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Original Article

Detection of growth hormone receptor on dendritic cells

Qiu-Liang Liu, Jing-Yao Gao, Jiao Zhang, Xin Liu

Department of Pediatric Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

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Abstract: Background: Malignant tumor is the leading killer of threat to human health. Dendritic cell vaccine-based immunotherapy can activate the immune response, through the identification of tumor associated antigen specific killing of tumor cells without harming normal cells. DCs, the most potent antigen-presenting cells, are the initiator of immune response and occupy a unique place in the induced immune response. But a small number of DCs in vivo are difficult to meet the need of clinical treatment and scientific research. To play a physiological role, GH, a protein hormone secreted by the anterior pituitary, must firstly combine with growth hormone receptor (GHR) on the surface of the target cell membranes to pass through the cell membrane. Methods: 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 5 ng/mlinterleukin-4 (IL-4) were used to stimulate differentiation of umbilical cord blood mononuclear cells (CBMCs) into the immature DCs and further into mature DCs. Cord blood T lymphocytes were also obtained as a control group. Fluorescence quantitative PCR, western blotting, immunofluorescence and immunohistochemistry (IHC) were used to detect the expression of GHR in DCs group and the control group. Results: After stimulated by GM-CSF and IL-4, CBMCs were differentiated into mature DCs. GHR mRNA expression was significantly elevated in DCs as compared to that in cord blood T lymphocytes. Immunofluorescence and western-blotting analysis revealed that GHR protein expression was also significantly increased in DCs as compared with that in cord blood T lymphocytes. Moreover, IHC results showed that GHR was highly expressed in cytoplasm and nucleus of DCs, which were positively stained brown. Conclusions: CBMCs can be stimulated by the cytokine combination in vitro into DCs. Strongly positive expression of GHR was detected on DCs.

Keywords: Dendrictic cells, growth hormone receptor, qRT-PCR, western blotting

Introduction

The treatment of malignant tumor is a comprehensive, individualized plan for patients' treatment. For earlier detection and development of tumors, the therapeutic efficacy of surgery is obvious, but it is relatively difficult to perform an operation on some areas, where tumors are not able to be completely removed with recognition of the high rates of local recurrence and potential metastasis as well as poor prognosis [1]. Biological therapy, often called immunotherapy, is a new cancer treatment, whose high efficiency and definite effect has been confirmed. Natural killer cells, dendritic cells (DCs) and cytokine-induced kill cells (CIK) are main effector cells that play important roles in adoptive inununotherapy. Specially, many efforts have been focused on utilization of the immunostimulatory power of fully activated DCs for immunotherapy of cancer [2, 3]. In fact, dendritic cell vaccine based immunotherapy can activate the immune response, through the identification of tumor associated antigen specific killing of tumor cells without harming normal cells. It also has strong growth inhibitory effects on tumor cells, achieving the purpose of preventing and controlling tumor recurrence and distant metastasis. DCs were discovered in 1973 by Ralph Steinman in the secondary lymphoid tissues of mice, as a previously undefined cell type in the mouse spleen [4].

Dendritic cells (DC) are the major antigen-presenting cells (APC) of the immune system and are probably the only APCs capable of initiating antigen-specific responses, inducing differentiation of naive T cells [5]. DCs not only instruct T- and B-lymphocytes, but also activate natural killer cells and produce interferons, thus bridge the innate and adaptive immunity [6]. They serve as the sentinels that capture antigens in

Table 1. Primers for quantitive RT-PCR

Genes	Primers	Sequences
GHR	Forward	5'-ATGGATCTCTGGCAGCTGCTGTT-3'
	Reverse	5'-ACTCCAGGGTGCTCTGCTAAGGAT-3'
GADPH	Forward	5'-AGAAGGCTGGGGCTCATTTG-3'
	Reverse	5'-AGGGGCCATCCACAGTCTTC-3'

the periphery, process them into peptides and present these to lymphocytes in lymph nodes, initiating primary T cell responses and linking innate and adaptive immune responses to pathogens [7, 8]. Dendritic cells (DCs) are important both in physiology and as tools for cell therapy programs, and the issue of their origins and differentiation has been addressed by many studies [9].

