubation, the wells were imaged on an inverted phase contrast microscope. HUVECs normally form a branching plexus of tubes on artificial extracellular matrix, such as Matrigel. We defined a tube forming node as one which had three or more branches coming from a common center. Quantification was blinded and carried out by counting each nodal branch point that had three or more branches. Branch point counts per image constituted the raw data for statistical analysis. There were four images per treatment group. Experiments were run in triplicate.

Rat aortic ring assay. Tissue culture plates (48-well) were coated with 100 ml of Matrigel and allowed to gel for 30 min at 37°C in 5% CO_2. Thoracic aortas were isolated from 6-week-old Sprague-Dawley rats and immediately placed in endothelial
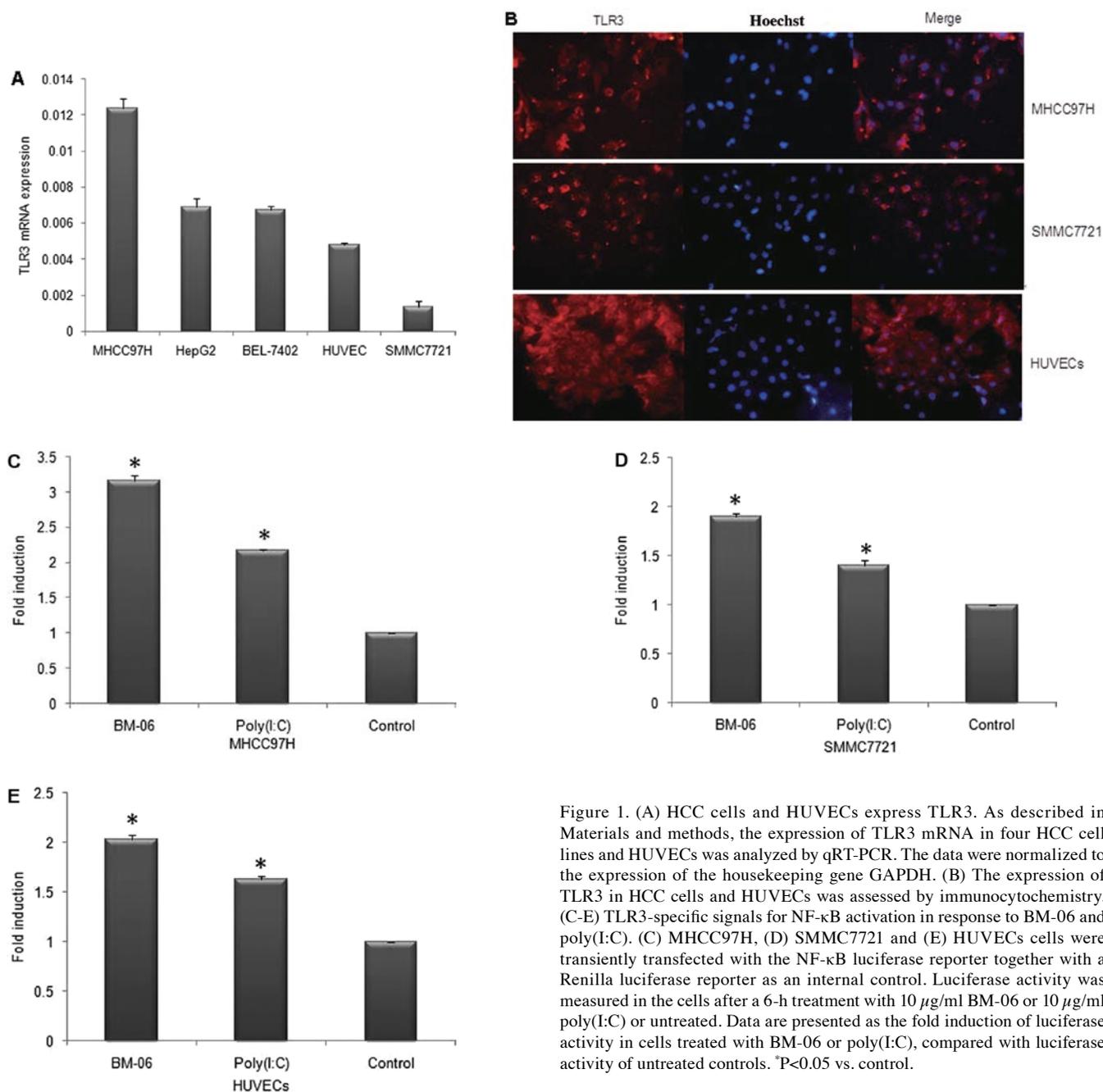
Figure 1. (A) HCC cells and HUVECs express TLR3. As described in Materials and methods, the expression of TLR3 mRNA in four HCC cell lines and HUVECs was analyzed by qRT-PCR. The data were normalized to the expression of the housekeeping gene GAPDH. (B) The expression of TLR3 in HCC cells and HUVECs was assessed by immunocytochemistry. (C-E) TLR3-specific signals for NF-κB activation in response to BM-06 and poly(I:C). (C) MHCC97H, (D) SMMC7721 and (E) HUVECs cells were transiently transfected with the NF-κB luciferase reporter together with a Renilla luciferase reporter as an internal control. Luciferase activity was measured in the cells after a 6-h treatment with 10 μg/ml BM-06 or 10 μg/ml poly(I:C) or untreated. Data are presented as the fold induction of luciferase activity in cells treated with BM-06 or poly(I:C), compared with luciferase activity of untreated controls. *P<0.05 vs. control.

