Identification of a protein associated with the activity of cytokine-induced killer cells

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Abstract. Cytokine-induced killer cells (CIKs) adoptive immunotherapy for efficient antitumor ability is used clinically, but details regarding the proteins associated with CIK activity remain unclear. In the current study, the cytotoxicity of CIKs on hepatoma was identified to be significantly downregulated by 1.61-fold following gentamicin treatment. Further research revealed that a differentially expressed protein (P43) was significantly downregulated by 1.22-fold using one-dimensional gel electrophoresis analysis. Of these, the P43 was identified as human haptoglobin using liquid chromatography-mass spectrometry. Western blotting demonstrated that the haptoglobin specifically reacted with rabbit anti-human-haptoglobin. Furthermore, western blotting results verified that the haptoglobin was significantly downregulated by 1.17-fold compared with the control group. In addition, the expression of haptoglobin mRNA was significantly downregulated by 1.73-fold following gentamicin treatment. Taken together, the results of the present study demonstrated that the expression of haptoglobin protein was associated with the activity of CIKs, and the results will be beneficial to the further investigation of CIK activity-enhancement mechanism.

Introduction

Adoptive immunotherapy is an effective and safe anticancer therapy (1), and currently used for various tumor treatments, including renal cell carcinoma (2), lung cancer (3), breast cancer (4), melanoma (5), and hepatoma (6).

As a valuable cancer therapeutic scheme, numerous studies focused on T cells in order to further improve the antitumor activity of adoptive immunotherapy (1). Rettinger *et al* (7) and Meng *et al* (8) demonstrated that IL-15 and IL-21, respectively were able to promote the proliferative, and cytotoxic activity of cytokine-induced killer cells (CIKs). Rutella *et al* (9) noted that thymoglobulin efficiently expanded the CIK population, and that the CIKs generated by thymoglobulin were feasible and safe for solid tumor treatment. Tan *et al* (10) verified that K-ras dendritic cells were able to enhance CIK proliferation and increase the killing effect on pancreatic cancer cells. Though the activity of CIKs may be enhanced by technological strategies, maneuvers to improve the antitumor efficacy through the use of T cells have been under exploration (11). However, the proteins associated with CIK activity remain unclear.

In the present study, it was demonstrated that the cytotoxicity of CIKs was significantly influenced following gentamicin treatment. Further research verified that a protein identified by proteomic and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) strategies was associated with CIK activity. The results of the current study have important implications for the investigation of the CIK activity-enhancement mechanism.

Materials and methods

CIK culture and protein extraction. Peripheral blood was donated from a 31-year-old male volunteer following written informed consent being obtained. The present study was approved by the Animal Welfare and Research Ethics Committee of the Institute of University of South China (Hengyang, China).

Lymphocytes were separated and cultured as reported by Pan *et al* (12), and Laport *et al* (13) with certain modifications. Equivalent volumes of peripheral blood and 0.9% physiological saline were mixed followed with ficoll gradient separation (LymphoPrep; PAA Laboratories; GE Healthcare, Chicago, IL, USA). Following centrifugation at 800 x g for 20 min at room temperature, the leukocyte layer was collected and washed twice with 0.9% physiological saline. Following centrifugation at 500 x g for 7 min at room temperature, the cells cultured with gentamicin (80 U/ml; Yichang Humanwell Pharmaceutical Co., Ltd., Yichang, China) were defined as the

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experiment group, and the cells cultured with no gentamicin were defined as the control group. The lymphocytes were cultured in GT-T551 medium (Takara Biotechnology Co., Ltd., Dalian, China) with 1,000 U/ml γ -interferon (Beijing Biocoen Biotechnology Co., Ltd., Beijing, China) and 10% autologous plasma added on day 0. Then, 50 μ g/ml CD3 monoclonal antibody (catalogue no. Mab-37; Skoda Biotechnology Co., Ltd., Shanghai, China) and 100 U/ml interleukin 1 α (PeproTech China, Suzhou, China) were added on day 1. Subsequently, 1,000 U/ml recombinant human interleukin 2 (SL Pharma Labs, Inc., Wilmington, DE, USA) and 2% autologous plasma were added to the medium from day 1 onward. The cells were cultured at 37°C with 5% CO₂ until the 11th day.

