Polyinosinic-polycytidylic acid liposome induces human hepatoma cells apoptosis which correlates to the up-regulation of RIG-I like receptors

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Toll-like receptor 3 and RIG-I like receptors (RLRs; MDA5, RIG-I) are involved in cell growth inhibition and apoptosis. However, the toll-like receptor 3-related apoptotic pathway is insensitive to direct polyinosinic-polycytidylic acid (dsRNA analog) stimulation in hepatoma cells. To determine whether the strategy of transferring polyinosinic-polycytidylic acid into cells (polyinosinic-polycytidylic acid-liposome) could induce apoptosis in hepatoma cells through cytoplasm receptors, we examined the responses of innate immune receptors RLRs and toll-like receptor 3 in response to different stimulation. We found that the apoptosis could exclusively be detected under polyinosinic-polycytidylic acid-liposome stimulation, which involved the activation of the caspase pathway. Besides, the expression of RIG-I, MDA5, IFNβ and interferon-stimulated gene 15 was increased significantly at an early stage. Moreover, the growth inhibition of polyinosinic-polycytidylic acid-liposome was confirmed in a mouse model. Taken together, these results suggest polyinosinic-polycytidylic acid-liposome could be used as a potential apoptotic agent in hepatocellular carcinoma cells and imply a potential therapeutic strategy. (*Cancer Sci* 2009; 100: 529–536)

Cancer is a major health problem worldwide. To define specific targets to enhance cell differentiation and to seek effective strategies for clinical tumor therapy have been the aims of many research groups. Polyinosinic-polycytidylic acid (PIC) is the analog of double strand RNA (dsRNA), which has been demonstrated to be efficacious in antitumor immunotherapy for a long time.[1,2] However, cells treated directly with PIC could not trigger cytotoxic activity against various tumor cell lines *in vitro*. Alternatively, the complex of PIC and cationic liposome (PIC-liposome) is effective on tumor growth inhibition,[3] but the mechanisms are controversial.

RLRs (RIG-I like receptors) were recently discovered as interferon-related innate immune receptors, which consist of three family members: retinoic acid inducible gene I (*RIG-I*), melanoma differentiation-associated gene 5 (*MDA5*) and the laboratory of genetics and physiology gene 2 (*LGP2*). They all have the unique structural combination of an N-terminal caspase recruitment domain (CARD) and a C-terminal DExD/H RNA helicase domain, except *LGP2*, which lacks the CARD homology and functions as a negative regulator of RIG-I and MDA5.[4,5] Double-stranded RNA (dsRNA)[4,6] and IFNβ[7] can induce the forced expression of RLRs both *in vivo* and *in vitro*. In contrast to toll-like receptor 3 (TLR3), which is expressed restrictedly to a specific cell type and binds to extracellular and endosomal dsRNA,[8] RLRs are expressed ubiquitously in different tissues and distributed in cytoplasm, recognizing intracellular dsRNA.[4–6,9] Cumulative evidence documented that ectopic expression of *MDA5* led to growth inhibition and/or apoptosis in various cancer cells.[7,10] The mouse homolog of *MDA5*, Helicard, might be involved in apoptosis by cleavage of Helicard between the CARD and helicase domain by caspases.[11] Similar cleavage was found in human *MDA5*.[12] *RIG-I* might also possess tumor suppressor properties regulated by tumor suppressor *IRF-1*.[13] In addition, another dsRNA sensor TLR3 has long been demonstrated to be participating in apoptosis through the activation of the Fas-Associated protein with Death Domain (FADD)–caspase-8 signaling pathway. TIR domain-containing adaptor inducing IFNβ (TRIF) over-expression alone may also lead to apoptosis by the same pathway.[14,15]

It has been reported that PIC-liposome suppresses the growth of melanoma both *in vitro* and *in vivo* while it exhibits no effect through extracellular delivery.[16] However, PIC could directly induce the expression of TNF-related apotosis-inducing ligand (TRAIL) and apoptosis in human intrahepatic biliary epithelial cell lines with constant expression of TLR3[17] whereas hepatocyte cell lines, HepG2 and Huh7, were deficient in signaling in response to direct PIC stimulation with a low abundance of TLR3 mRNA.[14,17,18] Thus, the proapoptotic properties of RLRs and TLR3 are still ambiguous in different types of tumor cells.

In this study, we demonstrated that PIC-liposome could induce apoptosis in PIC-insensitive hepatocellular carcinoma cells and up-regulate the mRNA levels of cytoplasm receptor RLRs. We provide evidence that RLRs might be involved in the programmed cell death of hepatocellular carcinoma cells, which implies a potential therapeutic application of PIC-liposome.

Materials and Methods

Cell lines. Human hepatoma cell line HepG2 was obtained from the Shanghai Cell Bank (Shanghai, China). Cells were grown in RPMI medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin in 95% air–5% CO_2, incubated at 37°C.

Animals. Male BALB/C nude mice of 3–4 weeks in age, weighing 10–15 g, were purchased from Shanghai Experimental Animal Center, Chinese Science Academy (Shanghai, China). The mice were maintained under controlled conditions (specific pathogen-free, 22°C, 55% humidity, 12-h day/night rhythm). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals.

