Native Soluble Carcinoembryonic Antigen Is Not Involved in the Impaired Activity of CD56^{dim} Natural Killer Cells in Malignant Pleural Effusion

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Key Words

Carcinoembryonic antigen · Natural killer cells · Malignant pleural effusion · Cytotoxic activity

Abstract

Background: Natural killer (NK) cells are lymphocytes of the innate immune system that play a crucial role in tumor immune surveillance. Accumulated data indicated that NK cells in the tumor microenvironment often display a suppressed function. However, the mechanism is not clear. **Objective:** In this study, the effects and relative mechanisms of malignant pleural effusion (MPE) from patients with lung cancer on NK cells were researched. **Methods:** MPE and peripheral blood (PB) samples were collected from patients with lung cancer. The cytotoxic activity of CD56^{dim} NK cells in PB and MPE mononuclear cells was analyzed by flow cytometry. **Results:** It was observed that the percentages of total NK cells and a CD56^{dim} NK subset in MPE reduced accompanying impaired cytotoxic activity compared with that in paired PB. Cell-free MPE treatment reduced both the proportion and cytotoxic activity of CD56^{dim} NK cells in PB from healthy donors. The suppression effects were not based on soluble carcinoembryonic antigen and the inhibitory cytokines interleukin-10 and transforming growth factor- β_1 , but were dependent on the factor with a molecular weight >100 kDa. **Conclusions:** These results demonstrated that native soluble carcinoem-

bryonic antigen does not suppress the activity of NK cells, and an unknown factor with a molecular weight >100 kDa plays a critical role in the impairment of CD56^{dim} NK cells in MPE, which might lead to tumor progression.

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Introduction

Human carcinoembryonic antigen (CEA), a highly glycosylated protein of about 200 kDa, is a well-characterized tumor-associated antigen that has been found overexpressed in a high percentage of human tumors, including 90% of gastrointestinal, colorectal and pancreatic cancers, 70% of non-small cell lung cancer cells and 50% of breast cancers [1, 2]. High levels of CEA have been implicated in enhanced metastasis and the development of malignancy [3]. It has been proven to be a suitable target antigen for the detection of primary and metastatic colorectal and some other carcinomas.

Natural killer (NK) cells are lymphocytes of the innate immune system that play a crucial role in tumor immune surveillance because of the ability to recognize and eliminate transformed cells. Human NK cells account for 10–

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20% of peripheral blood (PB) lymphocytes and are characterized by CD3⁻ and CD56⁺. NK cells can be divided into two main functional subsets according to the intensity of CD56 expression: CD56^{dim} NK cells, accounting for 90% of total NK cells in PB, have superior cytotoxic potential, whereas CD56^{bright} NK cells, comprising 10% of total NK cells in PB, have a greater ability to produce cytokines and are thought to be a regulatory subset [4, 5]. Accumulated data indicated that NK cells in the tumor microenvironment often displayed a suppressed function. Stern and his colleagues [6] had demonstrated that both the CEA-Ig recombinant protein and CEA in the context of mammalian cells by using cell transfectants inhibited NK cell killing via interaction with CEA-related cell adhesion molecule 1 (CEACAM1). However, the CEA used in their studies, but not the native CEA, was artificially modified. So far, the effect of native soluble CEA on NK cell killing was rarely reported. The relationship between native soluble CEA from the tumor microenvironment and NK cells is still mostly unknown. Malignant pleural effusion (MPE) is a common and distressing symptom in patients at advanced stage of various malignant diseases. Lung cancer is the most common etiology of MPE, accounting for 37% of all malignant effusions [7]. High levels of CEA are detected in squamous cell cancer and adenocarcinoma of the lung. Thus, MPE offers a good platform to study the interaction of CEA and NK cells in the tumor microenvironment. Early studies have confirmed the low cytolytic activity of NK cells against tumor cells in MPE [8, 9].

