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膠芽腫の低酸素環境を克服する HIF-1α ノックアウト同種 NK 細胞の開発

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Establishment of HIF-1α Knockout Allogeneic NK Cells To Overcome Hypoxic Environment In Glioblastoma

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NK 細胞は膠芽腫に対する免疫療法の理想的な候補である。腫瘍内低酸素環境は膠芽腫の一般的な特徴であり、腫瘍細胞および正常細胞は転写因子である低酸素誘導因子（HIF）-1α を増強することで腫瘍微小環境に適応するが、一方で NK 細胞を含む抗腫瘍免疫細胞の機能を抑止する可能性がある。本研究では CRISPR/Cas9 を用いてヒト初代 NK 細胞の HIF-1α をノックアウトし、膠芽腫に対する抗腫瘍効果を解析した。HIF1-α ノックアウト NK 細胞は膠芽腫患者における有望な免疫療法の選択肢となりうる。

NK cells are ideal candidates for immunotherapy of glioblastoma（GBM）. Hypoxia is a common feature of GBM, tumor cells and normal cells adapt to the tumor microenvironment by upregulating the transcription factor hypoxia-inducible factor（HIF）-1α, which can be detrimental to anti-tumor effector immune cell function. In this study, we knocked out HIF-1α in human primary NK cells using CRISPR/Cas9 and evaluated antitumor effects in GBM. HIF-1α knockout NK cells could be a promising immunotherapeutic alternative in patients with GBM.

1. 研究内容

1.1 Background

Natural killer（NK）cells are innate immune system effectors crucial in killing abnormal cells such as tumors and virus-infected cells independently of prior sensitization. The NK-cell-killing functions are regulated by activating and inhibitory receptors on the cell's surface, releasing small granules containing perforin（PFR）1 and granzymes（GZM）. Hypoxia is a significant TME feature that consistently pervades the tumor environment due to the rapid proliferation of tumor cells without sufficient blood support, caused by the inaccessibility of the oxygen source to the resident vasculature. Hypoxic conditions in the cancer microenvironment influence the NK cell phenotype, leading to tumor resistance and immune-suppressive cell production. The hypoxia-inducible factor（HIF）is a transcription factor instrumental in the ability of cells to sense and adapt to oxygen level changes. HIF-1α overexpression inhibits human NK cells by downregulating the NK-cell-activating receptors involved in the tumor killing. HIF-1α promotes multiple signaling activities and induced immune suppression, including that of NK cells. Based on the evidence that HIF-1α inhibition promotes NK cell function, permanently inhibiting HIF-1α

expression in NK cells could be a useful cancer immunotherapy modality. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) disturb the target gene via small RNAs that chaperone the Cas9 DNA nuclease to the target site through base pairing. This approach is extremely specific and efficient for engineering and disrupting eukaryotic genomes. CRISPR/Cas9 targeting of HIF-1α could be overcoming NK-cell-function-suppressive hypoxia circumstances in NK-cell-based immunotherapy for cancer, including glioblastoma (GBM). In the present study, we aimed to induce human NK cells with HIF-1α knockout by CRISPR/Cas9 and evaluate the NK cells. The cytotoxicity of the HIF-1α knockout NK cells (HIF KO NKP) against GBM cells under normoxic and hypoxic conditions was evaluated. We further detected in vivo antitumor effects using xenograft brain tumor mice.

1.2 Materials and Methods

1.2.1. Ethics

This study was conducted according to Nara Medical University guidelines. All procedures that involved human participants were conducted according to institutional and/or national research committee ethical standards and the 1964 Declaration of Helsinki and its subsequent alterations or equivalent ethical standards.

1.2.2. Cell Lines

T98G and U251MG GBM cells were from RIKEN BioResource Center and JCRB Cell Bank, respectively. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo) containing 100 μg/mL streptomycin, 100 U/mL penicillin (Thermo), and 10% heat-inactivated fetal bovine serum (FBS; MP Biomedicals at 37℃ in a humidified atmosphere with 5% CO_2).

1.2.3. Single Guide RNAs

Two sgRNAs targeting the human HIF-1α gene locus were designed according to the manufacturer's instructions (IDT, https://sg.idtdna.com/site/order/designtool/index/CRISPR_PREDE SIGN, accessed on 1 May 2022). The HIF-1α target sequences dHIF AB and dHIF AC were CCTCACACGCAAATAGCTGATGG and ACAGTAACCAACCTCAGTGTGGG, respectively. The negative control sgRNA was from IDT, and the sequence is as follows: rCrGrUrUrArArUr-CrGrCrGrUrArUrArArUrArCrGrGrUrUrUrUrArGrAr-GrCrUrArUrGrCrU.