cell basal medium (ECM) after careful removal of fibroadipose tissues. The aortas were cut into cross sections, 1-mm long, and placed on Matrigel-coated wells, and covered with an additional 100 μl of Matrigel. After the second layer of Matrigel had set, complete medium was added, and the rings were incubated for another 24 h. The next day, the medium was replaced with basal medium supplemented with 2% FBS. Aortic rings were treated daily with either the vehicle buffer (PBS), BM-06 (10 μg/ml) or poly(I:C) (10 μg/ml) for 6 days, and images were captured on day 6 using a x4 magnification. Experiments were repeated three times using aortas from six different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop CS3. Endothelial cells of the sprout grown from the rat aortic ring were identified by CD31 primary antibody which is a specific surface marker of vascular endothelial cells.

Annexin V/PI for cell apoptotic analysis. Cells were harvested with 0.25% trypsin without EDTA after application of BM-06 (10 μg/ml) or poly(I:C) (10 μg/ml) for 24 h, rinsed twice with PBS, and stained using the Annexin V-FITC apoptosis detection kit I (BD Biosciences). Analysis was performed on the FACSCalibur using CellQuest software.

Cell migration assays. Twenty-four transwell units with an 8-μm pore size were used for monitoring *in vitro* cell migration. HUVECs, after pre-treatment with the vehicle buffer (PBS) or the indicated dsRNA for 18 h, were added to the upper chamber at a density of 1x10^5 cells/well. dsRNA was continuously present during the experiments. After 6 h (HUVECs) or 24 h (for SMMC7721 and MHCC97H) of incubation at 37˚C, the non-migrated cells on the upper surface of the filter were removed by scraping. The cells that had migrated to the