Previous studies has revealed that interstitial type of DC can be generated from the precursor cells (monocytes) with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) for approximately 7 days which induce differentiation of the monocytes into immature DC (iDC) [10]. DC maturation is of crucial importance for the induction of immune responses in cancer patients in vivo [11], and DC number and tissue inflammation are critical parameters for DC-based vaccination [12]. However, DC present in peripheral blood comprises less than 1% of mononuclear cells [13]. Therefore, in order to prepare a DC based vaccine isolation and in vitro culturing of DC is required. Most of DCs precursor cells are derived from peripheral blood, bone marrow, and cord blood, including circulating CD34+ precursor cells from umbilical cord blood and bone marrow and CD14+ monocytes from peripheral blood [14]. Umbilical cord blood mononuclear cells (CBMCs) are derived from traditional peripheral blood collection with no anesthesia and patient pain as well as reduced side effects. Human umbilical cord blood contains an abundance of immature stem/progenitor cells, which have a strong ability of proliferation and multi-directional differentiation potency, and have widely used in the research. It provides a better choice for in vitro differentiation of DCs and has broad prospects for application as immuno-biologicals in clinic.

DCs from different sources may have different biological activity, which can thus induce CTL with different killing effect on tumor cells [15]. Previous studies on induction of the antitumor CTL effector killer response by dendritic cell vaccination against cancer demonstrated that the cytotoxic activity of CTL was low, even less than 40% reported in some research paper [16]. DCs derived from precursor cells or monocytes possess low functional activity and cannot present these antigens to T cells in lymphoid organs effectively, which contributes to the low cytotoxic activity of CTL induced by DCs. Thus, we hypothesize that there are some bioactive factors that can induce DCs and then activate T cells to produce CTL with a more powerful antitumor cytotoxic activity. Some researchers have been working on it for a long time. They used some cytokines, microbe, antibodies, drugs and the body metabolites to induce DCs, obtain CTL and sustain the antitumor CTL effector killer response [17, 18].

Growth hormone (GH) is a protein hormone composed of 191 amino acids that is secreted by the anterior pituitary and circulates throughout the body to exert important actions on growth and metabolism. Due to the membrane filtration, GH is unable to directly penetrate into the cells and acts on cell membrane. GH must firstly combine with growth hormone receptor (GHR) located on the surface of target cell membranes to exerts its pleiotropic effects [19]. Then by a transmembrane signal transduction system, the interaction triggers a series of enzymatic reactions and induces target cells to produce peptides with growth-promoting potency and insulin-like growth factors. Those peptides are considered to play critical roles in body/cellular growth, metabolism, and immune reaction [20].

The present study was aimed to test expression of GHR on DCs. Thus, after GM-CSF and IL-4 stimulation, expression of GHR on DCs was analyzed by fluorescence quantitative PCR, western blotting, immunofluorescence and immunohistochemistry, respectively.

Materials and methods

Cord blood DCs induction

Cord blood (60-100 ml) taken from 54 healthy puerperas from the First Affiliated Hospital of

