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Preliminary report

Low-doses of sequential-kinetic-activated interferon-γ enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study

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ABSTRACT

Natural killer (NK) cells are innate immune-system lymphocytes capable of killing tumor cells. They secrete cytokines, including interferon (IFN)-γ, which participate in shaping the initial inflammatory and downstream adaptive immune responses. Its potent immunoregulatory action means that IFN-γ might be beneficial in cases of tumor rejection, but its severe side-effects limit clinical applications. This pilot study compared low-dose IFN-γ prepared by sequential-kinetic-activation (SKA), with standard-dose recombinant (r) IFN-γ, in terms of ex-vivo cytotoxic activity of peripheral blood (PB)-NK cells from colorectal carcinoma (CRC) patients. This was tested against the NK-sensitive K562 cell line and the less-sensitive human CRC Caco-2 and HT-29 cell lines. Twenty primitive non-metastatic CRC patients, five metastatic CRC patients, and thirteen healthy donors were enrolled. PB lymphocytes (PBL) were exposed to medium alone, SKA-IFN-γ (0.25 fg/ml) or rIFN-γ (1 ng/ml). NK-cell cytolytic activity was examined via short-term ^{51}Cr-release. Pretreatment of PBL from non-metastatic patients with SKA-IFN-γ caused a significant increase in NK-cell cytotoxicity, compared to those from normal donors, although less markedly than pretreatment with rIFN-γ against all three cell lines. In contrast, PBL from metastatic CRC patients displayed significantly decreased NK-cell activity and responsiveness to both rIFN-γ and SKA-IFN-γ treatments. These results demonstrate in principle the immunomodulatory capacity of low-dose SKA-IFN-γ, and might open the door to the possibility of generating a novel, safe, and feasible approach to enhancing NK-cell antitumor activity in early-stage CRC patients.

1. Introduction

In vivo and in vitro studies have shown that NK cells, by eliminating malignant cells without the classical MHC restriction, play a crucial role in tumor immunosurveillance [1]. They also collaborate with antigen-presenting cells to amplify the immune response and induce T-cell-mediated antitumor immunity [2,3]. Interferon (IFN)-γ, produced as a result of initial tumor recognition by NK cells, chemoattracts innate immune effector cells. By producing additional IFN-γ these cells in turn activate the cytotoxic functions of tumor infiltrating NK cells and macrophages, leading to enhanced production of immunomodulatory cytokines, e.g. IL-12 and IL-18. The end result is the presence of sufficient numbers of dead tumor cells to activate the antigen-presenting machinery of the adaptive immune response. The IFN-γ- and IL-12-rich tumor environment inhibits T helper (h) 2 cell development, while promoting a Th1 anti-tumor response. This eventually gives rise to a specific cytotoxic $CD8^+$ anti-tumor cell response; it also induces MHC class I pathway protein expression in tumor cells, enhancing their immunogenicity [4]. The production of substantial levels of IFN-γ also indirectly enhances angiostasis, and directly causes tumor-cell anti-proliferative and pro-apoptotic responses [5,6]. However, this integrated and necessary link between innate and adaptive immune responses, whose goal is to elicit specific tumor rejection, is not usually efficient in controlling large solid tumors, because of the insufficient immune response and/or the active strategies employed by the tumor cells to escape immune attack [7].

Interest has been increasing, over the past three decades, in harnessing the immune system to eradicate cancer; this has been accompanied by heightened efforts to characterize cytokines and exploit their vast signaling networks, in the hope of developing cancer treatments [8]. Due to the extensive immunomodulatory functions in both innate and acquired immunity [9], and the proven pivotal role of endogenously-produced IFN-γ in animal models of antitumor immunity, IFN-γ quickly inspired clinical applications in a variety of disease conditions, including cancer. However, the clinical use of high-dose

Abbreviations: NK cell, natural killer cell; SKA, sequential-kinetic-activation; CRC, colorectal cancer; IFN, interferon; r, recombinant; PBL, peripheral blood lymphocyte; LU, lytic unit.

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IFN-γ as conventional chemotherapy against different types of tumor, including melanoma [10], glioma [11], small-cell lung [12], bladder [13], and breast [14] cancers, and in hematological [15], ovarian [16], renal [17], and gastrointestinal [18,19] malignancies, has had limited success, with severe dose-dependent toxicity. This suggests that in-vivo doses route, and/or administration schedule may be inappropriate in vivo. If the natural rapid, but transient production of IFN-γ is not reproduced therapeutically, a potentially-effective action on the functional organization of the immune responses to the tumor could be converted into a substantially negative one. Further studies are thus necessary to clarify the optimal clinical use of this cytokine, and to develop treatments affording potent, specific, and durable anti-tumor immunity, with limited adverse consequences.

Colorectal cancer (CRC), one of the leading causes of morbidity and mortality worldwide [20], is a highly treatable and often curable disease when localized to the bowel. Surgery is the primary form of treatment, and leads to a cure in approximately 50% of patients. Recurrence following surgery is a major problem, and is often the ultimate cause of death, although considerable progress has been made in treatment. An increasing body of evidence supports the hypothesis that the visibility of CRC to immune attack is substantial, and that it potentially could limit disease progression [21]. Despite some preclinical evidence of efficacy, clinical trials using IFN-γ have been generally disappointing, and the treatment is poorly tolerated [19,22].