Reagents. Polyinosinic-polycytidylic acid (PIC) was purchased from Amersham Pharmacia Biotech and reconstituted in PBS.

Cell stimulation. Cells were seeded onto 6-well plates at a density of 1.0×10^6 per well. PIC was either added directly to the medium at a final concentration of 50 μg/mL (PIC), or mixed with liposome (Lipofectamine 2000 reagent, Invitrogen, Carlsbad, CA, USA) for transfection at final concentrations of 2,

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5 and 10 μg/mL, respectively (PIC-liposome). PBS and empty liposome were used as mock treatments. Cells were assayed for PIC-induced responses with either route at 6 h for quantitative real-time PCR assay and after 6, 12, 24 and 48 h stimulation for apoptosis analysis.

Flow cytometry. For quantification of apoptosis in hepatoma cell lines HepG2, the cells were labeled with FITC-conjugated Annexin V (BD PharMingen, San Diego, CA, USA), which bound to phosphatidylserine exposed on the outer leaflet of cells undergoing apoptosis. The simultaneous application of the DNA stain propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) allowed discrimination of apoptotic from necrotic cells. Assays were performed following the manufacturer's protocol and results were expressed by comparing the apoptotic fluorescent cells with the total number of cells.

Caspase activity assay. Caspase-3/7 activity was measured as a marker of apoptosis using luminescent (Caspase-Glo 3/7 assay) methods according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, HepG2 cells were plated in 96-well plates at a density of 1.0×10^4 per well and treated as previous described. After 24 h, the cells were incubated with Caspase-Glo 3/7 reagent at ambient temperature for 1 h in the dark, and the luminescence was measured with a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The *n*-fold increase in protease activity was determined by normalizing to the PBS-only group. Equal numbers of cells were analyzed by counting a parallel set of cells and determining the total cell number for each sample.

Effects of Caspase Inhibitors. Cells were seeded onto 12-well plates at a density of 2.0×10^5 per well. After 1 day, cells were treated with z-VAD-fmk (Caspase Inhibitor I, CALBIOCHEM, Darmstadt, Germany) at a final concentration of 20 μM. PBS was used as control. PIC-liposome (5 μg/mL) and PIC (50 μg/mL) were added to the cells 2 h after treatment with inhibitors. Stimulation was continued for 24 h, and cell growth was measured by apoptosis analysis.

Quantitative real-time PCR. RNA was isolated using a nuclear acid purification kit (RNAfast200, Fastagen Biotech, Shanghai, China), followed by DNase I (MBI Fermentas) treatment according to the protocol provided by the manufacturer. Quantitative real-time PCR assays were performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative abundance of mRNA is obtained by the comparative threshold cycle (ΔCT) method using *GAPDH* for normalization as described by the manufacturer's protocol. Briefly, the difference in cycle times, ΔCT, was determined as the difference between the tested gene and the reference housekeeping gene, *GAPDH*. Then the formula was applied: relative abundance = $2^{-\Delta CT}$. Quantitative real-time PCR was performed in a 20-μL total reaction mixture in 96-well plates containing 2 μL of cDNA as template, 10 μL of SYBR green I (SYBR Premix Ex Taq, TAKARA BIO, Otsu, Japan). PCR primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China). Primer sequences were:[19–21] GPDH, sense, 5′-GAAGGTGAAGGTCGGAGTC-3′; antisense, 5′-GAAGATGGTGATGGGATTTC-3′; MDA5, sense, 5′-TGGTCTCGTCACCAATGAAA-3′, antisense, 5′-CTCCTGAACCACTGTGAGCA-3′; RIG-I, sense, 5′-CTCTGCAGAAAGTGCAAAGC-3′, antisense, 5′-GGCTTGGGATGTGGTCTACT-3′; LGP2, sense, 5′-CTTTGACTTCCTGCAGCATT-3′, antisense, 5′-CAATGAGGTGGTCAGTCCAG-3′; TLR3, sense, 5′-GTGCCAGAAACTTCCCATGT-3′, antisense, 5′-CTTCCAATTGCGTGAAAACA-3′ and IFNβ, sense, 5′-GACATCCCTGAGGAGATTAAGCA-3′, antisense, 5′-CAACAATAGTCTCATTCCAGCCA-3′.

Tumor inoculation and intratumoral administration. Human HepG2 cells were treated for 12 h with either route as the procedure of cell stimulation. Cells (1×10^6) were subcutaneously injected with 100 μL PBS into the shaved right flanks of naive nude mice. Tumor growth was detected every 5 days for 40 days. For the administration model, HepG2 cells were inoculated subcutaneously on day 0. PIC-liposome was injected intratumorally every 5 days for 30 days starting on day 5. Six mice per group were used. Tumor size was measured with a caliper and the volumes were estimated using the formula: $0.5 \times \text{length} \times \text{width}^2$.