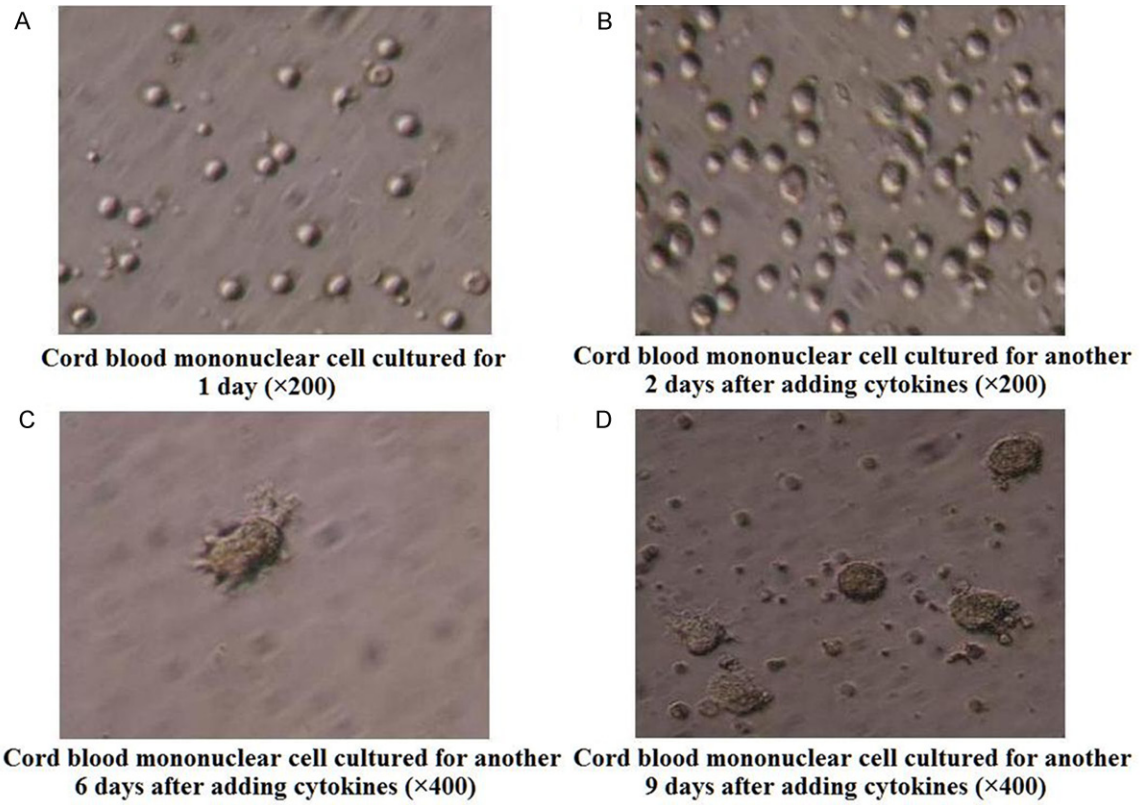

Figure 1. Morphology of DCs derived from CBMCs stimulated with rhIL-4 and rhGM-CSF. After 7-9 days of cultivation, the obtained DCs were identified using an inverted phase contrast microscope. Morphology of cells during primary culture for 24 h (Original magnification ×200) (A). When mononuclear cells were induced with rhIL-4 and rhGM-CSF for 2 days, cells exhibited good cell morphology with plump rounded shape and grew adherently (Original magnification ×200) (B). After 6 days of induction, cells exhibited a shuttle shape with a few protrusions and preliminary dendritic morphology (Original magnification ×400) (C). After 9 days of induction, a large amount of shuttle shape cells showed polygonal and mature DC morphology with obvious protrusions (Original magnification ×400) (D).

Zhengzhou University (Henan, China) from March 2013 to January 2014 was added with lydroxypropylmethyl cellulose (1:1.5, Tianjin Jinmai Gene Mapping Technology, China) before centrifuging at 2000 g for 25 min. 10 samples were prepared for subsequent experiments. The study was approved by the Medical ethics committee at the First Affiliated Hospital of Zhengzhou University. Written informed consents were obtained from all these participants.

Cord blood mononuclear cells (CBMCs) were collected from the superstratum of blood plasma, washed with RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) three times, and then diluted to 4×10^6 cells/ml with RPMI 1640 containing 10% autoserum. Subsequently, CBMCs were inoculated in a 24-well plate with 1 ml per well, and incubated at 37°C for 2 hours in an atmosphere of 5% CO_2. The T lymphocytes in suspension before the inducible expression were served as control. Remaining T lymphocytes in suspension were transferred into a culture bottle and IL-2 (10 ng/ml) was added. Both 5 ng/ml recombinant human interleukin (IL)-4 (rhIL-4, Peprotech, Rocky Hill, NJ, USA) and 10 ng/ml granulocyte-macrophage colony-stimulating factor (rhGM-CSF, Peprotech) were added to the adherent mononuclear cells obtained from the 24-well plates. Mononuclear cells after the inducible expression were designated as DCs group. Medium exchange was conducted every other day. T lymphocytes and DCs were re-supplemented after each medium exchange. After 7-9 days of cultivation, the obtained DCs were identified using an inverted phase contrast microscope (Olympus, Japan).

Quantitive real-time PCR (qRT-PCR)

Total RNA was extracted from using Trizol reagent (Ambion, CA, USA) and reversely transcribed into cDNA using the HiScript® II Reverse

