Molecular Immunology 54 (2013) 74–83

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Cimetidine suppresses lung tumor growth in mice through proapoptosis of myeloid-derived suppressor cells

Yisheng Zheng[a,1], Meng Xu[b,1], Xiao Li[c], Jinpeng Jia[b], Kexing Fan[d,**], Guoxiang Lai[a,*]

[a] Department of Respiratory and Critical Care Medicine, Fuzong Clinical College of Fujian Medical University, Fuzhou General Hospital, Fuzhou, Fujian 350000, PR China
[b] Department of Orthopaedics, Chinese PLA General Hospital, No. 28 Fuxing Road, Beijing 100853, PR China
[c] Department of ophthalmology, Chinese PLA General Hospital, No. 28 Fuxing Road, Beijing 100853, PR China
[d] International Joint Cancer Institute, The Second Military Medical University, No. 800 XiangYin Road, Shanghai 200433, PR China

ARTICLE INFO

Article history:
Received 31 May 2012
Received in revised form
18 September 2012
Accepted 12 October 2012
Available online 5 December 2012

Keyword:
Cimetidine
Myeloid derived-suppressive cell
Apoptosis
Tumor immune

ABSTRACT

Cimetidine, a histamine type-2 receptor antagonist, is known to inhibit the growth of several tumors in human and animals, however the mechanism of action underlying this effect remains largely unknown. Here, in the mice model of 3LL lung tumor, cimetidine showed significant inhibition of tumor growth. However, an in vitro study demonstrated that cimetidine showed no effect on proliferation, survival, migration and invasion of 3LL cells. We found that cimetidine reduced $CD11b^+Gr\text{-}1^+$ myeloid derived-suppressive cell (MDSC) accumulation in spleen, blood and tumor tissue of tumor-bearing mice. In vitro coculture assay showed that cimetidine reversed MDSC-mediated T-cell suppression, and improved IFN-γ production. Further investigation demonstrated that the NO production and arginase I expression of MDSCs were reduced, and MDSCs prone to apoptosis by cimetidine treatment. However, MDSC differentiation was not affect by cimetidine. Importantly, although histamine H2 receptor was expressed in MDSC surface, histamine could not reverse the proapoptosis of cimetidine. Moreover, famotidine also did not have this capacity. We found that cimetidine could induce Fas and FasL expression in MDSC surface, and sequentially regulate caspase-dependent apoptosis pathway. Thus, these findings revealed a novel mechanism for cimetidine to inhibit tumor via modulation of MDSC apoptosis.

1. Introduction

Cimetidine is the oldest histamine type-2 receptor antagonist and commonly prescribed to treat gastro-esophageal reflux diseases as well as gastric and duodenal ulcers (Freston and Cimetidine, 1982). Accumulating evidence suggests that cimetidine may improve survival of patients with malignant tumors, including gastric, colorectal and renal cell cancer, and melanoma (Tonnesen et al., 1988; Matsumoto, 1995; Hellstrand et al., 1994; Sagaster et al., 1995; Morris and Adams, 1995; Sasson et al., 1999). Cimetidine has been shown to inhibit tumor growth by several mechanisms, including inhibition of cancer cell proliferation (Adams et al., 1994a; Lawson et al., 1996; Reynolds et al., 1996), blockade of the tumor angiogenesis (Natori et al., 2005), and enhancement of immune activity (Adams et al., 1994b; Sahasrabudhe et al., 1987; McCarty, 1985). However, the exact mechanisms by which cimetidine affects tumor growth and progression remain poorly understood.

So far, little is known of the effect of cimetidine on lung cancer. Here, the aim of our study was to investigate the effect of cimetidine on 3LL lung tumor in C57BL/6 mice model, and explore the underlying mechanism. Consistent with that observed in other tumor models, cimetidine also exhibits a tumor-suppressive effect in lung tumor bearing mice. Interestingly, cimetidine showed no direct inhibitory effects on proliferation, survival, migration and invasion of lung tumor cell line 3LL in vitro. However, the accumulation of a novel immune-suppressive cell subset (Gabrilovich and Nagaraj, 2009), $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressive cell (MDSC) in spleen, blood and tumor tissue of tumor-bearing mice were significantly reduced after cimetidine treatment. The MDSC-mediated T cell suppressive response also was reversed by cimetidine-mediated MDSC cell death. The underlying mechanisms by which cimetidine induced MDSC apoptosis were explored.

2. Material and methods

2.1. Mice, cell lines, and reagents

C57BL/6J and Balb/C mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China) and used at

* Corresponding author. Tel.: +86 591 22859333; fax: +86 591 24937021.
** Corresponding author. Tel.: +86 21 81870801; fax: +86 21 65306667.
E-mail addresses: kexingfan@gmail.com (K. Fan), Laiguoxiang2007@163.com (G. Lai).
[1] These authors contributed equally to this work.

0161-5890/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.molimm.2012.10.035

the age of 6–8 wk. Lewis lung carcinoma cell line (3LL) were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% (v/v) heat-inactivated FBS (Gibco). Cimetidine was obtained from R&D Systems. The Abs for flow cytometry including Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (clone HL3), I-Ab (clone AF6-120.1), CD86 (clone GL1), CD80 (clone 16-10A1), Fas (clone Jo2), FasL (clone MFL3) and the respective isotype controls were obtained from BD PharMingen.