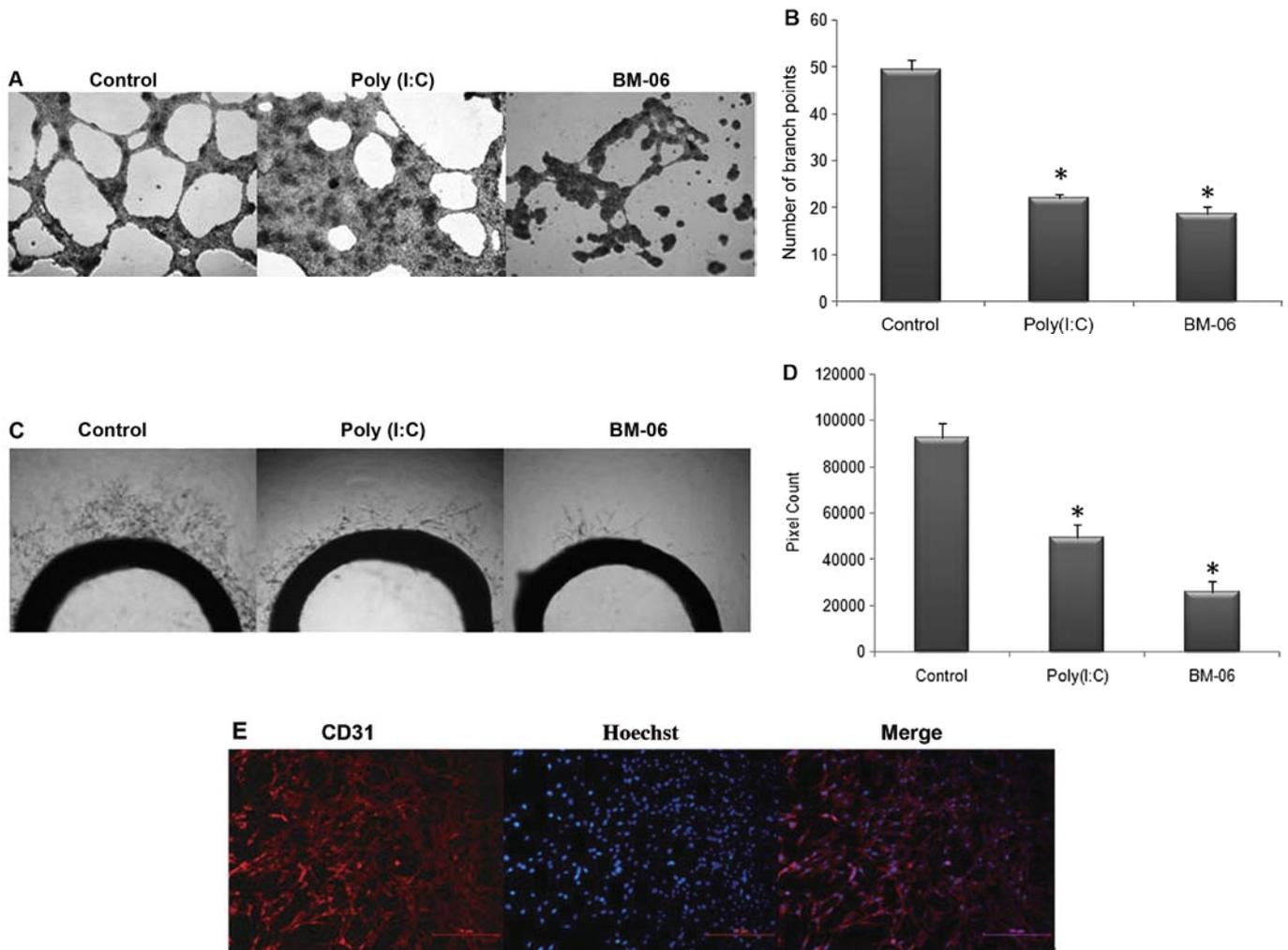
Figure 2. Effects of BM-06 and poly(I:C) on the tube formation and rat aortic ring models. (A) Representative images illustrating the ability of BM-06 and poly(I:C) to inhibit HUVEC tube formation on Matrigel. The first image represents tube formation of the control group, the second represents tube formation with poly(I:C) at 10 μg/ml and the third image represents tube formation with BM-06 at 10 μg/ml. (B) Quantification of the inhibitory activity of BM-06 and poly(I:C) on tube formation. (C) Representative rat aortic rings are shown from the treatment experiments. These rings were treated with BM-06 at 10 μg/ml, poly(I:C) at 10 μg/ml or vehicle alone. (D) Pixel counts representing the outgrowth of microvessels was quantified. Data shown are the mean $\pm$ SD from three independent experiments. (E) Vascular endothelial cells of the outgrown sprout were identified with the vascular endothelial cell-specific surface marker CD31 antibody using immunocytochemistry. *P<0.05 vs. control.

lower side of the filter were stained with crystal violet and photographed. To quantify the migrated cells, stained cells were counted in five random fields (x100 magnification) from each of the three transwell filters under each condition. Results were reported as the mean number of cells migrated per microscopic field.