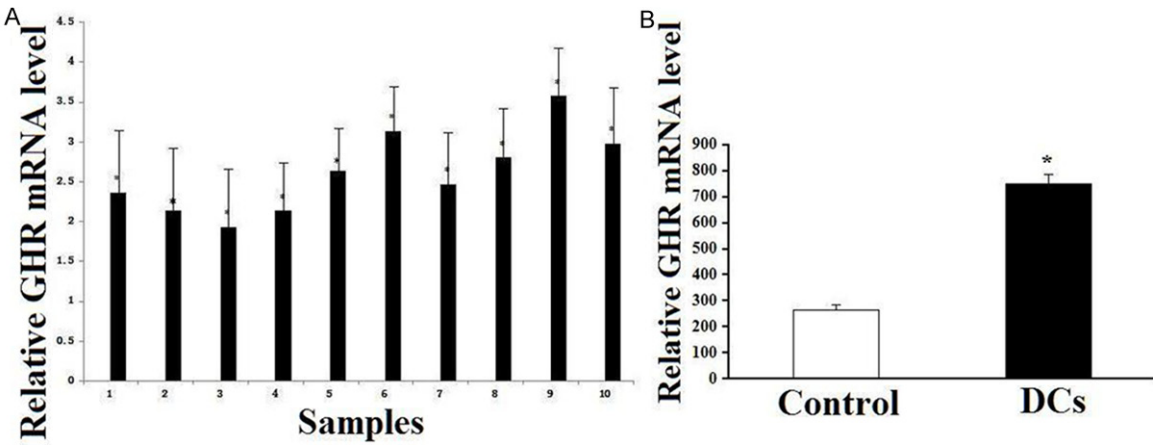

Figure 2. qRT-PCR analysis for GHR mRNA expression. GHR mRNA expression in control cells and DCs derived from CBMCs stimulated with IL-4 and GM-CSF of every sample measured by qRT-PCR (A). GHR mRNA expression in control cells and DCs of 10 samples determined by qRT-PCR (B). *P<0.05.

Transcriptase (Vazyme Biotech, NJ, USA) according to the manufacturer's protocols. GHR mRNA expression was determined by qRT-PCR using 2 μg of cDNA in a 20 μg reaction volume with an Ace Q™ SYBR Green Master Mix (Vazyme Biotech) and ABI 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA). PCR cycles were as follows: 95°C for 5 min, followed by a two-step PCR program consisting of 95°C for 10 s, 60°C for 30 s for 40 cycles, then 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The sequences of the primers for were summarized in **Table 1**. Reactions were run in triplicate and the relative RNA levels were normalized to GAPDH mRNA expression. Data were collected and quantitatively analyzed on the ABI GeneAmp 7500 SDS software (Applied Biosystems) using 2-delta delta CT method.

Western blotting for GHR

Western blot analyses were performed for the determination of GHR protein expression in T lymphocytes and DCs. Cells were rinsed with cold PBS, and lysed in cell lysis buffer containing 1 mm phenylmethanesulfonyl fluoride (PMSF) and homogenized subsequently. Samples from cell lysates was subjected to 10% SDS-PAGE and then transferred to PVDF membranes by 1.5 h electroblotting. Membranes were blocked with TBST containing 5% BSA overnight at 4°C, probed with an appropriate dilution of anti-GHR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a 1:1000 dilution of anti-β-actin (Santa Cruz Biotechnology) diluted in TBST for 1 h, and then incubated with goat anti-rabbit IgG/FITC secondary antibodies (Proteintech, Chicago, IL, USA) for 2 h. Finally, the blots were detected by the enhanced chemiluminescence (ECL) kit (Amersham Biosciences, NJ, USA) as recommended by the manufacturer's protocols. Each sample was replicated triple and total protein levels were normalized to β-actin.

Immunofluorescence

Immunofluorescence was performed on cells grown on four-chamber tissue culture slides for another 3 days. In brief, cells were grown to 60% confluence on plates, washed with PBS three times, fixed in 4% paraformaldehyde for 15 min and then washed three times in PBS. Subsequently, cells were permeabilized with 0.2% Triton X-100 for 10 min and then washed three times in PBS. Then, the cells were blocked for 1 hour 5% bovine serum albumin (BSA), washed three times in PBS, and incubated with the appropriate primary antibody, anti-GHR (1:300; Santa Cruz Biotechnology) overnight at 4°C. Cells were then washed with PBS three times, followed by incubation with secondary antibody, goat anti-rabbit IgG/FITC (1:500; Proteintech) for 1 h at room temperature (in the dark). Following another rinsing step with PBS three times, cells were incubated with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) to counterstain cell nuclei for 2 min at room temperature. When the reaction time ended, the coverslips were

