Aquaculture 402–403 (2013) 92–96

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Identification and functional characterization of a sTRAIL gene in mussel *Hyriopsis cumingii*

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ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 19 March 2013
Accepted 20 March 2013
Available online 29 March 2013

Keywords:
Hyriopsis cumingii
Soluble tumor necrosis factor-related apoptosis-inducing ligand
Immune response
Apoptosis
Caspase 3

ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) plays an important role in many biological processes including immune response and cell apoptosis. Here, we identified a soluble TRAIL homolog in a mussel species, *Hyriopsis cumingii* (designated Hc-sTRAIL), which shows significant structural and functional similarities to mammalian sTRAIL. Real-time PCR analysis shows that mussel TRAIL is ubiquitously expressed in various tissues and involved in the immune response of mussel. Study on its apoptotic effect indicates that Hc-sTRAIL can induce significant apoptosis in NCI-H446 cells and involved the caspase 3 pathway. This study provides new insight into the physiological function of Hc-sTRAIL.

1. Introduction

The apoptotic effects of tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL/Apo2L) or its extracellular domain—soluble TRAIL (sTRAIL) have been well studied in human (Pitti et al., 1996; Tang et al., 2011; Wiley et al., 1995; Yildiz et al., 2010). It can induce apoptosis in many kinds of cancer cells by binding to the death receptors (DR4 and DR5) (Yildiz et al., 2010). This interaction results in recruitment of adapter protein Fas-associated death domain (FADD) and procaspase 8, and leads to the formation of a protein complex called DISC (Death-Inducing Signaling Pathways). In this complex, procaspase 8 is proteolytically activated to caspase 8 (Choi et al., 2011; Shirley et al., 2011) and activated caspase 8 can induce cellular apoptosis through activation of the death receptor pathway and/or the mitochondrial pathway (Siegmund et al., 2011; Wang and El-Deiry, 2003). Both pathways eventually unite in the activation of caspase 3, the final executor of apoptosis, and result in cell death in various human cancer cell lines, while show little or no cytotoxicity to normal cells (Yildiz et al., 2010). Because of the selective cytotoxicity, TRAIL is regarded as a potential therapeutic molecule against cancer malignancies.

In addition, both TRAIL and sTRAIL play an important role in immune responses, and they can be induced by stimuli such as lipopolysaccharides (LPS), Rickettsia-like organism (RLO) and viruses (Collison et al., 2009; Halaas et al., 2000; Simons et al., 2007; Yang and Wu, 2010).

Despite the pro-apoptotic effects of TRAIL on human cancer cells and its roles in immune responses that have been well studied in various vertebrate species from fish to human (Abdalla et al., 2004; Gao et al., 2008; Wiley et al., 1995), little is known about its role in invertebrate mollusks (Yang and Wu, 2010). Particularly, there is no report about the pro-apoptotic effects of mollusk TRAIL on human cancer cells. On the contrary, several kinds of TNF members including Eiger, Wengen, TNF-α, FasL and LPS-induced TNF-a factor (LITAF) were identified in invertebrate species (De Zoysa et al., 2009; Kauppila et al., 2003; Park et al., 2008; Yang et al., 2012; Yu et al., 2007).

Hyriopsis cumingii (Lea) is an economically important freshwater mussel in China (Zhang et al., 2007). To understand the biological function of TRAIL in invertebrate species, a soluble form of TRAIL is cloned from *H. cumingii* (designated Hc-sTRAIL). Studies on the physiological functions of Hc-sTRAIL indicate that it plays a role in mussel immune system. Moreover, Hc-sTRAIL can induce apoptosis in human small cell lung cancer cell line NCI-H446 through caspase-3 apoptotic pathway.