Immunohistochemical staining. Sections from formalin-fixed and paraffin-embedded tumor tissue were stained with mouse monoclonal anti-PCNA antibody (Zhongshan Golden Bridge Biotechnology Co., Beijing, China, 1:100 dilution) at 4°C overnight. After incubation with biotin-labeled goat antimouse IgG secondary antibody at ambient temperature for 30 min, sections were incubated with peroxidase-labeled streptavidin under the same conditions. Finally, the sections were stained with peroxidase substrate 3,3-diamino-benzidine (DAB, DAKO Envision System, DAKO, Carpinteria, CA, USA) and counterstained with hematoxylin for 10 s.

Statistical analysis. Data were expressed as mean ± standard error of the mean (SEM). All experiments were performed at least three times. Data were compared for statistical significance by the Student's *t*-test with OriginPro 7.5 software. Tumor growth was tested by non-parametric Mann–Whitney test with Graphpad Prism software. Probability values of $P < 0.05$ were considered significant.

Results

Apoptosis was mediated by PIC-liposome complex *in vitro*. To test the effect of PIC-liposome on apoptosis induction of hepatoma cells, cells were exposed to either PIC or PIC-liposome stimulation. We found that hepatocyte lineage cells exhibited growth inhibition. Cells were shrinking and detaching from the plate apparently after 24 h under PIC-liposome treatment (Fig. 1a). Thereafter, for confirmation, cells were harvested at indicated times and different dosages (Fig. 1b). Apoptosis analysis was performed by Annexin V/PI double-staining, during which the percentage of cells in the apoptosis stage was calculated. Results showed that 5 μg/mL PIC-liposome led to a significant increase in the percentage of HepG2 cells in the apoptotic stage, reaching 30% at 24 h and 65% at 48 h (Fig. 1c). The percentage apoptosis increased in a PIC-liposome dose-dependent manner (Fig. 1d). Meanwhile, there was no evidence that PIC or liposome alone participated in the process (Fig. 1c). The results demonstrated that apoptosis was exclusively detected when challenging the cells to PIC-liposome, whereas the cells were not sensitive to PIC alone. Thus, we used 5 μg/mL PIC-liposome as the following treatment dosage.

Caspase was involved in PIC-liposome mediated apoptosis. Caspases are often used as early apoptotic indicators. The exposure of hepatoma cells to 20 μM caspase inhibitor Z-VAD-fmk partially suppressed PIC-liposome mediated apoptosis (Fig. 2a) and the inhibition rate was approximately 50% (Fig. 2b). Furthermore, caspase 3/7 activity was examined. The effector caspase activity increased twofold over that in PIC-alone treated cells in the PIC-liposome treated cells (Fig. 2c). Caspase activity did not extend past 48 h, due to the high level of cell death in the PIC-liposome treated cells. These studies demonstrated that caspase activation could be induced in PIC-liposome mediated apoptosis.

Expression of RLRs was up-regulated significantly in human hepatoma cells with PIC-liposome treatment but the response of TLR3 was slight. Transfection of PIC into cells using a liposome-mediated procedure has been addressed experimentally to trigger the cytosol receptor RLRs[6,18] while cells exposed to PIC alone are demonstrated to be recognized by TLR3. Moreover, it has been reported that dsRNA sensors TLR3 and RLRs lead to growth inhibition and apoptosis in different types of cancer

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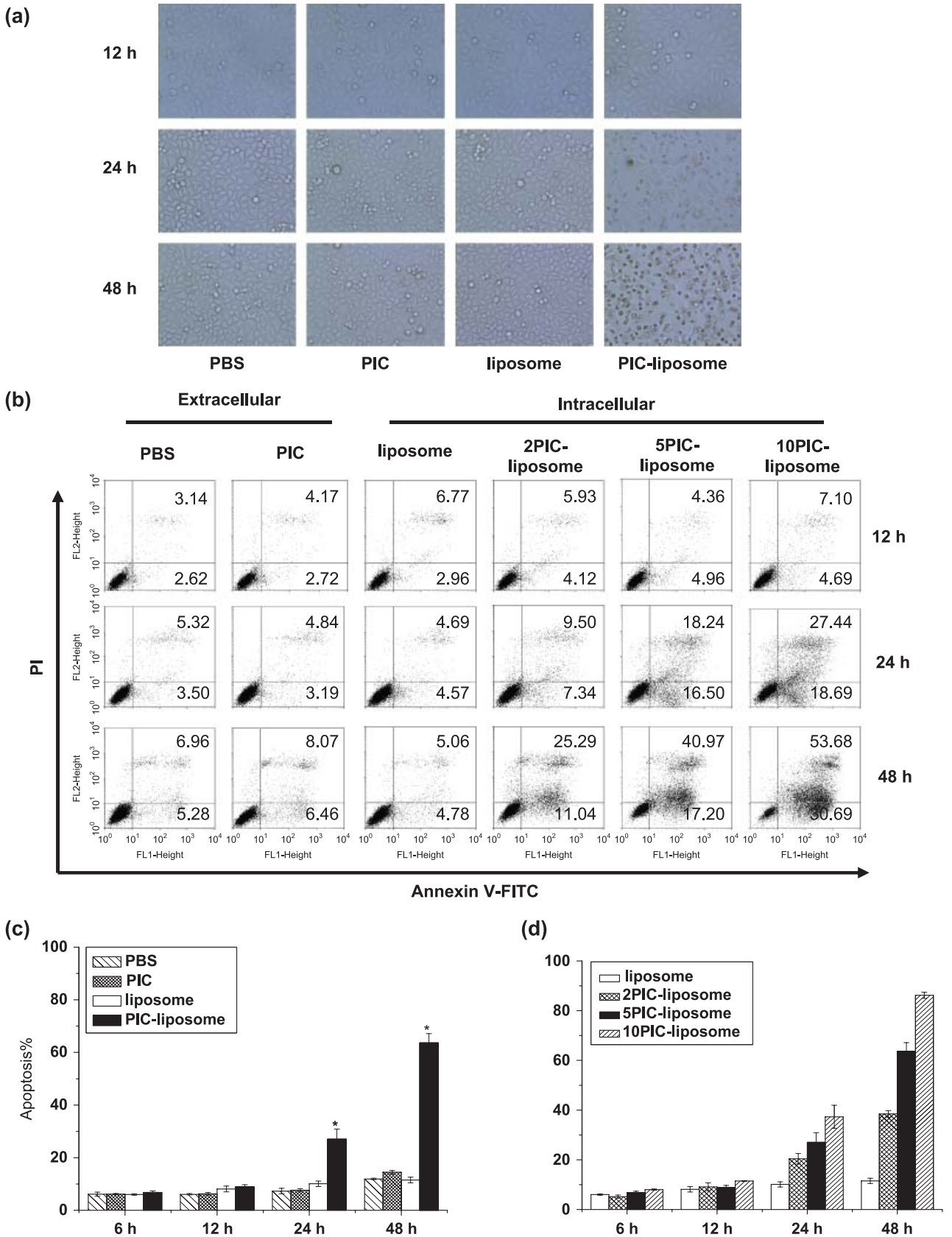