In this study, the effect of native soluble CEA within MPE on CD56^{dim} NK cells was investigated. It was observed that NK cells were accumulated in MPE; however, the percentages of total NK cells and the CD56^{dim} NK subset reduced the accompanying impaired cytotoxic activity in MPE compared with that in paired PB. Cell-free MPE (CFMPE) treatment reduced both the proportion and cytotoxic activity of CD56^{dim} NK cells in PB from healthy donors. CEA was not involved in the suppression of NK cells. Further, the suppression effects of CFMPE were not based on the inhibitory cytokines interleukin (IL)-10 and transforming growth factor (TGF)- β_1 , but were dependent on the factor with a molecular weight >100 kDa.

Materials and Methods

Subjects

Informed consent was obtained from patients according to the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, and the study protocol was also

approved by this institution. MPE samples were collected from 22 patients (age range 36–81 years, 10 males, 12 females) with newly diagnosed lung cancer. A diagnosis of MPE was established by the showing of malignant cells in pleural effusion and/or on closed pleural biopsy specimens. None of the patients had received any anticancer treatment, corticosteroids or other nonsteroid anti-inflammatory drugs at the time of sample collection. The levels of CEA in MPE ranged from 40.37 to 1,000 ng/ml.

Sample Collection and Processing

MPE samples were collected from each subject, using a standard thoracentesis technique. Ten milliliters of venous PB was drawn simultaneously. PB mononuclear cells (PBMCs) from patients and healthy donors were isolated by Ficoll-Hypaque gradient centrifugation. MPE samples were centrifuged, and the supernatant was collected and used as CFMPE. The cell pellets were resuspended in PBS, and mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation and described as MPE mononuclear cells. CFMPE was centrifuged at 12,000 rpm for 1 h at 4°C with different Amicon Ultra-15 Centrifugal Filter Units (Millipore) to be divided into different parts according to the size. The human erythroleukemia cell line K562 was cultured in RPMI-1640 culture medium supplemented with 10% heat-inactivated FBS.

Antibodies

The following fluorescent-labeled monoclonal antibodies were used. Alexa Flour488 anti-human CD66a/c/e, percp-Cy5.5-anti-CD3 and isotype controls were from eBioscience (San Diego, Calif., USA). FITC-anti-CD3, APC-anti-CD56, FITC-anti-CD107a and neutralizing antibodies anti-IL-10 were from BD Pharmingen (San Jose, Calif., USA). Neutralizing antibody of TGF- β_1 was from R&D Systems (Minneapolis, Minn., USA). Protein-A- and protein-G-conjugated agarose were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Anti-CEA polyclonal antibody was from Abcam (Hong Kong). Anti-CEA monoclonal antibody CC4 was a gift from the Institute of Biophysics, Chinese Academy of Sciences, Beijing, China [2].

Immunoprecipitation

Immunoprecipitation was performed to remove CEA from CFMPE. 40 μ g polyclonal antibody of CEA was mixed with 40 μ l protein-A- and 40 μ l protein-G-conjugated agarose at 4°C on a shaking platform overnight. Then the mixture was centrifuged and washed with PBS twice as well as incubated with CFMPE for 6 h at 4°C on a shaking platform. After that, the mixture was centrifuged at 3,000 rpm for 30 s at 4°C, and the supernatant was carefully collected for the indicative assay.

PBMCs from Healthy Donors Treated with CFMPE

PBMCs from healthy donors were adjusted to a density of 1×10^6 cells/ml in a 24-well plate and treated with CFMPE in indicated conditions for 24 h and were collected and resuspended in RPMI-1640 medium supplemented with 10% FBS for flow cytometry analysis or cytotoxicity assay.

Detection of Surface Molecules on NK Cells

PB or MPE mononuclear cells (1×10^6) were stained with FITC-anti-CD3 and APC-anti-CD56 for 30 min at 4°C, or 1×10^6 PB mononuclear cells from healthy donors were stained with

Fig. 1. Decreased percentage of NK cells in MPE from patients with lung cancer. **a** Representative flow cytometric dot plots showing percentages of different NK cell subsets in lymphocytes in MPE and paired PB. **b** Percentage of NK cells in gated lymphocytes in MPE and paired PB ($n = 15$). Lines indicate the mean values. **c** Proportion of CD56^{dim} and CD56^{bright} NK cells in MPE and paired PB. * $p < 0.05$.