Statistical analysis. Significance of the difference between groups was assessed with one-way ANOVA followed by LSD multiple comparison tests. P-value <0.05 was considered to be statistically significant. Statistical analysis was performed with SPSS software 17.0.

Results

HCC cells and HUVECs express TLR3, and the TLR3 ligand induces NF-κB activation. Recent reports have indicated that TLR3 plays important roles in many cancer types and endothelial cells, and it has recently been suggested as a possible therapeutic target in some cancer cell lines (9,10,20). The expression of TLR3 at both the mRNA and the protein level was evaluated by qRT-PCR and immunocytochemistry in four HCC cell lines and HUVECs. As shown in Fig. 1A and B, TLR3 was expressed in all of these cell lines. However, at various levels, the qRT-PCR result showed that of these four HCC cell lines TLR3 was expressed strongly in MHCC97H, moderately in HepG2 and BEL-7402 and weakly in SMMC7721. Therefore, MHCC97H and SMMC7721 HCC cell lines were selected for subsequent experiments. It has been demonstrated that when TLR3 is activated by its agonist it induces the activation of the classical NF-κB pathway in many different cell types (1,23). To verify whether TLR3 was functional after stimulation with BM-06 or poly(I:C) in MHCC97H, SMMC7721 and HUVEC cell lines, we incubated the cells with extracellular BM-06 or poly(I:C) and evaluated its ability to increase the NF-κB signaling pathway in pNF-κB-luciferase-transfected cells by measuring reporter gene activity. Results are shown in Fig. 1C-E. After a 6-h incubation with BM-06 or
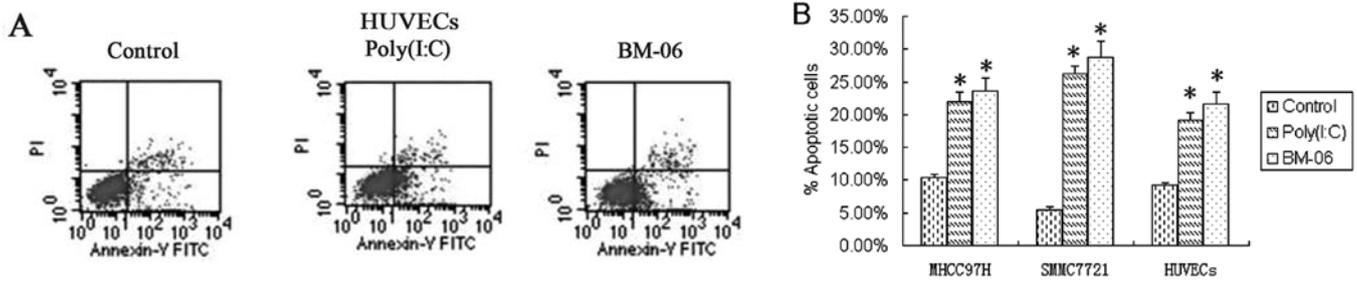
Figure 3. dsRNA induces apoptosis in MHCC97H and SMMC7721 cells and HUVECs. (A) Apoptosis of HUVECs after stimulation with BM-06 or poly(I:C). (B) Exposure of MHCC97H, SMMC7721 and HUVECs to BM-06 (10 μg/ml) or poly(I:C) (10 μg/ml) for 24 h induced an increase in apoptosis. *P<0.05 vs. control.
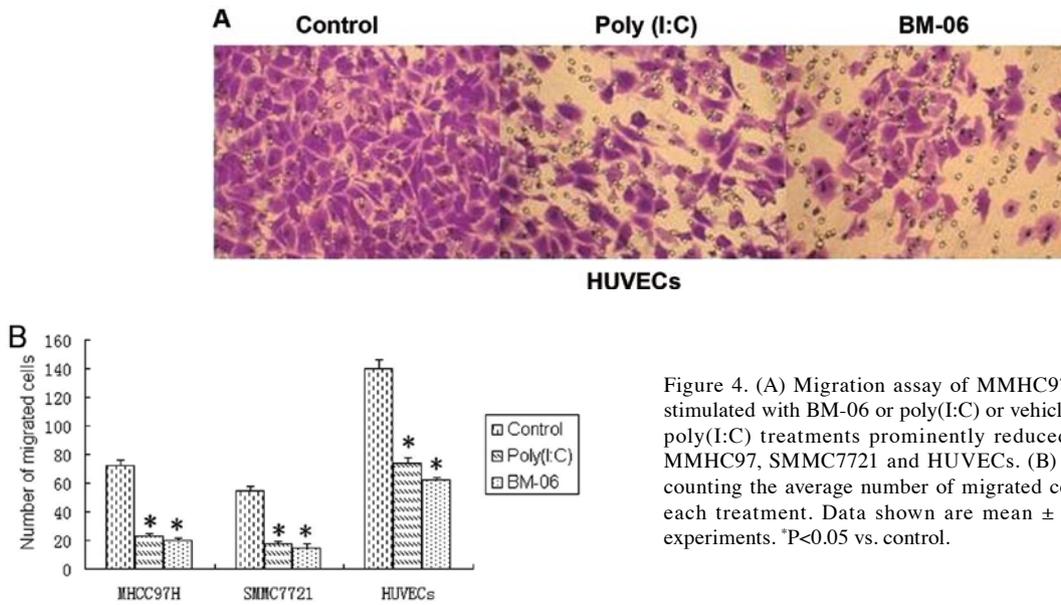