Fig. 1. Apoptosis mediated by PIC-liposome complex *in vitro*. (a) Cell morphology of human hepatoma cells HepG2 treated by 50 μg/mL PIC or 5 μg/mL PIC-liposome, magnification ×200. Cells stimulated with mock (PBS, liposome) or PIC (PIC, PIC-liposome). (b) Apoptosis analyzed by labeling apoptotic cells with FITC-conjugated Annexin V and propidium iodide at 6, 12, 24, 48 h with different concentrations of PIC (2PIC-liposome, 5PIC-liposome, 10PIC-liposome). (c,d) Rate of apoptotic cells (Annexin V$^+$ cells) expressed as the mean percentage ± SEM of apoptotic fluorescent cells *versus* the total number of cells from at least three independent experiments. *$P < 0.05$.

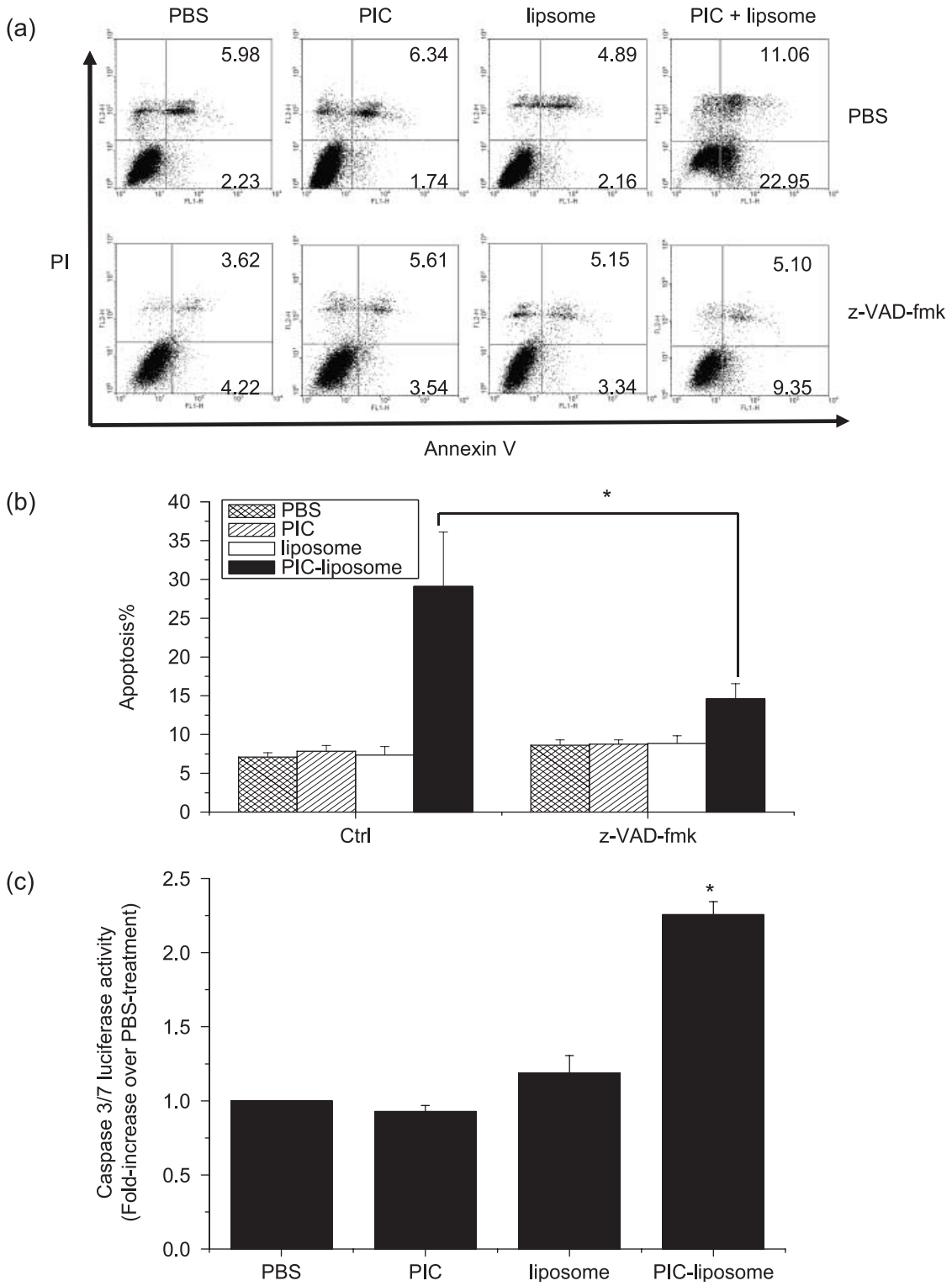

Fig. 2. Caspase involved in PIC-liposome mediated apoptosis. (a) Cells seeded onto 12-well plates at a density of 2.0×10^5 per well; after 24 h, inhibitors were added to each well 2 h before 24-h stimulation. (b) Statistical differences of apoptosis rates; data from at least three independent experiments. *$P < 0.05$. (b) Caspase activities measured at 24 h in mock-treatment (PBS, liposome) and PIC-treatment (PIC, PIC-liposome); the *n*-fold increase in protease activity was determined by normalizing to the PBS-only group. Data shown as mean values ± SEM of triplicate wells for each plate and duplicated in at least two plates. *$P < 0.05$.