The CIK protein was extracted as reported by Gao *et al* (14) with certain modifications. The CIKs were centrifuged at 150 x g for 10 min at 4°C, and the precipitate was washed with 1 ml 0.9% physiological saline three times. After resuspension in 0.1 ml 0.9% physiological saline, the CIKs were lysed by freezing-thawing from -80°C to room temperature five times, and then centrifuged at 13,000 x g for 10 min at 4°C. After determining the protein concentration using the Bradford method (15), the supernatant was collected and stored at -20°C with 1 Mm phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for subsequent analysis.

Flow cytometry analysis. A total of 1 ml CIK suspension was collected following centrifugation at 150 x g for 10 min at room temperature, the precipitate was resuspended in 1 ml of 0.9% physiological saline and centrifuged at 150 x g for 10 min at room temperature. The precipitate was then resuspended in 150 μ l 0.9% physiological saline and divided into two groups. One group as the isotype control with FITC mouse IgG 2 α (5 μ l; catalogue no. 555573; BD Biosciences, Franklin Lakes, NJ, USA) added, and the control or experiment groups had FITC mouse anti-human CD3 (5 μ l; catalogue no. 555339; BD Biosciences) added. The three groups were all incubated 15 min at room temperature, then resuspended in 1 ml 0.9% physiological saline, and centrifuged at 150 x g for 10 min at room temperature. Finally, the precipitate was resuspended in 0.2 ml 0.9% physiological saline, and prepared for analysis using a BD Accuri C6 flow cytometer (BD Biosciences).

MTT analysis. The HepG2 hepatoma cell line, which was purchased from the Advanced Research Center of Central South University (Changsha, China), was cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) at 37°C and in 5% CO₂. Hepatoma cells used as target cells were obtained at the logarithmic growth phase and the concentration was adjusted to 4x10⁴ cells/ml. CIKs cultured for 11 days were used as effector cells and mixed with target cells in the ratio 40:1 (effector cell:target cell). A total of 10 ml CIK culture medium was collected following centrifugation at 150 x g for 10 min at room temperature. The precipitate was resuspended in GT-T551 containing 2% autologous plasma and diluted to 1.6x10⁶ cells/ml. The suspension was divided into three groups: The effector-target group, 100 μ l effector and target cells; effector cell group, 100 μ l effector cells and GT-T551 culture medium; target cell group, 100 μ l target cells and GT-T551 culture medium. All groups

Table I. Nucleotide sequences of primers.

Primer ^a	Sequence (5'-3')
Haptoglobin-F	CAGCCAGAAACATAACCC
Haptoglobin-R	TCTACACCCTAACTACTCCC
β -actin-F	ATCGTGCCTGACATTAAGGAG
β -actin-R	TAGGTGCTTTGATGGAAGTTGAG

^aThe primers of haptoglobin were designed according to its cDNA sequence (GenBank: K00422.1). F, forward; R, reverse.

Figure 1. Cytokine-induced killer cells purity and activity analyzed by flow cytometry and MTT methods. The ratio of the CD3⁺ subtype in the (A) control group and (B) experiment group by flow cytometry. (C) Antitumor activity of T cells on hepatoma cells investigated using an MTT assay. Bars represent mean + standard deviation. (n=3). **P<0.01. CD, cluster of differentiation.

were cultured at 37°C and 5% CO₂ for 24 h. A total of five parallel experiments were used in each group. Then, 10 μ l MTT (5 mg/ml) was added and cultured at 37°C with 5% CO₂ for 4 h. Following centrifugation at 900 x g for 5 min at room temperature, the precipitate was dissolved with 100 μ l DMSO, agitated for 15 min at 37°C, and the optical density (OD) was detected at 490 nm. The killing rate (%) was calculated as follows: [1 - (OD_{effector-target cell well} - OD_{effector cell well}) / OD_{target cell well}] x 100%.