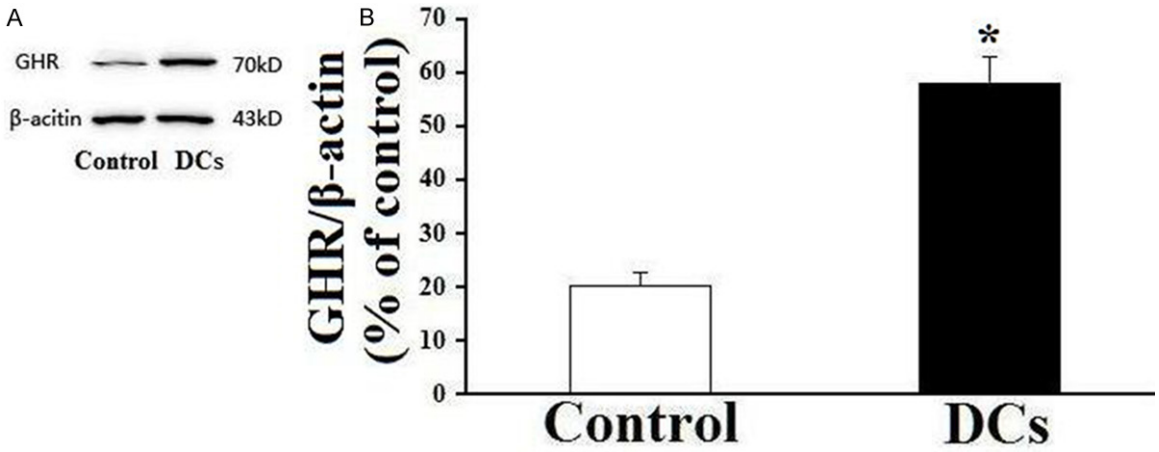

Figure 3. Western blotting analysis for GHR protein expression. GHR protein expression in control cells and DCs derived from CBMCs stimulated with IL-4 and GM-CSF assessed by western blotting (A). Densitometry of western blots to quantitate GHR protein expression in control cells and DCs (B). *P<0.05.

washed three times in PBS for 5 min each and dried at 37°C for 45 min. Before analyzing under the microscope, 10-15 μL Dabco mounting media (PBS, 50-60% glycerol, 2.5% 1,4 diazobicyclo (2,2,2)-octane) was added. Finally, coverslips were sealed by the transparent nail polish and examined under a TE-2000-E inverted fluorescence microscope (Nikon, Yokohama, Japan).

Immunohistochemistry (IHC)

DCs were then analyzed for GHR expression by immunohistochemistry (IHC). For fluorescence staining, cultured DCs were stained with anti-GHR antibody, followed by the secondary antibody, and were observed with a light microscope. Immunoreactions were processed using Ultra-Sensitive TM S-P Kit (Maixin-Bio, China) according to the supplier's protocols, and signals were visualized using the DAB substrate that stains the target protein yellow. Staining was scored by 3 independent pathologists and the pathologists blinded to the subject's clinical history and the results of the immunohistochemistry staining assay. Briefly, a proportion score was assigned that represents the estimated proportion of positive cells on the entire slide counted with an inverted phase contrast microscope (Olympus). Results of each IHC staining were evaluated as follows: the proportion of the stained cells was assessed on a five-tier scale (0, <10%; 1, 10-30%; 2, 31-51%; 3, 52-70%; and 4, >70% cells stained), and the staining intensity was graded on a four-point scale (0, negative; 1, weak/canary yellow; 2, moderate/yellow; and 3, strong/brown). We defined the immunoreactive score (IRS) as the product of the percentage of positive cells and the staining intensity [21]. Immunohistochemical results with an IRS of 0 were considered negative (-), 1-4 weak positive (+), 5-8 moderate positive (++) and 9-12 strong positive (+++).

Statistical analysis

SPSS ver 17.0 statistical software was used to detect the data, and all data are expressed as mean ± SD. Comparisons between two groups were tested by Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Induction of DCs in vitro

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) known that serve as the sentinels that capture antigens in the periphery, process them into peptides and present these to lymphocytes in lymph nodes [7]. In this study, the DCs were identified 7-9 days after cultivation using an inverted phase contrast microscope. No obvious changes were seen on within 1 day (**Figure 1A**). After 24 h of primary culture, CBMCs were then induced with rhIL-4 and rhGM-CSF for 2 days. Good cell morphology, more plump rounded cells and most adhered cells were observed (**Figure 1B**). When