cells[14,15] which are poorly defined in hepatocyte-derived cells. Thus, we detected the expression of RLRs and TLR3 in various cell lines. Coinciding with the reported data, we found that hepatocyte lineage cells express relatively low levels of RLRs and TLR3 mRNA (data not shown). To characterize the responses of RLRs and TLR3 in hepatoma cells, the cells were challenged by either PIC or PIC-liposome. When PIC was added to the culture medium, there were no induced changes of

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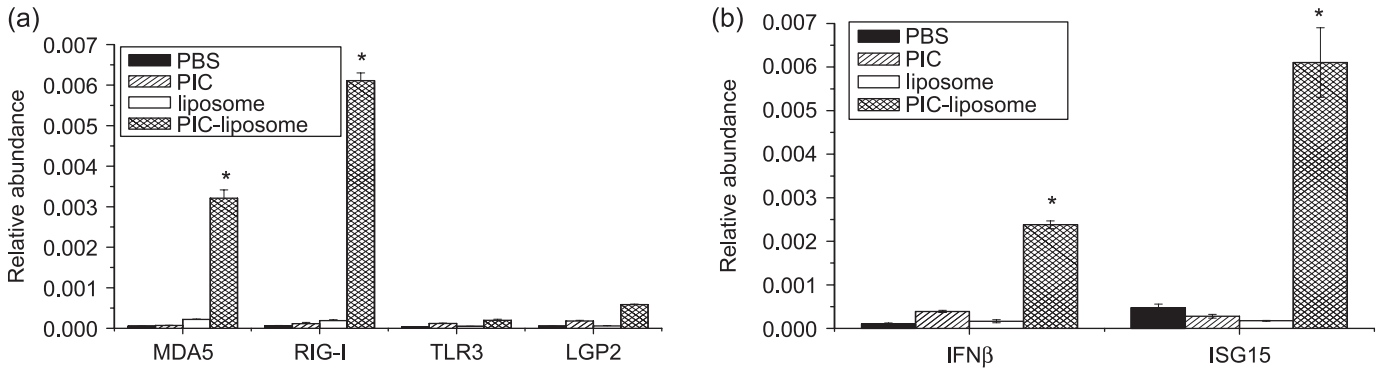


Fig. 3. Expression of RLRs up-regulated significantly in human hepatoma cells with PIC-liposome treatment but the response of TLR3 is slight. Cells grown in 6-well plates mock-treated (PBS, liposome) or treated with PIC for 6 h (PIC, PIC-liposome). Total RNA was isolated and relative mRNA abundances of (a) *MDA5*, *RIG-I*, *LGP2*, TLR3 and (b) IFNβ and ISG15 quantified by real-time PCR. Data shown as mean values ± SEM for at least three independent experiments. *$P < 0.05$.