2. Material and methods

2.1. Animals, tissue sampling, and immune challenge

Healthy mussels (*H. cumingii*) of body weight ranges from 150 to 180 g, were collected from a pearl mussel farm in Zhuji city (Zhejiang,

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http://dx.doi.org/10.1016/j.aquaculture.2013.03.021

China), and were cultured and fed with diatoms in a circular freshwater system for 7 days at 20 ± 2 °C. Ten mussels were used in each experimental condition. Mussels were challenged, by injecting into the adductor muscle, 100 μl of *Aeromonas hydrophila* (10^9 bacteria/ml, Institute of Hydrobiology, Chinese Academy of Sciences; diluted in sterile saline: 0.65% sodium chloride) with a syringe, or 100 μl of sterile saline (0.65% sodium chloride) as control into their adductor muscle. Hemocytes were extracted from the pericardial cavity with a syringe at different timepoints (0 h, 6 h, 12 h, 18 h, 24 h) post-injection by centrifuged at 800 *g* for 10 min at 4 °C. Gill, mantle, gonad and digestive gland were also collected from healthy mussel without injection. Total RNA was isolated using the TRIzol reagent as described (Invitrogen, USA) (Yang et al., 2012), and the cDNA was synthesized with a First-Strand System Kit (Promega, USA) according to the manufacturer's instructions.

2.2. Gene cloning and sequence analysis

Partial cDNA sequence of TRAIL was obtained by 3′ Rapid Amplification of cDNA Ends (RACE) PCR when cloning of immune-related gene from mussel *H. cumingii*. PCR amplification was carried out under the following conditions: denaturation at 95 °C for 5 min, followed by 35 cycles (95 °C for 1 min, 68 °C for 45 s, 72 °C for 90 s), with final cycle of 10 min at 72 °C. The primers for hcf and Adaptorp were listed below: hcf (5′-GAKTGTTCYGAGCCTAMTCCATC-3′); Adaptorp (5′-CTG ATC TAG AGG TAC CGG ATC C TTTTTTTTTTTTTT-3′).

Nucleotide and deduced amino acid sequences were analyzed using Blast software by NCBI. Multiple sequence alignment and phylogenetic tree were performed using neighbor-joining method with MEGA version 4.0.

2.3. Quantitative real-time PCR

Quantitative real-time PCR was carried out as described previously (Yang et al., 2012) to examine the distribution and the potential physiological functions of mussel Hc-sTRAIL. Total RNA and cDNA from mussels were prepared as mentioned. The primers for Hc-sTRAIL are hcsTRLf1 (5′-CTGACCCTATAT TGTTGATG-3′) and hcsTRLr1 (5′-GTCCATG TCTATCAAGTGC-3′). For beta-actin (GenBank accession no. HM045420, NCBI) are BACTf (5′-GATGATATGGAGAAGATCTG-3′) and BACTr (5′-CATCACCAGAGTCTAAGACA-3′). Real-time PCR was conducted in a final volume of 25 μl containing 1 × SYBR Premix Ex TaqTM Kit (Takara, Japan), 0.2 μM of each primer and 1 μl of cDNA on an iCycler iQ™ Real-Time PCR System (Bio-Rad, USA), using the following amplification protocol: 3 min initial denaturation at 94 °C, followed by 45 cycles of amplification (94 °C for 25 s, 56 °C for 40 s).

2.4. Protein expression, purification and western blot analysis

The open reading frame (ORF) sequence of Hc-sTRAIL gene was amplified from cDNA template prepared as above using primers HcsOf (5′-GACCATATGGTGAGAGAAAGAG-3′) and HcsOr (5′-GCCTCGAGTTAGC CAACTTAAAAG-3′), underlined b.p. represents the restriction sites for *NdeI* and *XhoI* respectively. The PCR product was then inserted into *Nde*I and *Xho*I site of pET28a (Novagen, Germany). The recombinant plasmids were transformed into BL21 (DE3) competent cells and induced with 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at 30 °C. After induction, the recombinant proteins were purified by His-bind nickel column chromatography (Qiagen, Germany), and the elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH5.9) was used according to the manufacturer's instruction. Western blotting analysis was conducted as previously described to verify the above product (Yang et al., 2012).

