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Original article

Notch signaling pathway dampens tumor-infiltrating CD8$^+$ T cells activity in patients with colorectal carcinoma

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ABSTRACT

CD8$^+$ T cells play critical role in controlling the metastasis and prognosis of cancer. Controversy remains as to the contribution of Notch signaling pathway in modulation of CD8$^+$ T cells activity and development of tumorigenesis. Thus, the aim of the current study was to investigate the immunoregulatory role of Notch signaling pathway to peripheral and tumor-infiltrating CD8$^+$ T cells in patients with colorectal carcinoma. A total of 46 patients with colorectal carcinoma and 20 health individuals were enrolled, and CD8$^+$ T cells were purified from both peripheral bloods and carcinoma specimens. Cytolytic and noncytolytic functions of CD8$^+$ T cells in response to Notch signaling inhibition were evaluated by measurements of lactate dehydrogenase release and proinflammatory cytokines production in both direct and indirect contact co-culture system to target HT29 cells. Cellular proliferation and inhibitory receptors expression in CD8$^+$ T cells were also assessed by CCK-8 method and flow cytometry. There was no remarkable difference in percentage of CD8$^+$ T cells between healthy individuals and patients with colorectal carcinoma. Notch1/2 and Hes1/5 mRNAs were elevated expressed in tumor-infiltrating CD8$^+$ T cells in patients with colorectal carcinoma, however, did not correlated with tumor differentiation or stages. CD8$^+$ T cells from healthy individuals presented stronger cytotoxicity, which was not affected by Notch signaling inhibitor. Inhibition of Notch signaling pathway not only promoted cytotoxicity of tumor-infiltrating CD8$^+$ T cells, but also enhanced proinflammatory cytokines (including IFN-γ, TNF-α, IL-1β, IL-6, and IL-8) production by CD8$^+$ T cells from patients with colorectal carcinoma. This process was accompanied by decreased expression of PD-1 in CD8$^+$ T cells without influencing cellular proliferation. Our results indicated a potential immunosuppressive property of Notch signaling pathway, which dampened both cytolytic and noncytolytic functions of CD8$^+$ T cells probably *via* induction of PD-1 in patients with colorectal carcinoma.

1. Introduction

Colorectal cancer is the third most common cancer in male and the second most common cancer in female all over the world [1,2], with approximate 1 million people affected annually, which results in more than 700,000 deaths globally [3]. Although the incidence and mortality of colorectal cancer is declining over the past decade due to effective screening programs [4] and increased therapeutic options [5,6], many colorectal cancers remain incurable and incidence remains on the rise probably owing to changes of lifestyle and lack of health-care intervention [1]. Thus, it is vital to better understand the biological mechanism for progression of colorectal cancer and clinical relevant insights for disease management.

Evolution and progression of cancer always leads to CD8$^+$ T cells dysfunction or exhaustion, which contributes to the immunotolerance and correlates with metastasis and prognosis [7]. This process involves elevated expression of inhibitory receptors, such as programmed death-1 (PD-1), cytotoxic T-lymphocyte associated protein-4 (CTLA-4), and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), thereby favoring tolerogenic environment [8]. Restoration of CD8$^+$ T cells functions by disrupting these negative signaling is currently the focus of therapeutic approaches. Antibodies targeting PD-1 and CTLA-4 have been already approved in cancer therapy, which opened new immune checkpoint inhibitors for cancer therapies [9]. Antibodies targeting

Abbreviations: CCK-8, cell counting kit-8; CTLA-4, cytotoxic T-lymphocyte associated protein-4; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenyl glycinet-butyl ester; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; LDH, lactate dehydrogenase; NCs, normal controls; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death-1; RT-PCR, real-time polymerase chain reaction; SPSS, statistical product and service solutions; TILs, tumor-infiltrating lymphocytes; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; TNF-α, tumor necrosis factor-α; UICC, union internationale contre le cancer; VEGF, vascular endothelial growth factor

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other inhibitory receptors or activating co-stimulatory molecules are also under evaluation [10,11]. Furthermore, tumor-infiltrating lymphocytes (TILs) rather than peripheral lymphocytes are more important for reflection of immune response in tumor microenvironments, resulting in tumoral immune attack or escape and affecting clinical outcomes [12].

Notch receptors are transmembrane epidermal growth factor-like repeat-containing proteins, which are important for regulation of cell proliferation, differentiation, and apoptosis [13]. Notch receptors have been demonstrated to be up-regualted in prostate cancer [14,15], lung cancer [16], and pancreatic cancer [17]. However, controversy remains as to the Notch signaling pathway in the development of tumorigenesis depending on different cell types [18]. More importantly, inhibition of Notch signaling pathway down-regulated PD-1 expression in activated $CD8^+$ T cells [19], indicating a potential immunosuppressive characteristic of Notch signaling in chronic viral infection and cancer. Owing to the differential expression profiling of Notch1 and Notch2 in colorectal carcinoma specimens [20,21], we hypothesized that Notch signaling contributes to $CD8^+$ T cells dysfunction in the pathogenesis of colorectal carcinoma. To test this possibility, cytolytic and noncytolytic functions of peripheral bloods and tumor-infiltrating $CD8^+$ T cells purified from patients with colorectal carcinoma were measured in direct and indirect contact co-culture system *in vitro*.