FITC-anti-CD3, APC-anti-CD56 and Alexa Flour488-anti-human CD66a/c/e for 30 min at 4°C. After that, cells were washed twice in PBS-1% FBS and analyzed by flow cytometry. Labeled isotype controls were also used to evaluate non-specific staining.

Analysis of Cytotoxic Activity of CD56^{dim} NK Cells

PB or MPE mononuclear cells were coincubated with target cells K562 at a ratio of 1:1 in a final volume of 100 μ l in round-bottomed 96-well plates for 4 h. Then cells were stained with CD3, CD56 and CD107a antibodies for 30 min at 4°C. After that, cells were washed twice in PBS-1% FBS and analyzed by flow cytometry. Results are expressed as the percentage of CD107a-positive cells in CD56^{dim} NK cells.

Statistics

Data are presented as the mean $\pm$ SEM. Statistical analyses were performed with GraphPad Software. Paired data comparisons were made using a Wilcoxon signed-rank test, and data from different groups of treatments were analyzed using the t test. A p value < 0.05 was considered significant.

Results

NK Cells Appeared in MPE

Mononuclear cells in 15 pairs of MPE and PB were first assayed by flow cytometry. As shown in figure 1a and b, NK cells appeared in MPE. The average percentage of NK

cells (CD3⁺ CD56⁺) in MPE mononuclear cells was 7.7%, i.e. significantly lower than that in paired PBMCs (15.2%). The proportion of CD56^{dim} NK cells in MPE was also lower compared with that in paired PB (fig. 1a, c). In contrast, the percentage of CD56^{bright} NK cells in MPE was relatively higher (fig. 1a, c).

Impaired Cytotoxic Activity of CD56^{dim} NK Cells in MPE

The surface expression of CD107a, which is present in the cytotoxic activity of NK cells, on CD56^{dim} NK cells was detected by flow cytometry after target K562 cell encounter for 4 h. As illustrated in figure 2, in the MPE group, not only the frequency of CD107a⁺ CD56^{dim} NK cells (fig. 2a, b) but also the mean fluorescence intensity of CD107a on CD56^{dim} NK cells (fig. 2c) was significantly lower compared with that in paired PB.

Proportion and Cytotoxic Activity of CD56^{dim} NK Cells from a Healthy Donor Decreased after Incubation with CFMPE

To confirm the association between the impaired amount and function of CD56^{dim} NK cells and MPE, PBMCs from 1 healthy donor were treated with CFMPE from 2 patients for 72 h. As shown in figure 3a, after treat-

Fig. 2. Impaired cytotoxic activity of CD56^{dim} NK cells in MPE. **a** Representative flow cytometric dot plots showing surface expression of CD107a on CD56^{dim} NK cells. SSC = Side scatter. **b** Percentage of surface CD107a⁺ CD56^{dim} NK cells after coincubation with K562 for 4 h. **c** Mean fluorescence intensity (MFI) of surface CD107a on CD56^{dim} NK cells in MPE and paired PB (n = 6). * p < 0.05.

ment, at 24-, 48- and 72-hour time points, both the percentage of NK cells (CD3⁺ CD56⁺ cells) and the proportion of CD56^{dim} NK cells were more significantly reduced than those in the medium. Furthermore, treating the same healthy PBMCs with CFMPE from 7 patients depressed the surface expression of CD107a on CD56^{dim} NK cells (fig. 3b). Treating PBMCs from 3 donors with the same CFMPE got similar results (fig. 3c).