One-dimensional gel electrophoresis (1-DE) analysis. 1-DE was performed using a 5% stacking gel (pH 6.8) and an 8% separating gel (pH 8.9) in Tris-glycine buffer (pH 8.3), and 10 μ g protein was analyzed. The gels were run at 60 V for 45 min, then 120 V for 2-3 h per gel in an ice-bath until the bromophenol blue dye had migrated to <1 cm from the bottom of the gel. Subsequently, The gels were stained with coomassie

Figure 2. 1-DE was used to identify differentially expressed proteins in cytokine-induced killer cells. (A) 1-DE analysis of P43 protein: Lane 1, molecular mass markers; lane 2, control group; lane 3, experiment group. (B) The histogram analysis of gray values. Bars represent mean + standard deviation. (n=3). *P<0.05. 1-DE, One-dimensional gel electrophoresis.

brilliant blue R-250 at room temperature for 15 min. The bands was analyzed using Quantity One 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Mass spectrometry analysis. Differentially expressed proteins bands were excised from 1-DE gels for LC-MS/MS mass spectrometry analysis. Briefly, after the gel plug was digested with trypsin, 10 μ l of the peptide mixture was separated with a linear gradient of 5-80% buffer B [100% acetonitrile, 0.1% formic acid (FA)] at a flow rate of 400 nl/min on a C₁₈-reversed phase column packed in-house with Eprogen-Pur C₁₈-AQ 5 μ m resin in buffer A (100% H₂O, 0.1% FA). A prominence nano 2D chromatography system (Shimadzu Corp., Kyoto, Japan) was coupled online to the micrOTOF-QII (Bruker Corporation, Billerica, MA, USA). The data was collected using BrukerDaltonicsmicrOTOFcontrol software 3.2 (Bruker Corporation) with the conditions 50-2,200 m/z scan range, 1,500 V capillary voltages, and 150°C drying argon gas temperature. Finally, the selected peptide masses were analyzed using DataAnalysis software 4.1 (Bruker Corporation) and searched using the Mascot search engine version 2.3.01 (http://www.bgi-proteomics.cn/tocsam/cgi/master_results_2.pl?file=20140317%2FF044726.dat;pr.eh=3%2C3p#tc:rf:hits:3).

Western blot analysis. Following 1-DE, the gel was separately transferred to a polyvinylidene fluoride (PVDF) membrane for 90 min at 300 mA in transfer buffer (25 mM Tris, 0.1 M glycine, and 20% methanol). Subsequently, the membranes were blocked for 1 h in TBS-Tween 20 (TBST; 0.05% Tween-20, 20 mM Tris, 150 mM NaCl, pH 7.4) containing 5% skim milk at room temperature. After washing three times with TBS for 5 min each, the PVDF membrane was incubated with rabbit anti-human haptoglobin (catalogue no. BA3744; Wuhan Boster Biological Technology, Ltd., Wuhan, China) at a dilution of 1:200 in TBST containing 5% skim milk at room temperature for 1 h. The membrane was washed three times with TBS for 20 min and incubated with goat anti-rabbit IgG-horseradish peroxidase antibody (catalogue no. MBS856805; Sino-American Bio. Corp., Luoyang, China) at a dilution of 1:1,000 in TBST containing 5% skim milk at room temperature for 40 min. Next, the membrane was

washed two times with TBS for 20 min and analyzed using the ECL Plus (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) chemiluminescence detection method. Finally, the results were analyzed using Quantity One 4.6.2 (Bio-Rad Laboratories, Inc.).