Figure 4. (A) Migration assay of MMHC97, SMMC7721 and HUVECs stimulated with BM-06 or poly(I:C) or vehicle alone as control. BM-06 and poly(I:C) treatments prominently reduced the migration potential of MMHC97, SMMC7721 and HUVECs. (B) Migration was quantified by counting the average number of migrated cells in five different fields for each treatment. Data shown are mean ± SD from three independent experiments. *P<0.05 vs. control.

poly(I:C), NF-κB luciferase activity was significantly higher (P<0.05) in the three cell lines compared with the unstimulated cells.

dsRNA modulates tube formation and vascular sprouting. We investigated whether dsRNA alters the endothelial tube-forming activity of HUVECs, often referred to as an *in vitro* angiogenesis assay (24,25). To do this, HUVECs were plated on Matrigel and treated with BM-06, poly(I:C), or vehicle alone as control. Within 18 h, untreated cells formed an organized network of endothelial tubes. In contrast, tube formation in BM-06- or poly(I:C)-treated cells markedly decreased (Fig. 2A and B) (P<0.05). Furthermore, the effects of BM-06 and poly(I:C) in the rat aortic ring assay were evaluated by an *ex vivo* assay of vascular sprouting that depends on the function of a variety of angiogenic growth factors and their receptors (26). Sections of rat aorta were cultured in growth factor-reduced Matrigel and treated with BM-06 or poly(I:C) or vehicle alone, and the area of vascular sprouts was measured on day 6. Compared with the control rings, BM-06 or poly(I:C) induced a statistically significant reduction in the area of vascular sprouts (Fig. 2C and D) (P<0.05). Taken together with the tube formation assays, these results indicate that dsRNA had a potent inhibitory effect on angiogenesis.

TLR3 agonist dsRNA directly triggers apoptosis in HCC cells and HUVECs. Recently, a direct apoptotic effect of poly(I:C) on breast and prostate cancer cells was demonstrated (9,20). To investigate whether BM-06 and poly(I:C) are able to induce apoptosis in MHCC97H, SMMC7721 and HUVECs, the cells were cultured with 10 μg/ml of the dsRNA analog BM-06 or poly(I:C) or vehicle alone as control for 24 h. The percentages of apoptotic cells in the MHCC97H cell line were 23.62, 22.02 and 10.43%; for SMMC7721 cells, 28.66, 26.32 and 5.45%; and for HUVECs, 21.63, 19.20 and 9.24% following BM-06 or poly(I:C) stimulation or vehicle, respectively (Fig. 3A and B). The percentages of apoptotic cells in both the BM-06 and poly(I:C) groups in these three cell lines were higher than that in the control group (P<0.05). Collectively, these data demonstrated that TLR3 agonists directly trigger the apoptosis of MHCC97H and SMMC7721 cells as well as HUVECs.