Soluble Factors >100 kDa in CFMPE Impaired the Cytotoxic Activity of CD56^{dim} NK Cells

To determine whether native soluble CEA which was dramatically elevated in MPE was the critical factor for suppression of cytotoxicity of CD56^{dim} NK cells, the blocking experiment was performed. As shown in figure 4a, CD56^{dim} NK cells in healthy donors express CEA receptors CD66a/c/e. The molecular weight of CEA is

about 180 kDa, so the >100-kDa part in CFMPE was separated by ultrafiltration centrifugation to be used in the following experiments. As shown in figure 4b, treatment of the >100-kDa part significantly impaired the cytotoxicity of CD56^{dim} NK cells from healthy donors, and neutralizing antibody CC4 did not rescue the changes. The effect of treatment of the >100-kDa part on the percentage of NK cells was of marginal significance (p = 0.06, n = 3; fig. 4c). Removing CEA with immunoprecipitation (fig. 4d, e) had no significant effect on the ability of the >100-kDa part to bring about the changes in NK cells. The data suggested that CEA had no significant effects on the changes of NK cells in CFMPE.

To identify the component in CFMPE which impairs the amount and function of CD56^{dim} NK cells, the effects of TGF- β ₁ and IL-10, which are potent immunosuppressive factors and significantly elevated in MPE [10, 11], on

Fig. 3. Decreased proportion and cytotoxic activity of CD56^{dim} NK cells after treatment with CFMPE. **a** Percentage of NK cells and proportion of the CD56^{dim} NK subset in gated lymphocytes. Medium = RPMI-1640 medium; CFMPE = 50% (v/v) CFMPE in RPMI-1640 medium. **b, c** Expression of CD107a on CD56^{dim} NK cells stimulated with K562 for 4 h. PBMCs from a healthy donor was treated with CFMPE from 7 patients for 24 h (**b**). PBMCs from 3 healthy donors (HD) were treated with the same CFMPE for 24 h (**c**). *** $p < 0.001$ ($n = 7$).

NK cells were assayed. As shown in figure 4f and g, neither TGF- β_1 - nor IL-10-neutralizing antibody resumed the cytotoxicity of CD56^{dim} NK cells treated with CFMPE. In addition, blocking TGF- β_1 or IL-10 had no effect on the ability of CFMPE to bring about the decrease in the percentage of NK cells (online suppl. fig 1A, B; for all online supplementary material, see www.karger.com/doi/10.1159/000345214).

To identify the component impairing the cytotoxicity of CD56^{dim} NK cells in MPE, ultrafiltration tubes with several sizes were used to divide CFMPE into different parts. Compared with the medium, the full supernatant and the parts >100 kDa in CFMPE impaired the percentage of NK cells and the cytotoxicity of CD56^{dim} NK cells ($n = 7$; fig. 4h, i). However, the parts <100 kDa or <10, <30 and <50 kDa had no impairment on NK cells (online suppl. fig. 1C, D).

Discussion

MPE is a common and distressing symptom in patients at advanced stage of various malignant diseases. In a study of patients presenting with MPE, lung cancer was the most frequent cause [12]. In this study, a significant reduction in NK cells was observed in MPE compared

with paired PB from lung cancer patients. This was consistent with an early study by Okubo et al. [9]. Furthermore, the proportion of the CD56^{dim} NK subset in MPE was much lower than that in paired PB, while the CD56^{bright} NK subset was higher. The altered proportion of NK subsets was also described in several other tissues, as well as in some human diseases [13–15]. There were various interpretations about this finding, including apoptosis of the CD56^{dim} NK cell subset or recruitment of the CD56^{bright} NK subset [14, 16, 17]. As CD56^{dim} NK cells is the main subset to kill tumor cells, the decreased cytolytic activity of NK cells in MPE might attribute to the reduced proportion of the CD56^{dim} NK subset. Therefore, CD56^{dim} NK cells in MPE were investigated by our team. CD107a is considered a functional marker for the identification of activity of NK cells [18]. Consequently, in this study, the cytotoxic activity of CD56^{dim} NK cells was evaluated by detecting the surface expression of CD107a after cocubation with target cells. It was found that the cytotoxic activity of CD56^{dim} NK cells was considerably decreased in MPE compared with paired PB.