2.2. Animal experiments

All protocols involving experimental animals were in accordance with the institutional guidelines for animal care of Fuzong Clinical College of Fujian Medical University. 5×106 murine syngeneic lung cancer cells (3LL) were suspended in 0.1 mL of PBS and injected subcutaneously into the left flank fold of the C57BL/6J mice. Each group of mice received either saline or cimetidine (10 or 20 mg/kg/day) intraperitoneally on days 0, 2, 4, 6, 8, 10, 12 and 14 after the tumor inoculation. Tumor size was measured with calipers everyday and tumor volume was calculated as $\text{width}^2 \times \text{length} \times 0.52$. When tumors reached 2.0 cm × 2.0 cm, the duration of survival was recorded, the mouse was euthanized.

2.3. In vitro tumor cell proliferation and survival assay

3LL cells (1×10^5/well) were cultured in a 24-well culture plate, and treated with various concentrations of cimetidine for various time, followed by the addition of 1 mL DMEM containing 0.05 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (MTT; Sigma). After incubation at 37 °C for 1 h, the media were removed and the formazan crystals in the cells were dissolved in 1 mL DMSO for OD (optical density) reading at 490 nm using a spectrophotometer. To determine the effect of cimetidine on tumor cell apoptosis, various concentrations of cimetidine were added into the culture. After 24 and 48 h, cells were harvested, and then stained for Annexin V and 7-amino-actinomycin D (7-AAD) for 15 min and ran for FACS.

2.4. In vitro tumor cell migration and invasion assays

The motility and invasiveness of 3LL cells were evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-μm sized pores (Costar 3422, Corning Inc., NY). 5×10^4 cells suspended in 100 μL of DMEM–0.1% BSA were plated into the top chamber. DMEM–10% FBS was placed into the bottom chamber to act as a chemoattractant. Various concentrations of cimetidine were added into the culture for 24 h. The cells that migrated through the 8-μm sized pores and adhered to the lower surface of the membrane were fixed with 3.7% paraformaldehyde, stained with 0.2% crystal violet and washed with 1× PBS three times. The dye was eluted using 30% acetic acid and quantification of cell number was performed using colorimetric analysis with a microplate reader (absorbance at 590 nm). The absorbance units obtained for cimetidine-treated cells were each divided by the absorbance units obtained for untreated cells and expressed as a migration index. By definition, untreated cells were assigned an index of 1.

In a similar fashion, the invasiveness of 3LL cells were evaluated in Matrigel™ (Collaborative Biomedical Products, Bedford, MA) coated 24-well transwell chambers. Matrigel was used at a concentration of 0.4 mg/mL. Cells, media, experimental conditions and analysis performed were similar to those of migration assays. Triplicate assays were performed for each group of cells in both migration and invasion assays.

2.5. Fluorescence-activated cell sorting analyses of MDSC

Tumor-bearing mice were sacrificed at various times. Spleen, lymph node, blood and tumors were harvested, and single cell suspensions were prepared. MDSCs were stained with anti-CD11b, and Gr-1 antibodies for 30 min at 4 °C. After one wash with cold PBS, they were analyzed by flow cytometry.

2.6. Isolation and purification of MDSCs

Single cell suspensions of splenocytes from normal or tumor-bearing mice were prepared. $CD11b^+Gr\text{-}1^+$ MDSCs were prepared as previously described (Le et al., 2009). Cells were incubated with biotinylated anti-Gr-1 and anti-CD11b microbeads. $CD11b^+Gr\text{-}1^+$ cells were positively selected. The purity of $CD11b^+Gr\text{-}1^+$ cells was confirmed by flow cytometry. The purity of MDSCs was about 99%.

2.7. Analysis of T cell proliferation and IFN-γ production

For studies examining the mechanism of MDSC-mediated T-cell suppression, and the effect of cimetidine on this, T cells and MDSCs isolated as before were cocultured 1:1 in the presence of 2 mM L-NMMA, or 0.5 mM nor-NOHA, or various concentrations of cimetidine, and T cells were stimulated with anti-CD3/CD28-coated beads for 72 h. Proliferation was assayed with tritiated thymidine incorporation. Intracellular IFN-γ was detected by FACS staining for IFN-γ and CD3.

2.8. In vitro MDSC viability and differentiation assays

To measure the effect of cimetidine on MDSC viability, MDSCs were incubated in complete RPMI 1640 with 10% FBS and 50 ng/mL GM-CSF to support cell viability over 48 h. Various concentrations of cimetidine were added into the culture, and after 48 h, cells were harvested, and then stained for Annexin V and 7-amino-actinomycin D (7-AAD) for 15 min and ran for FACS. In some experiments, zVAD-fmk or blocking anti-FasL mAb was added into the culture, respectively.

For studies examining the effect of cimetidine on MDSC differentiation, MDSCs were isolated and incubated in GM-CSF, with the addition of 50 ng/mL IL-4, with or without cimetidine at various concentrations. Half the medium was replaced after 3 days, and after 6 days, the cells were harvested and analyzed for the expression of CD11 C, I-Ab, CD80, and CD86 by FACS. Remaining cells were irradiated at 3000 rad and used as stimulators in mixed lymphocyte reactions with allogeneic, fresh T cells from Balb/C mice. T-cell proliferation was determined after 3 days by the incorporation of tritiated thymidine.

2.9. Assay of MDSCs for NO production

For analysis of NO production from MDSCs, purified MDSCs were incubated for 12 h with 1 μg/mL lipopolysaccharide and 100 IU/mL 1 IFN-γ in the presence of various concentrations of cimetidine. Supernatants were assayed for NO using the Parameter Total NO/Nitrite/Nitrate Assay Kit (R&D Systems), as per the manufacturer's instructions.

2.10. Western-blot analysis

Total cell lysate was prepared in 1 × SDS buffer. Proteins at the same amount were separated by SDS-PAGE and transferred onto PDVF membranes. After probing with individual antibodies, antigen-antibody complex was visualized by enhanced chemiluminescence's reagents Supersignal (Pierce Biotechnology). The