dsRNA inhibits HCC and HUVEC migration. The effect of dsRNA on MMHC97, SMMC7721 and HUVEC migration was investigated by using a transwell migration assay. The migrated cells were quantified over 24 h. The number of migrated cells treated with BM-06 or poly(I:C) or vehicle was 20, 23, 72 for MHCC97H; 15, 18, 55, for SMMC7721; and 62,

74, 140 for HUVECs, respectively (Fig. 4A and B). Cell migration was significantly inhibited after the cells were treated with BM-06 or poly(I:C) compared with the control group (P<0.05).

Discussion

The results of the present study demonstrated that the four human HCC cell lines and HUVECs express TLR3 at the mRNA and protein level. It is well accepted that long dsRNA, such as poly(I:C) or those of a viral origin, bind TLR3 (1). The precise minimum length of dsRNA required to interact with and activate TLR3 has been the focus of intense investigation. Kleinman *et al* suggest that at least 21 nucleotides are required to activate TLR3 (7). In this study, the length of the designed dsRNA analog BM-06 was 25 nt. We found that the TLR3 agonist BM-06 and poly(I:C) were able to inhibit angiogenesis, and our results confirmed previous studies of Kleinman *et al* (7) and Cho *et al* (6) who demonstrated that 21-nucleotide or longer dsRNAs exhibited an anti-angiogenic effect in mice not through RNAi but via TLR3 (7). Cho *et al* (6) also reported that naked, unmodified 21-nt siRNA was not internalized by mouse or human lymphatic endothelial cells (LECs) and BECs, and this naked 21-nt siRNA suppressed both blood and lymphatic vessels by activating cell surface TLR3 regardless of the target.

Moreover, we showed that the TLR3 agonist BM-06 and poly(I:C) were able to induce significant *in vitro* cell apoptosis and inhibit migration through TLR3 signaling in these HCC cells and HUVECs. These data are consistent with recent observations on the pro-apoptotic properties of poly(I:C) upon TLR3 engagement in other human cancer cell lines, such as human melanoma, human head and neck cancer, and human cervical cancer cells (21,27,28).

To date, the signaling pathway through which TLR3 mediates cancer cell apoptosis has still not been clarified. To elucidate the mechanism of TLR3-mediated tumor cell apoptosis, researchers have deciphered the molecular mechanisms of dsRNAs which exhibit apoptotic function in cancer cells, however, there is no uniform conclusion. Rasschaert *et al* (29) reported that dsRNA plus IFN-γ triggered β-cell apoptosis by two complementary pathways, namely TLR3-TRIF-NF-κB and STAT-1. Salaun *et al* (9) also reported that TLR3 triggered apoptosis in breast cancer cell lines directly via the TRIF-dependent pathway, whereas apoptosis of endothelial cells stimulated with exogenous dsRNA was mostly dependent on the extrinsic caspase pathway (10,30). In addition, Nomi *et al* (28) identified that stimulation of TLR3 by poly(I:C) induced cell apoptosis in human head and neck squamous carcinomas through survivin-mediated pathways. Taken together, the mechanism for TLR3-induced cancer cell apoptosis requires further studies.

HCC is currently the fifth most common cancer in the world and the third cause of cancer-related mortality (31). The treatment of HCC has traditionally consisted of locoregional strategies: surgery (partial resection or transplantation) or interventional radiologic procedures, such as chemoembolization or ablative techniques. Although major advances in HCC therapy have been achieved, none are fully effective. Thus ongoing strategies to treat HCC are needed. TLR3

agonists have been used in the past to treat cancer patients, demonstrating a good safety and tolerability profile (32). Together with our results, the use of TLR3 agonists with multifunctional effects including angiogenesis suppression which can alter the tumor microenvironment and directly sacrifice cancerous cells and simultaneously inhibit cancer cell migration is promising. This is may provide new clinical perspectives for the use of TLR3 agonists as multifunctional agents.

Acknowledgements

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