2.5. Cell line

The human small cell lung cancer cell line NCI-H446 (Kaiji, China) was cultured at 37 °C in RPMI 1640 medium (Sijiqing, China) supplemented with 10% fetal calf serum (Sijiqing, China) and 200 U/ml of Streptomycin. Cells were treated with or without recombinant Hc-sTRAIL proteins (100 ng/ml), and were cultured for 24 h at 37 °C as described above.

2.6. Apoptosis assay

2.6.1. Annexin-V/PI staining and flow cytometry analysis

NCI-H446 cells (5×10^5) were collected by centrifugation (500 *g* for 15 min), and were incubated with annexin-V fluorescein and propidium iodide (PI) staining (Annexin V-FITC Apoptosis Detection Kit, Merck, Germany) respectively according to the manufacturer's instruction. FITC and PI-fluorescence were used for analysis to distinguish among live, early apoptotic, late apoptotic or necrotic cells by FACStar plus flow cytometer (Becton Dickinson, USA) using the *CellQuest* Software (Becton Dickinson, USA).

2.6.2. DNA fragmentation

Genomic DNA of NCI-H446 cells treated with or without recombinant Hc-sTRAIL proteins was extracted by apoptotic DNA isolation kit (Beyotime, China) according to manufacturer's instructions and subjected to electrophoresis in a 2% agarose gel to detect the formation of DNA ladder.

```
  1 M  V  R  E  R  G  P  Q  R  V  A  A  H  I  T  G  T  R  G  R
  1 ATGGTGAGAGAAAGAGGTCCTCAGAGAGTAGCAGCTCACATAACTGGGACCAGAGGAAGA
 21 S  N  T  L  S  S  P  N  S  K  N  E  K  A  L  G  R  K  I  N
 61 AGCAACACATTGTCTTCTCCAAACTCCAAGAATGAAAAGGCTCTGGGCCGCAAAATAAAC
 41 S  W  E  S  S  R  S  G  H  S  F  Q  S  N  L  H  S  R  N  G
121 TCCTGGGAATCATCAAGGAGTGGGCATTCATTCCAGAGCAACTTGCACTCGAGGAATGGT
 61 E  L  V  I  H  E  K  G  F  Y  Y  I  Y  S  Q  T  Y  F  R  F
181 GAACTGGTCATCCATGAAAAAGGGTTTTACTACATCTATTCCCAAACATACTTTCGATTT
 81 Q  E  E  I  K  E  N  A  K  N  D  K  Q  M  V  Q  Y  I  Y  K
241 CAGGAGGAAATAAAAGAAAACGCAAAGAACGACAAACAAATGGTCCAATATATTTACAAA
101 Y  T  S  Y  P  D  P  I  L  L  M  K  S  A  R  N  S  C  W  S
301 TACACAAGTTATCCTGACCCTATATTGTTGATGAAAAGTGCTAGAAATAGTTGTTGGTCT
121 K  D  A  E  Y  G  L  Y  S  I  Y  Q  G  G  I  F  E  L  K  E
361 AAAGATGCAGAATATGGACTCTATTCCATCTATCAAGGGGGAATATTTGAGCTTAAGGAA
141 N  D  R  I  F  V  S  V  T  N  E  H  L  I  D  M  D  H  E  A
421 AATGACAGAATTTTTGTTTCTGTAACAAATGAGCACTTGATAGACATGGACCATGAAGCC