1.2.4. Induction of HIF-1α Knockout NK Cells Including Populations from Human Peripheral Blood

The highly purified human NK cells were expanded. Peripheral blood (PB) was obtained from 16 mL heparinized peripheral blood from healthy volunteers. The PB mononuclear cell (PBMC) CD3 fraction was depleted using RosetteSep™ Human CD3 Depletion Cocktail (STEMCELL Technologies). The CD3-depleted PBMCs (2×10^6 cells) were placed for 7 days in a 6-well plate (Corning) coated with anti-NKp46 (clone 195314, R&D Systems) and anti-CD16 antibody (clone 3G8, Thermo) (both 5 μg/mL) and containing 2 mL AIM-V medium (Life Technologies) supplemented with 50 ng/mL recombinant human IL-18 (rhIL-18, MBL), 10% autologous plasma, and 3000 IU/mL rhIL-2 (COREFRONT) at 37℃ in a humidified atmosphere with 5% CO_2. The AIM-V containing 3000 IU/mL

rhIL-2 was refilled as required. The Expanded NK cells (3×10^6) were electroporated to RNP complexes (targeted sgRNA/tracrRNA and recombinant Cas9, IDT) using Human NK Cell Nucleofector Kit (Lonza) and electroporated by program X-001 of Nucleofector (LONZA). The cells were resuspended in AIM-V containing 10% autologous plasma and 3000 IU/mL rhIL-2 and placed for 4 days in a 12-well plate (Corning) at 37 ℃ in a humidified atmosphere with 5% CO_2 and 20% or 1% O_2.

1.2.5. Western Blotting

10^6 NK cells were dissolved in radioimmunoprecipitation assay lysis and extraction buffer with Halt protease inhibitor cocktail (Thermo) and sonicated using the ultrasound-based Sonifier 250 homogenizer (Branson). The primary antibodies were rabbit polyclonal IgG against HIF-1α (clone D1S7W, Cell Signaling) and GAPDH (clone 14C10, Cell Signaling). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling). The blots were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo). The signal intensity was determined using FUSION Solo 7S Edge (Vilber Bio Imaging).

1.2.6. Growth Inhibition Assays

The inhibitory effects of the genome-edited NK cells on GBM cells were examined using xCELLigence RTCA (real-time cell analysis) S16 and DP instruments (ACEA Biosciences) in electrical impedance-based assessment. Briefly, 100 μL complete medium was added to each well on an E-plate 16 (ACEA Biosciences). The data were analyzed using RTCA version 1.2 (ACEA Biosciences), and the normalized cell growth was calculated.

1.2.7. In vivo Orthotopic Xenograft Assays

We purchased non-obese diabetes/severe combined immunodeficiency/γc null (NOG) mice from the Central Institute for Experimental Animals. We anesthetized the mice and infused the mice stereotactically with 2 μL native Hank's buffered salt solution (HBSS) that contained 10^5 U87MG cells into the right thalamus with a Hamilton syringe (33 S-gauge needle) mounted on an infusion syringe pump (Harvard Apparatus, Holliston, MA, USA). The mice were randomly assigned to three intracranial infusion groups: negative background (NB, HBSS only), NK mock, and HIF KO NKP (10^6 cells). The mice were directly infused intracranially with the cells and reagents prepared using the aforementioned settings and using the infusion syringe pump via the same burr hole used for implanting the U87MG. The infusion speed for both the U87MG and NK cells was 1 μL/min.

1.3 Results

1.3.1. Induction of HIF1α Knockout Human PB-Derived NK Cells

The RNP electroporation and transduction demonstrated no morphological changes on day four after electroporation in individual cellular populations. Western blotting revealed that hypoxia tended to upregulate HIF-1α expression in controlled NK cells more than in the normoxic conditions. The transduction of both sgRNAs inhibited HIF-1α expression almost completely in normoxic and hypoxic conditions.

1.3.2. Anti-Tumor Effects of HIF-1α Knockout Human Primary NK Cell Populations on Allogeneic GBM Cells in Normoxic and Hypoxic Conditions

The functional aspects of the HIF-1α knockout human primary NK cell populations (HIF KO NKP) were evaluated using cell lines derived from allogeneic GBM cells in vitro through real-time cell analyzer-based growth inhibition assays. In normoxic conditions (20% O_2), the control NK cells, HIF KO NKP significantly inhibited the T98G and U251MG growth time dependently compared to the target GBM cells only. Compared to control NK cells, HIF KO NKP did not significantly alter the growth inhibition of the GBM cells. In hypoxic conditions (1% O_2), HIF KO NKP significantly inhibited T98G and U251MG cell growth time dependently compared to the target cells. Interestingly, HIF KO NKP significantly inhibited the growth inhibition of the GBM cells compared to control NK cells in hypoxia. Furthermore, the growth inhibition of the control NK cells did not significantly alter the GBM cell growth inhibition in normoxic and hypoxic conditions.

1.3.3. HIF-1α Knockout Enhanced NKP Anti-tumor Activity in The Allogeneic Brain Tumor Model

We tested the anti-tumor effects of allogeneic HIF KO NKP in intracranial orthotopic xenografts derived from U87MG using NOG mice. U87MG were implanted into NOG mouse brains, followed by intracranial infusion through the same burr hole used for U87MG implantation. The control NK mock group was significantly associated with longer survival time compared to the NB group. The HIF KO NKP group exhibited prolonged overall survival compared to the NK mock group.

1.4 Discussion

Our data demonstrate that the loss of HIF-1α enhanced the NK-cell-killing-mediated GBM cell growth inhibition in the hypoxia environment but did not affect under normoxic culture conditions. The data suggest that HIF-1α strongly suppresses the mechanism by which NK-cell-killing is enhanced under hypoxia. HIF KO NKP showed antitumor effects against GBM *in vivo*. The HIF KO NKP could be an effective and feasible cell-based immunotherapy for GBM.

2. 発表（研究成果の発表）

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