RNA isolation and RT-qPCR analysis. Total RNA was extracted from CIKs using the RNAsimple Total RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. A total of 1 μ g RNA was synthesized to cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following cDNA synthesis, a RT-qPCR assay was performed as Livak and Schmittgen (16) reported with some modification. A segment of 141 and 221 bp was amplified using primer sets Haptoglobin-F/Haptoglobin-R (Table I) and β -actin-F/ β -actin-R (Table I), respectively. RT-qPCR was performed in a volume of 20 μ l containing 10 μ l 2X Master mix (Maxima SYBR Green/ROX qPCR Master Mix kit; Thermo Fisher Scientific, Inc.), 0.3 μ l of 10 μ M Forward Primer, 0.3 μ l of 10 μ M Reverse Primer, 0.8 μ l cDNA and 8.6 μ l dH₂O. Next, the 20- μ l mixture was analyzed using ABI Stepone Plus (Thermo Fisher Scientific, Inc.), under the following thermocycling conditions: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec and a final cycle of 60°C for 1 min and 95°C for 15 sec. Data were normalized according to the $2^{-\Delta\Delta C_q}$ method (16). The assays were performed in three independent experiments.

Statistical analysis. The data were presented as the mean $\pm$ standard deviation. Statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Differences were determined using the Student's t-test. P<0.05 or P<0.01 were respectively considered to indicate significant or very significant differences. All experiments were repeated at least three times.

Results

Ratio and activity analyzed for CIKs. In order to investigate the associated protein activity of CIKs cultured *in vitro*,

Table II. Identification of the differentially expressed protein in cytokine-induced killer cells using liquid chromatography-mass spectrometry.

Protein	Accession name	Description	Species	Mr (Da)	pI	Trends in expression
P43	P00738	Haptoglobin	<i>Homo sapiens</i>	45861	6.13	↓

pI, isoelectric point; Mr, molecular weight.

Table III. Matched amino acid sequences of haptoglobin (P00738) protein identified using liquid chromatography-mass spectrometry.

Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Start-end	Sequence
709.9410	1417.8673	1417.8181	0.0493	1	56	216-228	DIAPTLTLYVGKK
854.4398	1706.8649	1706.8120	0.0529	0	27	298-311	YVMLPVADQDQCIR
724.7164	2171.1274	2171.0504	0.0770	0	34	326-345	SPVGVQPILNEHTFC AGMSK
602.3429	1202.6712	1202.6295	0.0417	0	42	392-401	VTSIQDWVQK

Mr, molecular weight; expt, expected; calc, calculated.

Figure 3. Identification and analysis of haptoglobin. The rabbit anti-haptoglobin (1:200) or rabbit anti- β -actin (1:200), and goat anti-rabbit IgG-horseradish peroxidase (1:1,000) were used as the primary and secondary antibodies, respectively. (A) Haptoglobin identified by 1-DE and western blotting. Lane 1, 1-DE of T cell proteins; lane 2, western blotting. (B) Western blotting of haptoglobin from T cells. Lane 1, control group; Lane 2, experiment group for haptoglobin and β -actin. (C) The histogram analysis of gray values, gray value ratio=the gray value of haptoglobin/the gray value of β -actin. Bars represent mean $\pm$ standard deviation. (n=3). * $P<0.05$. 1-DE, One-dimensional gel electrophoresis.

Figure 4. Reverse transcription-quantitative polymerase chain reaction analysis of haptoglobin mRNA transcripts in cytokine-induced killer cells. Haptoglobin mRNA was normalized against β -actin mRNA levels. Bars represent mean $\pm$ standard deviation. (n=3). ** $P<0.01$.

gentamicin was selected as an activity inhibitor. Following stimulation, the ratio of CIKs in the control and experiment groups were 98.2 and 98.3%, respectively (Fig. 1A and B). However, the activity of CIKs in the experiment group (34.3%) was very significantly lower compared with the control group (55.2%; $P<0.01$; Fig. 1C) because of the effect of gentamicin.