161 S  F  F  G  A  F  *
481 AGTTTTTTCGGGGCCTTTTAA
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Fig. 1. Nucleotide and amino acid sequence of Hc-sTRAIL. The initial nucleotide and amino acid numbers are indicated as guanine and valine, respectively. Position of conserved TNF domain of the TNF superfamily was underlined. The cysteine residue (C117) was indicated in a box. Asterisk indicates the stop codon.

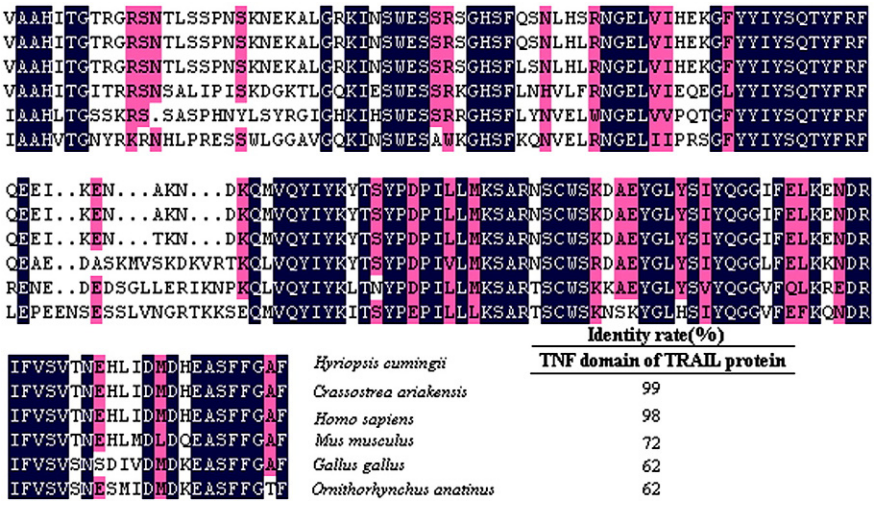

Fig. 2. Comparison on TNF domain from Hc-sTRAIL with other TRAIL proteins and their identity rates.

2.7. Roles of caspase 3 in Hc-sTRAIL-mediated apoptosis pathway

NCI-H446 cells were collected and divided into three groups: Hc-sTRAIL untreated, Hc-sTRAIL treated and caspase 3 inhibitor groups. Cells were treated with or without 100 ng/ml Hc-sTRAIL recombinant proteins for 24 h. In the caspase 3 inhibitor group, cells were pre-treated with 5 μmol/l Z-DEVD-FMK (R&D Systems, USA) for 1 h followed by Hc-sTRAIL treatment for 24 h. Cellular apoptosis was analyzed using Annexin-V/PI staining and flow cytometry analysis as stated above.

2.8. Statistical analysis

The relative expression levels of Hc-sTRAIL were detected using real-time PCR and were calculated according to the formula: $2^{(CT\ housekeeping\ gene - CT\ target\ gene)}$. Data were presented as the standard errors of the mean (S.E.M.). Differences were considered statistically significant when p values were less than 0.05.

3. Results and discussion

3.1. Cloning and sequence analysis of Hc-sTRAIL

Partial cDNA sequence of TRAIL gene (GenBank accession no. GU984232, NCBI) was cloned from the mussel species, *H. cumingii* (designated Hc-sTRAIL). TRAIL contains an ORF of 501 bp encoding for a putative protein of 167 amino acids, which displays a molecular mass of 18.4 kDa (Fig. 1). It has a TNF domain (from 9 aa to 166 aa) which is conserved among TNF superfamily members, and the cysteine residue (C117) is conserved in human sTRAIL, which is essential for its structure and pro-apoptotic activity (Bodmer et al., 2000). ClustalW pairwise comparison showed that the TNF domain of Hc-sTRAIL has over 98% similarity with either oyster CasTRAIL or human sTRAIL; and 62–72% similarity with sTRAIL from other vertebrates (Figs. 2, 3a). But it showed only 15–20% similarity with other types of TNF domains from invertebrate species (Fig. 3b). These results indicated that Hc-sTRAIL highly homologous to human sTRAIL, which is consistent with the study of CasTRAIL from oyster (Yang and Wu, 2010).