Several lines of evidence suggest that low-doses of cytokines are adequate in many different models [23]. A recent in vitro study showed that low-doses of IL-12 modulate functional activities of T cell subpopulations from non small cell lung cancer patients [24]. In particular, Gariboldi et al. [25] employed a murine model of allergic asthma to demonstrate that cytokines activated by the pharmaceutical preparation process known as "sequential kinetic activation" (SKA) retain their functional activities even at physiological low-dose concentrations.

These results encouraged us to investigate whether this specific method might render cytokines at low-doses as active in the treatment of the human diseases as the high concentrations normally used in clinical pharmacology, but without the side effects typical of high doses.

This explorative study utilized peripheral blood (PB)-NK effector cells, from healthy donors and from non-metastatic and metastatic CRC patients, to assess whether ex-vivo exposure to a very low-dose of SKA-IFN-γ might enhance these cells' antitumoral activity, compared to the normally-administered conventional dose of r-IFN-γ.

2. Materials and methods

2.1. Reagents

SKA-IFN-γ was prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy) using the standardized method. IFN-γ underwent a shaking process (vertical shaking; 10 cm motion range; shaking speed corresponding to 100 oscillations over 10 s), sequentially diluted in saline solution (serial dilution 1:100) and kinetically energized by a mechanically-applied force [25]. The preparation was supplied at a concentration of 10^{-8} μg/ml. Recombinant human IFN-γ was obtained from PeproTech Inc. (Rocky Hill, NJ, USA).

2.2. Patients

The study group comprised 25 patients who had received a diagnosis of CRC (15 males and 10 females, median age 70.5 years, range 57–84) from the Department of Surgical Medical Sciences at "Città della Salute e della Scienza" Hospital, Turin (Italy) between April 2011 and September 2013. Twenty patients had histopathologically-confirmed primary CRC and were staged by Dukes' system, revised by Astler and Coller (8 Dukes' A and 12 Dukes' B) [26]. Entry criteria were: primary CRC indicative of surgery with no preoperative evidence of distant metastasis. Five patients had histopathologically-confirmed metastatic CRC (3 Dukes' C with lymph node metastasis, and 2 Dukes' D with hepatic metastasis). None of the patients had undergone surgical or other anticancer treatment at the time of blood sampling. All patients provided their informed consent prior to entering the study. The study procedures complied with the Helsinki Declaration. A group of thirteen healthy donors were used as controls (7 males and 6 females, median age 68, range 48–90).

2.3. Cell isolation and treatment

Peripheral blood (PB) samples (15 ml) were collected in anticoagulant-coated tubes from CRC patients and healthy donors. PB mononuclear cells (PBMC) were isolated using Ficoll–Hypaque density gradient centrifugation. PB lymphocytes (PBL) were obtained from PBMC depleted of $CD14^+$ cells by means of immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). PBL were cultured at 1×10^6/ml for 24 h in 5 ml of RPMI 1640 medium, containing 10% fetal calf serum (FCS) (complete medium) (Sigma Aldrich, St. Louis, MO, USA), in the absence or presence of the previously-determined optimal dose of SKA-IFN-γ (0.25 fg/ml) or of rIFN-γ (1 ng/ml).

2.4. Cytotoxicity assays

NK cell activity against the NK-sensitive human myelogenous leukemia cell line K562 (ATCC, Manassas, VA) and against two CRC cell lines, Caco-2 [27] and HT-29 (ATCC) [28], of untreated- and rIFN-γ or SKA-IFN-γ-treated PBL was tested in a standard short-term (4-h) in vitro cytotoxic assay. Briefly, tumor targets were incubated with 100 μCi of sodium [^{51}Cr] chromate (PerkinElmer, Waltham, MA, USA) for 60 min at 37 °C, and washed three times to remove excess isotope. A quantity amounting to 5×10^3 target cells/well was added to different numbers of effector PBL in triplicate, in 96-well U-bottomed microtiter plates (Costar, Cambridge MA, USA), to assess effector-to-target (E:T) final ratios of 40:1, 20:1, 10:1 and 5:1. After 4 h incubation at 37 °C in a CO_2 incubator, 100 μl of supernatant was removed from each well for isotope counting in a γ-counter (Packard, Downers Grove, IL, USA). Spontaneous and maximum release values were determined, respectively, by incubating targets in medium alone, or in medium plus 1% Triton-X-100. Spontaneous release in no case exceeded 15% of maximum release. In all cases, cultures were set up in triplicate and the % specific target cell lysis was calculated from the following formula: $(E - S) / (M - S) \times 100$, where E is the mean cpm release in the presence of effector cells, S is the mean cpm released spontaneously by target cells incubated with medium alone, and M is the cpm release of 100 μl of Triton-X-100-treated cells. NK-cell activity was expressed in terms of the number of lytic units 30% (LU_{30}) per 1×10^6 cells for all groups. LU_{30} was defined as the number of effectors required to produce 30% specific cytotoxicity of 5×10^3 target cells [29]. This method, which uses 4 E:T ratios, is the preferred method for establishing the lytic potential of effector cells, and is preferable to calculating a single percent cytotoxicity value, as it relies on the slope of the graph of E:T ratio versus radioactivity released into the supernatant at the end of incubation.

2.5. Statistical analysis

For matched pairs, the paired Student's t-test was applied. For intergroup comparisons, variables were analyzed by means of one-way ANOVA followed by the Student–Newman–Keuls method, using Sigmastat 3.1 software (Jandel Scientific, San Rafael, CA, USA). Significance was set at $p < 0.05$.