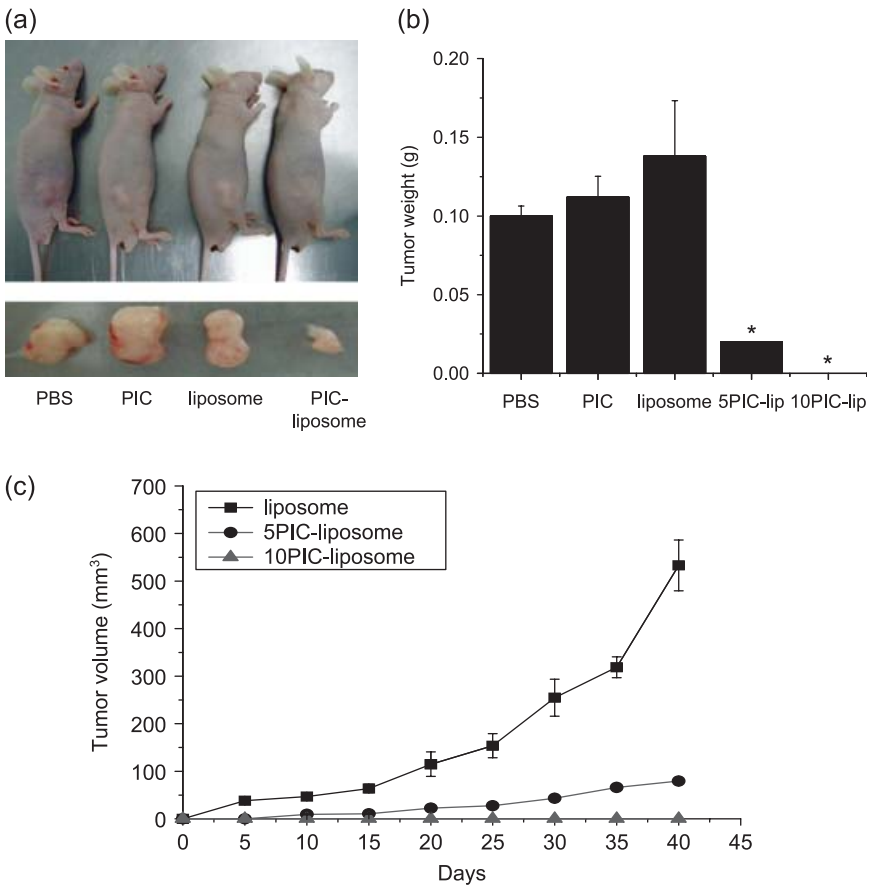
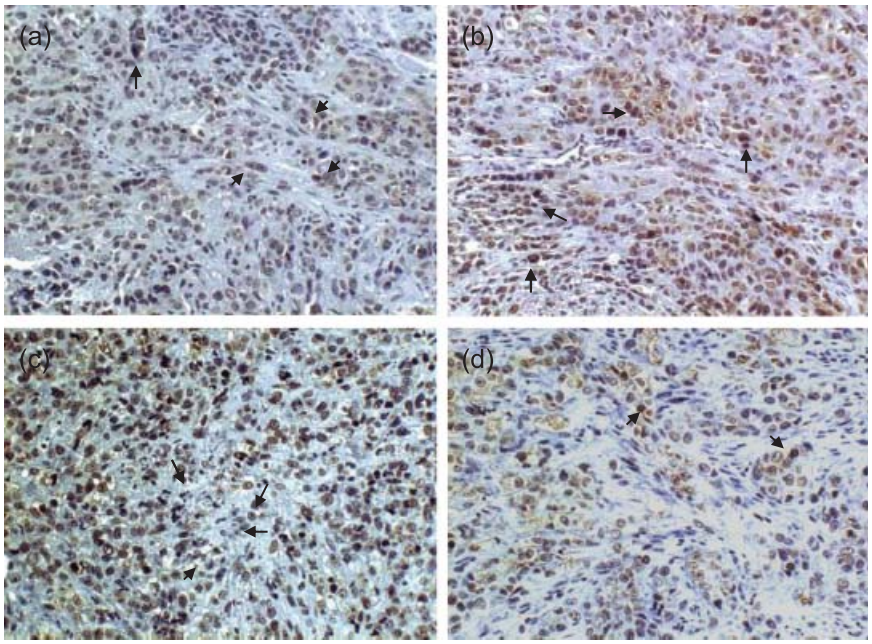


Fig. 4. Growth of PIC-liposome treated hepatoma cells inhibited *in vivo*. (a) Tumors from cells treated with either mock (PBS, liposome) or PIC (PIC, 5PIC-liposome, 10PIC-liposome) at 40 days after subcutaneous inoculation in nude mice. (b) Tumor weight; mean ± SEM of five mice is shown. *$P < 0.05$. (c) Time course for measuring the inhibitory effects of PIC-liposome complex on the growth of treated HepG2 cancer nodule; treated HepG2 cells inoculated in the right flanks of mice and the growth of tumors was monitored using calipers. Data shown as mean values ± SEM of five mice (Mann–Whitney *U*-test, $P < 0.05$).