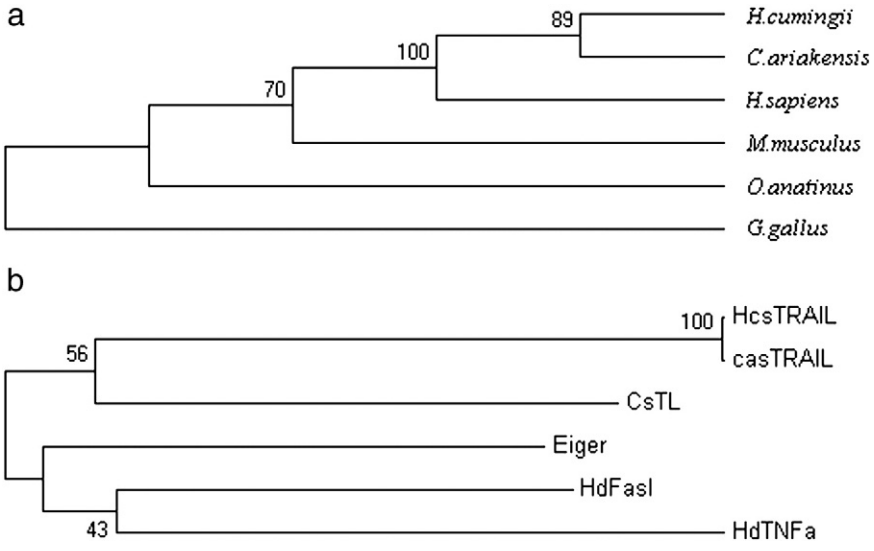

Fig. 3. Phylogenetic analysis of Hc-sTRAIL. (a) Phylogenetic tree for Hc-sTRAIL and other TRAIL proteins. The unrooted tree was built using the NJ method based on the alignment of TNF domain sequences. (b) Phylogenetic tree for Hc-sTRAIL and other TNF member proteins of invertebrates.

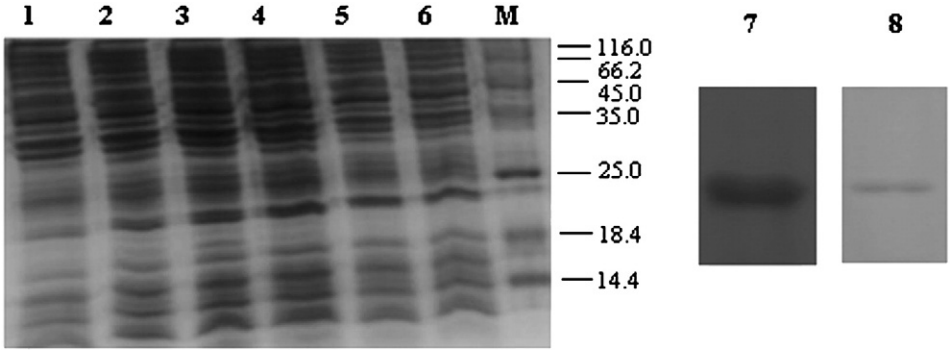

Fig. 4. SDS-PAGE and western blot analysis of Hc-sTRAIL. Bacterial proteins and recombinant fusion proteins were separated on 10% SDS-PAGE gels. A band with a molecular weight of about 20 kDa was detected using anti-His tag antibody. Lane 1, non-induced; Lanes 2–6, after induction by 0.01, 0.025, 0.05, 0.1 and 0.2 mM IPTG, respectively; M, molecular weight marker; Lane 7, purified recombinant proteins; Lane 8, western blotting of purified recombinant proteins using anti-His tag antibody.

3.2. Recombinant protein and western blotting

The Hc-sTRAIL recombinant protein was expressed in *Escherichia coli* BL21 (DE3) and purified with Ni-NTA affinity columns. Western blotting using anti-His tag antibody confirmed the presence of Hc-sTRAIL (~20 kDa) (Fig. 4).

3.3. Tissue distribution and expression analysis of Hc-sTRAIL

To determine the potential functions of Hc-sTRAIL, we examine the distribution of Hc-sTRAIL in various normal tissues (including hemocytes, gill, mantle, gonad and digestive gland) by quantitative real-time PCR. Results showed that Hc-sTRAIL was ubiquitously expressed in all examined tissues, and relatively higher expression levels were observed in hemocytes, gills and mantle (Fig. 5a), which is consistent with the fact that sTRAIL is involved in a broad range of important biological processes (Collison et al., 2009; Thorburn, 2007; Wiley et al., 1995; Yildiz et al., 2010). Interestingly, the expression level of Hc-sTRAIL mRNA in hemocytes increased sharply after *A. hydrophila* challenge and reached a peak level at 12 h (Fig. 5b). Meanwhile, no significant changes were detected in the control groups. In mussel and other invertebrates, hemocytes play a key