Differentially expressed protein analyzed. Based on the changes in CIK activity, an unknown differential expression protein (P43) was identified using 1-DE (Fig. 2A). As presented in Fig. 2A, the P43 molecular weight was ~43 kDa, and the expression of P43 was significantly downregulated by 1.22-fold compared with the control group ($P<0.05$; Fig. 2B).

Identification of P43. For further investigation on the P43 protein in CIKs, the P43 foci was detected as *Homo sapien*

haptoglobin protein (P00738) using LC-MS/MS (Tables II and III). As Tables II and III demonstrates, the matched amino acid sequences of haptoglobin attained an overall score of 159, and the molecular and isoelectric point was 45861 Da, and 6.13, respectively. Following a 1-DE separation and western blotting, the P43 protein in CIKs lysis was demonstrated to specifically react with the rabbit anti-human-haptoglobin (Fig. 3A). Together, these results suggested that the P43 protein was haptoglobin.

Haptoglobin expression analyzed. To analyze the expression of haptoglobin protein, western blotting was performed (Fig. 3). In the CIK protein, only haptoglobin could specifically reacted with rabbit anti-haptoglobin (Fig. 3A). Further investigation revealed that the expression level of β -actin in the control and experiment groups was similar and the haptoglobin was significantly downregulated by 1.17-fold compared with the control group ($P < 0.05$; Fig. 3B and C).

Haptoglobin mRNA analyzed. Based on the proteomic results, a RT-qPCR assay was performed to determine the transcription levels of haptoglobin in CIKs. As presented in Fig. 4, SpHMC mRNA in the experiment group was significantly downregulated by 1.73-fold compared with the control group ($P < 0.01$; Fig. 4).

Discussion

CIKs adoptive immunotherapy is used in tumor treatment due to its efficiently antitumor activity (17-20). Gentamicin is a broad-spectrum antibiotic used to preserve the aseptic culture of cells (21-23). In the present study, it was demonstrated that the CIK activity was significant inhibited by gentamicin, and further research revealed that a protein P43 was associated with CIK activity.

To determine the identity of the P43 protein, 1-DE with LC-MS/MC was performed (24,25). The P43 protein was successfully identified as human haptoglobin. This was similar to the findings that an unknown protein spot identified as haptoglobin in patient sera using the mass spectra strategy (26-28). For further verification of the mass spectra results, western blotting was used (29), and the results demonstrated that haptoglobin protein was specificity connected with rabbit anti-human-haptoglobin. Therefore, it was concluded that the P43 protein was haptoglobin.

Haptoglobin is a hemoglobin-binding protein expressed by a genetic polymorphism for prevented oxidative damage in plasma (30-32). However, research has identified that haptoglobin was also involved in the immune response (33,34). Delanghe *et al* (35) demonstrated that haptoglobin phenotypes may influence T cells activation and effect the interplay of lymphocytes. Shen *et al* (36) reported that haptoglobin activates the innate immunity. Kreisel and Goldstein (37) noted that haptoglobin was a novel protein activator of the innate immune system and involved in immune modulatory as a known acute phase protein. These results demonstrated that the concentration of haptoglobin in CIKs treated with gentamicin was significantly downregulated by 1.17 using western blotting. Thus, it was deduced that the haptoglobin was associated with the enhancement of CIK activity.

To further illustrate the association of haptoglobin with CIKs activity, a quantitative RT-qPCR assay was performed. As a result, the mRNA level of haptoglobin in CIKs treated with gentamicin was significantly downregulated by 1.73-fold compared with the control group. This is similar to the finding that haptoglobin expression signal was decreased 5.1-fold in peripheral blood mononuclear cells from healthy participants compared with methotrexate-resistant patients with rheumatoid arthritis (38). Herein, these results suggest that haptoglobin was involved in the activity-enhancement of CIKs.

In conclusion, it was identified that haptoglobin in CIKs was an activity-enhancement-associated protein. To the best of our knowledge, this is the first study to demonstrate that haptoglobin may be associated with the activity-enhancement of CIKs. Further investigation is required to explore its exact role of the signaling pathway associated with an increase in CIK activity.

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