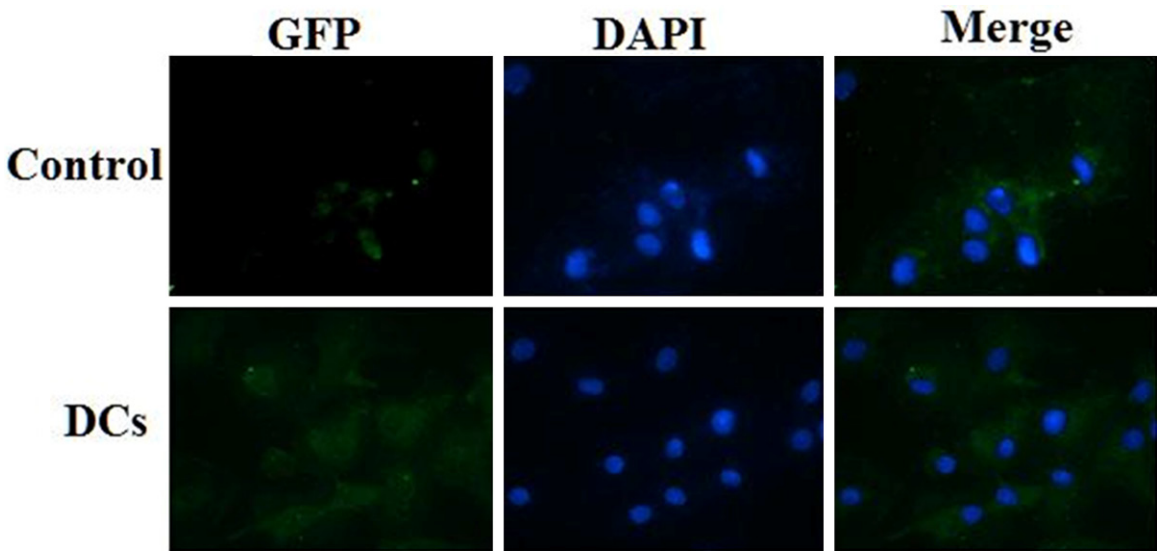

Figure 4. GHR protein expression in T lymphocytes and DCs. T lymphocytes (upper panel) and DCs (bottom panel) were assessed by immunofluorescent staining with anti-GHR antibody, goat anti-rabbit IgG/FITC (green), Green fluorescent protein (GFP) (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue). Green stains and red stains indicate the accumulation of GFP and that of DAPI in nuclei of the cells, respectively.

mononuclear cells were induced with rhIL-4 and rhGM-CSF for 6 days, more cells presented with shuttle shape and adherent growth with cell volume slightly enlarged, and spindled edges of multiple cells started to spread out and preliminarily formed dendritic morphology (**Figure 1C**). After 9 days of induction, a large amount of cells exhibited mature DC morphology, most of which had obvious protrusions, indicating successful induction of DCs with rhIL-4 and rhGM-CSF (**Figure 1D**).

GHR mRNA expression is significantly elevated in DCs

GH is a protein hormone composed of 191 amino acids that is secreted into the circulation by the anterior pituitary, which is a major regulatory factor for overall body growth as evidenced by the height extremes in people with abnormal circulating GH levels or GH receptor (GHR) disruptions [20]. In this study, results demonstrated that GHR mRNA expression was significantly increased in DCs derived from CBMCs stimulated with IL-4 and GM-CSF as compared to that in control cells (T lymphocytes in suspension before the inducible expression) (**Figure 2**).

GHR protein expression detected by western blotting is significantly increased in DCs

To further explore GHR protein expression in DCs derived from CBMCs stimulated with IL-4 and GM-CSF, western blotting analysis was performed in the current study. As shown in **Figure 3**, DCs derived from CBMCs stimulated with IL-4 and GM-CSF showed a dramatically enhanced GHR protein expression when compared with control cells.

GHR expression measured by immunofluorescence is higher in DCs

The subcellular localization of GHR was investigated using immunofluorescence. Green fluorescent protein (GFP) and DAPI were localized sparsely in T lymphocytes (control; **Figure 4**, upper panel), whereas DCs presented more intensive GFP and DAPI (**Figure 4**, bottom panel). The fluorescence intensity and the number of fluorescent protein were higher in DCs, suggesting a higher expression of GHR on DCs after the induction.

Immunohistochemical analysis of GHR expression

GHR expression on DCs was also heterogeneously distributed in respect to the proportion of positive tumor cells and staining intensity. As shown in **Figure 5**, GHR was highly expressed in cytoplasm and nucleus of DCs, which were positively stained brown. Moreover, staining intensity of GHR in cytoplasm and nucleus of DCs was gradually increased with positive staining rate of 81.3%.