role in the innate immunity system in which they act against microorganisms (Bachere et al., 2004; Canesi et al., 2002). A relatively high expression level of Hc-sTRAIL in normal hemocytes indicated that Hc-sTRAIL might be involved in the immune response of mussel. This hypothesis was supported by the observation of mussel challenged by *A. hydrophila* (Fig. 5b). Our previous study on CasTRAIL from oyster *Crassostrea ariakensis* indicated that oyster TRAIL might play key roles in transferring the stimuli of Rickettsia-like organism (RLO), an obligate intracellular Gram negative bacterium, to activate the p38 and then the transcription factor NF-κB, and NF-κB triggered the expression of subsequent cytokines and other molecules to against the RLO infection (Yang and Wu, 2010). But whether the similar mechanisms exist in the processes against *A. hydrophila* infection of Hc-sTRAIL, still needed to be clarified.

3.4. The role of Hc-sTRAIL in inducing cancer cell apoptosis

To investigate whether Hc-sTRAIL can induce apoptosis in mammalian cells, a cancer cell line, NCI-H446 (Fig. 6a), was treated with 100 ng/ml Hc-sTRAIL proteins. Cell viability assay showed that Hc-sTRAIL proteins caused a marked reduction in the cell number of NCI-H446 cells and reached to an average apoptotic rate of 23.7% at 24 h (Fig. 6c), which is similar to the results of human sTRAIL on NCI-H446 cells (Liu et al., 2007); Apoptosis of NCI-H446 was also verified by the presence of DNA fragmentation, the hallmark of apoptosis, within 24 h (Fig. 6b).

Previous studies showed that either TRAIL or sTRAIL can specifically induce apoptosis in various tumor cell lines through binding and activation of the death receptor pathway and/or mitochondrial pathway (Siegmund et al., 2011; Wang and El-Deiry, 2003). In both pathways, caspase 3 is ultimately activated and results in cell death (Qi et al., 2004; Yang et al., 2001). To further illuminate the molecular mechanisms underlying Hc-sTRAIL-induced apoptosis in NCI-H446 cells, Z-DEVD-FMK, a specific inhibitor of caspase 3 was used to examine the role of caspase 3 in Hc-sTRAIL-mediated apoptosis. As expected, cytotoxicity induced by Hc-sTRAIL on NCI-H446 cells could be blocked by Z-DEVD-FMK, in which the average apoptotic rate was significantly decreased to about 7.6% ($P < 0.05$) (Fig. 6c). Similar to human sTRAIL (Liu et al., 2007), Hc-sTRAIL could induce cell death in NCI-H446 cells, and caspase 3 plays a key role in Hc-sTRAIL-mediated apoptotic pathway.