RLRs (Fig. 3a). Meanwhile, TLR3 revealed a slight response upon exposure to PIC (Fig. 3a). To trigger the signaling pathway of the cytoplasm receptors, we also introduced PIC into cells using a liposome mediated procedure, which resulted in much more potent stimulation of the RLRs expression, leading to 32- and 15-fold increases of *RIG-I* and *MDA5* mRNA levels, respectively (Fig. 3a). We concluded from these data that cultured hepatoma cells generally responded significantly to PIC-liposome and resulted in massive up-regulation of the levels of intracellular RLRs compared to the PIC mediated response via TLR3.

Consistent with the reported data, PIC-liposome treatment resulted in significant increase of IFNβ (15-fold induction), ISG15 (35-fold induction) expression in HepG2 cells (Fig. 3b) but PIC treatment alone was not effective (Fig. 3b)

Growth of PIC-liposome treated hepatoma cells was inhibited *in vivo*. We examined whether the treatment with PIC or PIC-liposome could influence the growth of hepatoma cells *in vivo*. Cells treated by either route were inoculated subcutaneously into the right flanks of nude mice. As shown in Fig. 4(a), the growth of PIC-liposome treated cells was markedly attenuated compared to those treated with either PIC or empty liposome



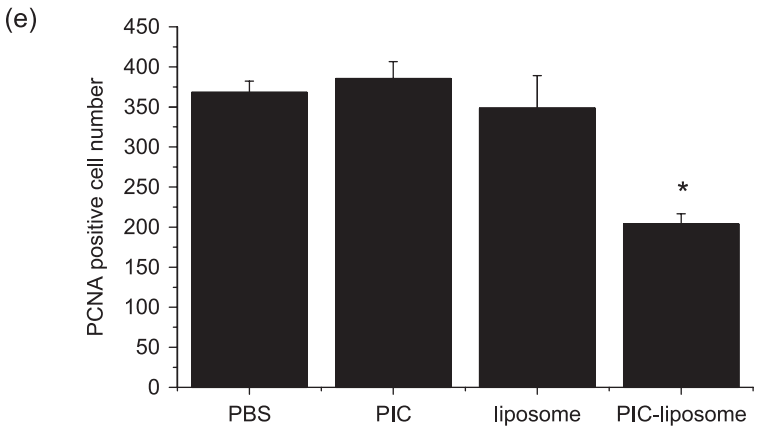


Fig. 5. Tumor cell proliferation of PIC-liposome treated hepatoma cells was inhibited. Immunohistochemical staining for PCNA in tumor nodule from (a) PBS, (b) PIC, (c) empty liposome and (d) PIC-liposome treated cells at a magnification of ×200, showing PCNA-positive cells (brownish spots) and nuclear (blue spots). (e) PCNA-positive cell counts in the histological sections. *$P < 0.05$).

alone (Mann–Whitney *U*-test, $P < 0.05$). In addition, the tumor cell growth inhibition was in a PIC-liposome dose dependent manner (Fig. 4b,c).

To further confirm the growth inhibition, the *in vivo* tumor cell proliferation was evaluated by the cells positive for PCNA, a marker of cell proliferation. As shown in Fig. 5, the level of PCNA protein was significantly down-regulated in tumors from PIC-liposome treated cells; whereas there were no changes in PIC-treated cells compared with mock-treated cells. Our results demonstrated that the inhibition of PIC-liposome treated cells *in vivo* at least in part relied on down-regulation of PCNA. Coinciding with the *in vitro* data, the results further suggest that PIC-liposome exerts stronger antitumor effects than PIC alone.

PIC-liposome exerted antitumor activity in HepG2-bearing mice. Finally, we evaluated the therapeutic effect of PIC-liposome in mouse models by direct injection on HepG2-bearing mice, and 100 μg/mL PIC-liposome was injected intratumorally. Corresponding to previous data, PIC-liposome affected tumor growth inhibition compared with control groups. A significant inhibition of tumor volume at day 35 was observed in the PIC-liposome treated group compared with the control group (Fig. 6, Mann–Whitney *U*-test, $P < 0.05$). The results implied potential therapeutic application.

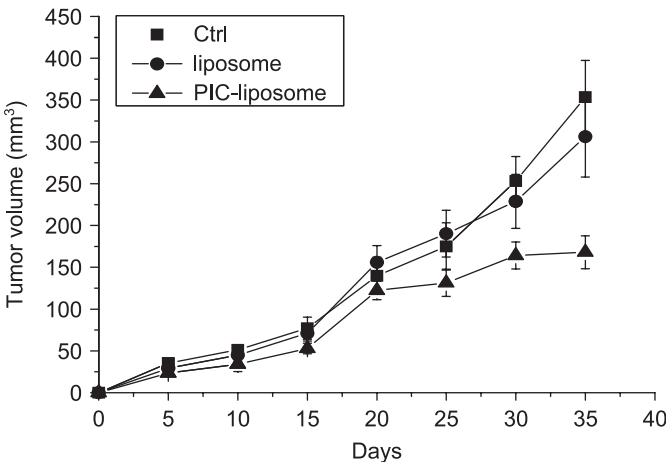


Fig. 6. PIC-liposome antitumor activity in HepG2-bearing mice. HepG2 cells were inoculated subcutaneously on day 0. PIC-liposome was injected intratumorally every 5 days for 30 days starting on day 5. Data shown as mean values ± SEM for six mice (Mann–Whitney *U*-test, $P < 0.05$).