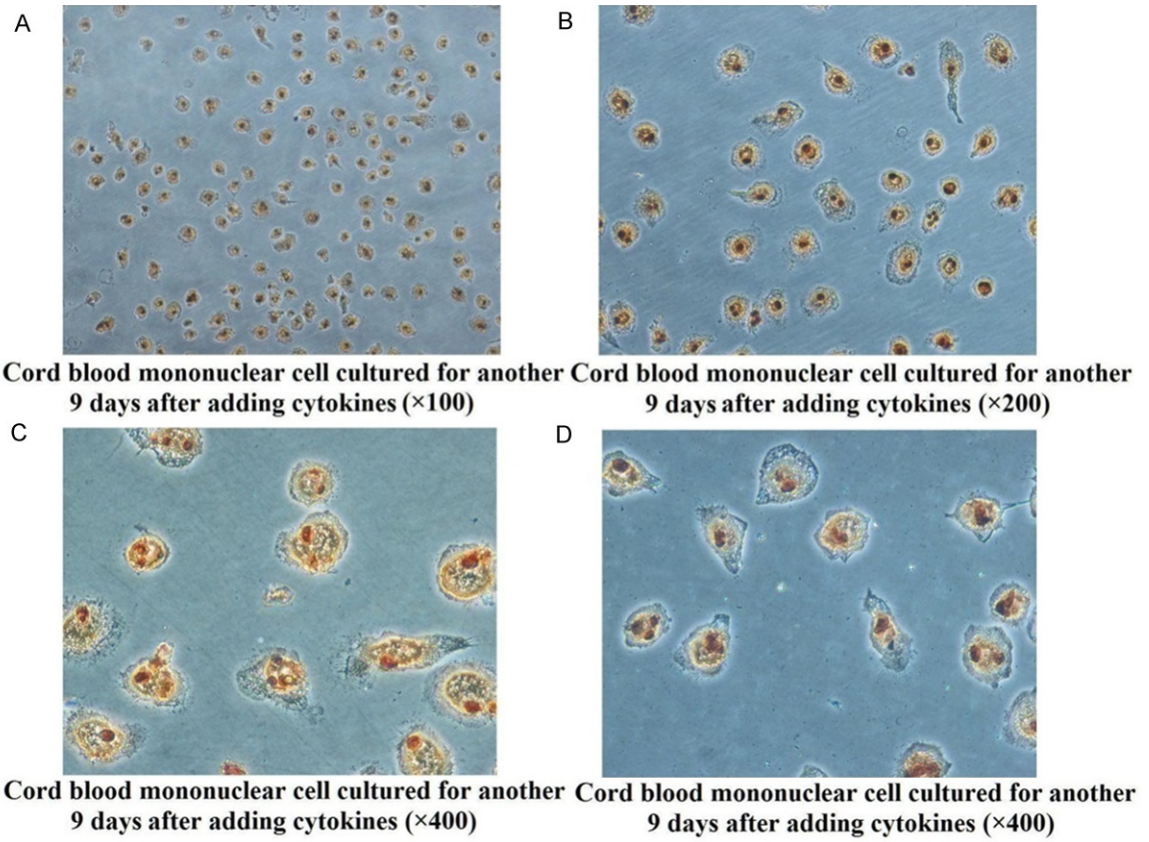

Figure 5. GHR expression and protein status analyzed by IHC in DCs. When mononuclear cells were induced with rhIL-4 and rhGM-CSF for 9 days, immunohistochemical expression of GHR protein was shown at original magnification ×100 (A), original magnification ×200 (B) and original magnification ×400 (C and D).

Discussion

Adoptive immunotherapy of cancer is a novel therapeutic approach that is carried out by promoting differentiation of stem/progenitor cells into specific antigens that a Th1 immune response and also activate CTLs in order to eliminate the tumor [22].

Adaptive immune responses are orchestrated by specialized professional antigen-presenting cells (APCs), the DCs, which play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses [23]. Unfortunately, the unique capabilities of DCs to control immune responses are sometimes exploited by tumors, allowing the uncontrolled growth of potentially immunogenic tumors. However, the peripheral tolerance of tumor-directed T cells induced by the growing tumor can be broken by providing appropriate signals that target and activate DCs. For instance, after CD40-triggering on DCs, or injection of Toll-like-receptor-activating agents such as CpG or LPS could induce a strong systemic CTL-reaction that was able to eradicate established tumors [24]. Indeed, the goal of utilizing DC to deliver a cancer vaccine is to induce a Th1 immune response and also activate CTLs in order to eliminate the tumor. Therefore, induction of the antitumor CTL effector killer response by dendritic cell vaccination against cancer has become a hot area of research on adoptive immune response during biological therapy [25].