2. Patients, materials and methods

2.1. Subjects

Blood samples, fresh colorectal carcimoma specimens, and patient-matched normal tissues were obtained from 46 patients (age, 31–82 years; male/female gender, 33/13) who underwent surgery at the Department of General Surgery of Baoji Municipal Central Hospital between January 2016 and January 2017. No patients received chemotherapy or radiotherapy prior to surgery. Histomorphology of all specimens was confirmed by Department of Pathology of Baoji Municipal Central Hospital. All colorectal carcinoma specimens were divided into three groups: well differentiated (n = 9), moderately differentiated (n = 25), and poorly differentiated (n = 12). The stage of tumor was also determined according to the Union Internationale Contre le Cancer (UICC) classification system. For normal controls (NCs), 20 of sex and age-matched healthy individuals (age, 26–65 years; male/female gender, 14/6) were also enrolled for blood sampling. The study was approved by the Ethics Committee of the Baoji Municipal Central Hospital, and written consent form was obtained from each enrolled subjects.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs) and TILs, and purification of $CD8^+$ T cells

PBMCs were isolated using Ficoll-Hypaque (Sigma, St. Louis, MO, USA) density gradient centrifugation from peripheral bloods. TILs were purified from tissue specimens by passage through 70 μm-pore strainers, and were treated with Collagenase D (0.5 mg/mL) at 37 °C for 30 min. Cells were then resuspended in 44% Percoll in RPMI 1640 (vol/vol), and layered over 56% Percoll in PBS (vol/vol). The gradient was centrifuged for 30 min at 850 × *g* for 30 min. The interphase, which contained TILs, was collected and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. $CD8^+$ T cells were purified using human $CD8^+$ T cells Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions. The purity of enriched $CD8^+$ T cells was > 95% based on flow cytometry analysis.

2.3. Cell culture

The human colon carcinoma cell line HT29 cells were cultured in RPMI 1640 supplemented with 10% FBS at 37 °C under 5% CO_2 condition. $CD8^+$ T cells purified from patients with colorectal carcimoma were stimulated with γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenyl glycinet-butyl ester (DAPT; Notch signaling inhibitor, 75 μmol/L; Selleck Chemicals, Huston, TX, USA) for 12 h in presence of anti-CD3/CD28 (1 μg/mL; eBioscience, San Diego, CA, USA). $CD8^+$ T cells were then co-cultured in direct contact and in parallel in indirect contact system with target HT29 cells (effector cells to target cells = 1: 4) in presence of anti-CD3/CD28 for 48 h. In the indirect contact co-culture system, effector and target cells were separated by a 0.4 μm-pore membrane in a Transwell culture plate inserts (Corning, Corning, NY, USA), which allowed the passage of soluble factors only [22]. The supernatants were harvested for further studies.

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA in $CD8^+$ T cells was isolated using RNeasy Minikit (Qiagen, Hilden, Germany). RNA was reversely transcribed using PrimeScript RT reagent Kit (TaKaRa, Dalian, Liaoning Province, China). The real-time PCR was performed using SYBR Green Premix Ex Taq (TaKaRa). The sequences of the primers for Notch1, Notch2, Hes1, Hes5, and 18S rRNA, as well as the amplification program were described previously [20,21,23]. Relative gene expression was quantified by $\Delta\Delta C_T$ method using 7500 System Sequence Detection software (Applied Biosystems, Foster, CA, USA).

2.5. Lactate dehydrogenase (LDH) assay

The cytotoxicity of target cells were assessed by measuring LDH expression in the supernatants at the end of incubation using LDH Cytotoxicity Assay Kit (Beyotime Biotech, Wuhan, Hubei Province, China) following manufacturer's instructions. The percentage of cytotoxicity was calculated as described previously. Briefly, a low-level LDH control was represented by HT29 cells cultured alone, while a high-level LDH control was represented by HT29 cells which was treated with Triton X-100 to lyse the cells and induce maximum LDH release. The resulting value was calculated in the following equation: (experimental value-low control)/(high control-low control) × 100% [22].

2.6. Cytokine assay

Levels of twelve cytokines [granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF)] in the cultured supernatants were measured by Human Mag Luminex Performance Assay Base Kit, HS cytokine A (R & D System, Minneapolis, MN, USA) using Luminex LX-200 Instrument with xPONENT 3.1 (R & D System) following the instructions of manufacturer.

2.7. Cellular proliferation assay

Cellular proliferation was measured using Cell Counting Kit-8 (CCK-8, Beyotime Biotech) following manufacturer's instructions.

2.8. Flow cytometry

Cells were transferred into FACS tubes, and then anti-CD3-PerCP (BD Bioscience, San Jose, CA, USA), anti-CD8-APC Cy7 (eBioscience) and anti-CD279 (PD-1)-FITC (eBioscience) were added for a 20-min incubation at 4 °C in the dark. Cells were then fixed by administration of 100 μL Fixation & Permeabilization Medium A (Caltag Lab, Invitrogen, Carisbad, CA, USA) for a 15-min incubation at room temperature in the dark. 100 μL Fixation & Permeabilization Medium B (Caltag Lab, Invitrogen) containing anti-CD152 (CTLA-4)-PE (eBioscience) were added for another 20-min incubation. Isotype