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Discussion

In this study, we demonstrated that the complex of PIC-liposome could be sensed by RLRs in hepatoma cells, resulting in corresponding up-regulation of the mRNA levels and subsequent apoptosis. In addition, evidence was provided that the growth of hepatoma cells under PIC-liposome treatment was inhibited *in vivo*.

PIC alone does not easily enter into the cytoplasm, which could be recognized by TLR3, except for special cells like macrophages and dendritic cell (DC). Moreover, cumulative evidence documented that various cancer cells are not sensitive to PIC treatment alone *in vitro*. In our study, the same phenomenon was observed in hepatoma cells, which could be attributed to the following two reasons. First, hepatocyte lineage cells express relatively low levels of TLR3 mRNA.[14] Second, the mRNA abundance of TLR3 revealed slight up-regulation in response to PIC stimulation, which suggested that defects might be present in the TLR3-related cellular apoptotic machinery. We took the method of using liposome to transfer PIC across the cell membrane, triggering the response of cytosol RLRs.[6,18] Although the constitutive expression of RLRs was relatively low in hepatoma cells, they could be up-regulated significantly when exposed to PIC-liposome. The results imply that in hepatoma cells, cytoplasm receptors were more critical than the extracellular pathway in inducing apoptosis.

Molecular events involved in the death of hepatoma cells induced by PIC-liposome are controversial. Upon interaction with the RLRs agonists, the RNA-dependent ATPase activity of *MDA5* and *RIG-I* triggers a transduction cascade and induces the expressions of interferon and interferon-stimulated gene (ISG)s.[22,23] Interferon and ISGs have been demonstrated to be involved in growth inhibition, differentiation and apoptosis. In our study, the up-regulation of the mRNA levels of IFNβ and ISG15 could be detected before the apoptosis of hepatoma cells. However, whether interferon and ISGs could induce the apoptosis of hepatoma cells directly requires more evidence.

On the other hand, the above data suggested that intracellular dsRNA has a direct proapoptotic effect on hepatoma cells, which might be related to the unique structures of *RIG-I* and *MDA5*.[4,5] The CARD superfamily is a member of the death domain family, including death domains (DDs), death effector domains (DEDs) and pyrin domains (PYDs), which are related to inflammatory or cell-death pathways. CARD-containing proteins always transfer the signal through homotypic interactions within a death domain subfamily.[24,25] Furthermore, RNA helicase, through its ATP-dependent unwinding of RNA, seems to have growth suppressive properties and may also be involved in apoptosis. Upon apoptotic stimuli, Helicard (the mouse homolog of *MDA5*) is cleaved by caspases, thereby separating the CARD domain from the helicase domain, which enters into the nucleus for DNA degeneration during apoptosis.[11] The same process of cleavage is also found in human *MDA5*.[12] The Ras/Raf/MEK/ERK signaling pathway has been reported in related to MDA5-mediated growth inhibition and apoptosis.[10] *RIG-I* may also function as tumor suppressor regulated by *IRF-1*.[13] However, description of detailed mechanisms needs more evidence.

Our data showed that caspase was involved in PIC-liposome-mediated apoptosis. However, apoptosis could not be abolished completely, which suggested the participation of another pathway. We also found that the nuclease inhibitor could have some effect (data not shown). Furthermore, the activation of caspases 3/7 was induced in response to PIC-liposome stimulation. The caspase-8 activity, which was reported to participate in the TLR3-related apoptotic pathway, could not be detected (data not shown). In addition, another involvement of apoptosis-related pathways, such as mitochondrial cytochrome-*c* release and caspase-9 activation remains a possibility in our study.

Besides TLR3 and RLRs, dsRNA-dependent protein kinase (PKR) was described to initiate a cellular response to dsRNA. Thus, we can not completely exclude the possibility that RKR contributed to the recognition of PIC-liposome in this study. However, unpublished results of our study on the apoptosis in various tumor cell lines suggested that the RLRs may play a major role in the growth inhibition and apoptosis of hepatoma cells.

The therapeutic effect of PIC-liposome to inhibit the metastatic liver cancer growth and melanoma growth was demonstrated in mouse model.[3,16] Consistent with these previous reports, we found that the PIC-liposome treated cells revealed tumor growth inhibition *in vivo*. In our study, we also administered PIC-liposome to the HepG2-bearing mice by intratumoral injection to trigger the apoptosis signaling *in vivo*. An antitumor effect was observed, although it was not as significant as *in vitro* study. We could not neglect that the *in vivo* microenvironment which influences the growth of the tumor and its ability to progress might alter drug metabolism, contributing to the development of drug resistance and making the tumors hard to destroy. Thus, to improve the therapeutics of PIC-liposome in clinical research, further consideration was needed, such as reasonable delivery methods or combination of adjuvants targeting stromal elements.

In conclusion, our results demonstrate that PIC-liposome could induce apoptosis and up-regulate cytoplasm receptors *RIG-I* and *MDA5* in hepatoma cells, which open a new range of therapeutic applications for PIC-liposome as cytotoxic agents in hepatoma cells.

Acknowledgments

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