Although Stern et al. [6, 19] reported that recombinant CEA and CEA transfected in mammalian cells inhibited the cytotoxicity of NK cells via CEACAM1, which is one of the receptors of CEA, in this study, we found that native soluble CEA in MPE did not play a role in the impair-

Fig. 4. Factors with a molecular weight >100 kDa played a critical role in the impaired cytotoxicity of CD56^{dim} NK cells in MPE. **a** Expression of receptors of CEA (CD66a/c/e) on CD56^{dim} NK cells in healthy donors (HD). Each color of the thread stands for an individual. **b, c** PBMCs (1×10^6) from a healthy donor were treated with the >100-kDa part of CFMPE supplemented with the indicated concentration of CC4 antibody for 24 h; the level of CEA is 100 ng/ml in the CFMPE. **d, e** PBMCs (1×10^6) from a healthy donor were treated with the >100-kDa part of CFMPE (CEA⁺ MPE) or with CEA-free MPE (CEA⁻ MPE). In CEA⁺ MPE, CEA is 110 ng/ml, and in CEA⁻ MPE, 90% CEA was removed by immunoprecipitation,

which is lower than the clinically significant level. **f, g** TGF- β_1 - and IL-10-neutralizing antibodies had no effect on the expression of CD107a on CD56^{dim} NK cells. Healthy PBMCs (1×10^6) were treated with CFMPE supplemented with anti-TGF- β_1 (α TGF- β_1 , 5 μ g) or anti-IL-10 (α IL-10, 0.1 μ g) antibody for 24 h. **h, i** Impairment of different parts of CFMPE on NK cell percentage and the cytotoxicity of CD56^{dim} NK cells. CFMPE was centrifuged with 100-kDa Centrifugal Filter Units to be divided into two parts according to size. <100 kDa = Factors with a molecular weight <100 kDa in CFMPE; >100 kDa = factors with a molecular weight >100 kDa in CFMPE; n.s. = no significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

ment of NK cell cytotoxicity. Native CEA is a highly glycosylated macromolecule, and this glycosylation plays a critical role in its function [20]. That may explain why the effect of native CEA on NK cells is totally different from that of recombinant CEA. In the experiment system of

Stern et al. [6], NK cells expressed a high level of CD56; thus, we analyzed the effect of CEA on CD56^{bright} NK cells in our study as well. As shown in online supplementary figure 2, using CD107a as the marker of degranulation, CFMPE decreased the degranulation of CD56^{bright}

NK cells. CEA blockade or depletion and TGF- β_1 or IL-10 blockade were not able to modulate the decrease. Because Stern et al. [6] used a CEACAM1+ NK cell clone and we used primary NK cells in the experiment system, we concluded that NK cells from different origins may display a different response to CEA stimulation. It suggests that the real effect of native CEA on the killing ability of NK cells needs to be further investigated and reevaluated.

Previous studies compared cytokines and other soluble factors between MPE and paired PB [21], among them, TGF- β_1 and IL-10 which were considered to inhibit the cytotoxic activity of NK cells which was highly increased in MPE compared with paired PB [22, 23]. However, our study demonstrated that neither TGF- β_1 nor IL-10 played a role in the impairment of cytotoxic activity of CD56^{dim} NK in MPE.

Further study indicated that factors in MPE >100 kDa depressed the cytotoxicity of CD56^{dim} NK cells. The components of MPE are complicated. Both immune cells and abundant tumor cells are infiltrated in MPE although the pathophysiology of MPE is multifactorial and still incompletely understood. Because chemokines and cytokines from immunocytes abnormally accumulated in MPE are usually small molecular proteins [21], we specu-

lated that the factors which impact the cytotoxicity of NK cells and are >100 kDa are released by tumor cells.

In summary, CD56^{dim} NK cells displayed a series of alteration in MPE compared with paired PB, including the reduced proportion and impairment of cytotoxic activity which appeared to be affected by a component >100 kDa in cell-free pleural effusion from MPE. These results are helpful for understanding the mechanism that may lead to impaired NK cell-mediated tumor rejection in lung cancer and provide clues for immune therapy by NK cells. Components >100 kDa in CFMPE need to be further investigated and identified.

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Financial Disclosure and Conflicts of Interest

The authors have no financial conflicts of interest.

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