According to the different morphology, biological and functional characteristics, DCs can be divided into Immature DCs and mature DCs. Immature DCs have the ability to constantly sample their environment in search of a danger signal or pathogen. Thereafter the DCs migrate to the draining lymph node where they meet and activate cognate T cells [26]. After receiving an activation signal, DCs migrate to organized lymphoid tissues, they lose their ability to

internalize antigens and mature into potent APCs that acquire the capacity to present antigens to naive T cells, a process referred to as DCs maturation or activation [27]. There are many stimuli that can initiate DCs maturation process, including bacterial lipopolysaccharide (LPS), bacterial toxins, inflammatory cytokines, prostaglandins, as well as calcium mobilization. The maturation process involves more secretion of cytokines, the upregulation of surface MHC II and costimulatory molecules (CD80, CD86, and CD40), and increase of the ability to stimulate T cells [28, 29]. DCs activation results in the enhanced ability to stimulate and polarize T cells in vitro and in vivo [30]. For biological therapy of cancer, more mature DCs are expected to implement the immunotherapeutic effect.

DC are commonly cultivated from CD34+ progenitor cells or peripheral blood mononuclear cells (PBMC) [28]. DC present in peripheral blood comprises less than 1% of mononuclear cells and human umbilical cord blood exerted a high differentiation rate. Thus, in this study, we used DCs derived from cord blood mononuclear cells (CBMCs) to test growth hormone receptor on DCs. Moreover, different types of DC can be obtained for which the development is mediated by specific cytokines, including granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF) [31], transforming growth factor (TGF) [32], FL [33].

As part of the haematopoiets in family, GM-CSF is a DC-activating cytokine inducing differentiation, proliferation and activation of macrophages and dendritic cells [34], which enhances the oxidative metabolism, cytotoxicity and antibody-dependent phagocytosis [35, 36]. Previous studies on DCs in vitro culture indicated that CBMCs differentiated into macrophages by only adding GM-CSF into medium, whereas monocyte-derived DCs (MDDCs) can be produced by culturing monocytes with GM-CSF and interleukin-4 (IL-4) for 7 to 9 days, which are phenotypically equivalent to immature DCs residing in peripheral tissues [37].

When MDDCs are incubated with activating agonists such as LPS, TNF-α or cholera toxin (CT), they undergo maturation and take on the phenotypic characteristics of mature DCs found in secondary lymphoid tissues [37]. In the current study, after culturing with GM-CSF and L-4 for 7 to 9 days, monocytes harvested from cord blood have successfully differentiated into DCs in vitro, the morphology of which could be observed using an inverted phase contrast microscope (**Figure 1**).

GH is a peptide hormone that is secreted and synthesized by cells called somatrophs in the anterior pituitary gland and acts upon various target tissues expressing GHR. GH is a key regulator of postnatal growth and metabolism and plays a physiological role by interaction with the GH receptor (GHR) on the surface of target cells [38]. Previous studies confirmed that global disruption of the GH receptor in mice (GHR-/-) produces a large and reproducible extension in lifespan [39]. Moreover, GH and GHR are pivotal in feto-placental development and pregnancy maintenance [40], and the GHR mediates metabolic and somatogenic actions of GH [41]. In the present study, GHR mRNA and protein expressions measured by qRT-PCR (**Figure 2**), western blotting (**Figure 3**) and immunofluorescence (**Figure 4**) were significantly elevated in DCs derived from CBMCs stimulated with IL-4 and GM-CSF as compared to that in the control cells. Tumor tissue section was mostly used at home and abroad as a source for induction of DCs, but the testing of GHR expression on in vitro induced DCs from CBMCs was rarely reported. Our study indicated that GH could stimulate further maturation of DCs and several analyses at different levels confirmed the existence of GHR on DCs. Moreover, immunohistochemical analysis of GHR expression demonstrated that GHR was highly expressed both in DCs nucleus and cytoplasm (**Figure 5**). So we proved that GH was capable of modifying DCs differentiation process and stimulating the differentiation of cord blood lymphocytes into mature DCs. And on this basis we are supposed to carry out further research on signaling pathways and underlying mechanisms of GH on DCs.

Conclusion

In summary, our results demonstrated that more mature DCs were obtained from umbilical cord blood mononuclear cells stimulated by IL-4 and GM-CSF. In addition, GHR mRNA and protein expressions measured by qRT-PCR, western blotting and immunofluorescence were significantly elevated in these DCs. Moreover,

after induction, GHR was more highly expressed both in DCs nucleus and cytoplasm as compared with that in the control cells, which could induce more immunologic effector cells with powerful immune function and enhance their killing effect on tumor cells.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qiu-Liang Liu, Department of Pediatric Surgery, The First Affiliated Hospital of Zhengzhou University, 1 Jianshedong Rd., Zhengzhou 450052, People's Republic of China. Tel: +86-371-67967252; E-mail: qliangliu@163.com

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