In human, normal cells are believed to be resistant to TRAIL because of expressing higher levels of two TRAIL decoy receptors DcR1

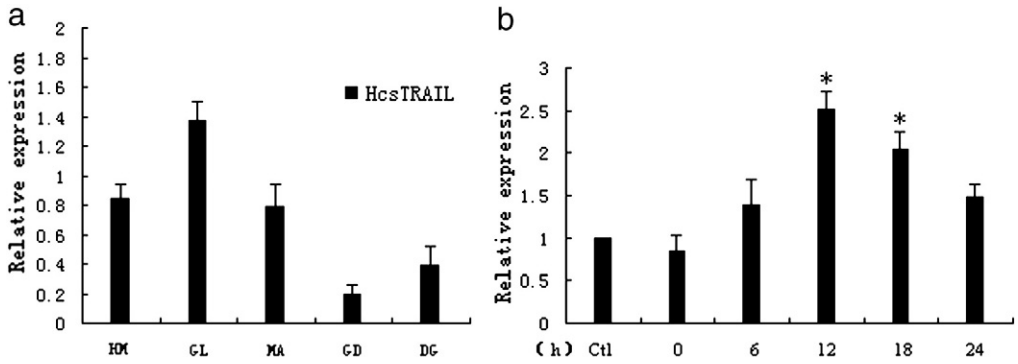

Fig. 5. Distribution and expression of Hc-sTRAIL. (a) Distribution of Hc-sTRAIL in various tissues. HM, hemocytes; GL, gill; MA, mantle; GD, gonad; DG, digestive gland. (b) Real-time RT-PCR analysis of the expression of Hc-sTRAIL in hemocytes with *Aeromonas hydrophila* challenge. Values presented as mean ± S.E. of independent experiments done in triplicates and analyzed by Student's *t*-test; *$P \leq 0.05$ when compared to control values.

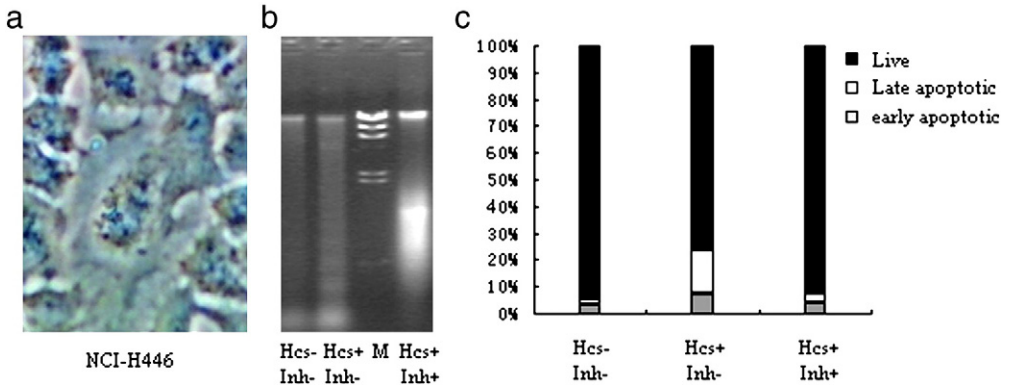

Fig. 6. Cytotoxicity of Hc-sTRAIL on tumor cells. (a) Culture of NCI-H446 cells. (b) DNA electrophoresis to detect the formation of DNA ladder at 24 h after Hc-sTRAIL addition with or without caspase 3 inhibitor group. (c) Flow cytometry analysis of apoptosis in Hc-sTRAIL untreated, Hc-sTRAIL treated and caspase 3 inhibitor groups. These experimental groups were represented with two parallels, and the average apoptotic rates in these groups were 4.5%, 23.3% and 7.6% respectively.

or DcR2 on their cell surface (Van Noesel et al., 2002). Our previous study showed that oyster sTRAIL (CasTRAIL) couldn't induce apoptosis in oyster hemocytes (Yang and Wu, 2010). This finding and the data in the present paper suggest that Hc-sTRAIL might also have no obvious cytotoxicity to normal mussel hemocytes.

In conclusion, a novel human sTRAIL homolog, Hc-sTRAIL, was identified from the mussel species, *H. cumingii*. Study on its physiological function indicated that Hc-sTRAIL was involved in the immune response of mussel and can induce cell death in cancer cells through the caspase 3 apoptotic pathway.

Acknowledgments

This work was supported by the China Postdoctoral Science Foundation (20100481431), the Key Scientific Program of Shaoxing City, Zhejiang Province, China (2010A22019) and the Zhejiang Provincial Natural Science Foundation of China (LY12C19010).

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