伯喹的大鼠肝微粒体和线粒体体外代谢

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提要 以硅胶薄层色谱法及反相高压液相色谱法研究了伯喹在肝微粒体和线粒体体外代谢的主要代谢产物. 结果提示，微粒体或线粒体制品均可同时产生5-羟基伯喹和羧基伯喹；但定量研究表明微粒体产生的5-羟基伯喹量比线粒体的大19倍，而前者产生的羧基伯喹仅为后者的1/34.

关键词 伯喹；肝微粒体；肝线粒体；薄层色谱法；高压液相色谱法；药物代谢解毒 大鼠

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Antitumor effects of new-type recombinant interleukin-2[1]

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ABSTRACT Two new human recombinant interleukin-2 (rIL-2), 125-Ser-rIL-2, and 125-Ala-rIL-2, were generated by protein engineering technique. Both of them maintained the proliferation of natural killer (NK) cells, CTLL-2 cells and their long-term propagations. The mutated new rIL-2 also enhanced the bioactivity of NK cells and the cytotoxicity of tumor-infiltrating lymphocytes (TIL) against the target tumor cells. The above results were all compared with that of the native rIL-2 and a similarity between them was found, which indicates that new type rIL-2 could be used for adoptive immunotherapy of malignant diseases.

KEY WORDS interleukin-2; protein engineering; natural killer cells; tumor-infiltrating lymphocytes; cultured tumor cells

Interleukin-2 (IL-2) is an immunoregulatory lymphokine secreted by antigen-stimulated T cells which can enhance NK activity, stimulate its differentiation and proliferation, induce lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes[1-3]. Since native IL-2 from cell culture is difficult to be obtained and purified, the study for clinical application of IL-2 has been quite limited. Fortunately the development of recombinant IL-2 (rIL-2) has greatly changed this situation[4]. Native rIL-2 contains 3 Cys. Among them 58-Cys and 105-Cys form a disulfide bond which is necessary for its activity[5, 6]. The 125-Cys is free and is liable to mismatch with other Cys. To prevent this mismatch 125-Cys was substituted with Ser and Ala by means of protein engineering in Shanghai Institute of Biochemistry, Chinese Academy of Sciences (CAS) and two new type of rIL-2 with 125-Ser and 125- Ala was elaborated. In this report the biological features, especially the antitumor effects, of the 2 new rIL-2 were studied and compared with that of the native one.

MATERIALS AND METHODS

C3H mice, weighing 15.0± *s* 0.8 g, were provided by the Experimental Animal Center of Shanghai

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Medical University (SMU). YAC-1 cells and CTLL-2 cells were obtained from the Department of Immunology, SMU. H22 ascitic liver cancer cells were obtained from the Shanghai Institute of Materia Medica, CAS. Recombinant native rIL-2 was used as control. Two new types of rIL-2 were made by site-directed mutation to replace 125-Cys of native IL-2 with Ser or Ala (125-Ser and 125-Ala rIL-2). These 3 kinds of rIL-2 were all produced in the Shanghai Institute of Biochemistry. They were all of > 96% purity with specific activity of 1×10^7 IU · mg^{-1}. Human anti-rIL-2 monoclonal antibody was provided by the Department of Immunology of the Institute of Oncology, Chinese Academy of Medical Sciences. RPMI 1640 medium (Gibco-BRL Co) [^{3}H]TdR (Shanghai Institute of Nuclear Science, CAS). $Na_2^{51}CrO_4$ (Amersham).

NK cell preparation and proliferation[1,7] C3H mouse splenocyes prepared under aseptic condition. After the erythrocytes were destroyed by Tris-NH_4Cl, cell suspensions were placed on the top of discontinuous density gradient (composed of 60%, 55%, 50%, 45%, 40%, of Percoll), and centrifuged at $400 \times g$ for 25 min. The cells located on 2 interfaces of 45-40% and 50-45% were collected, washed 3 times, and the cell concentration was adjusted to 1×10^6 cells · ml^{-1}, then rIL-2 was added (finally to 500 IU · ml^{-1}). Cell growth and tumoricidal activities were assayed.

Preparation of TIL Subcutaneous nodules of tumor from tumor-bearing mice of 10-d inoculating H22 liver cells were excised out. The tumor explants removed of necrotic parts were disaggregated into single cell suspension (5×10^7 cells · ml^{-1}) by a combination of mechanical and enzymatic methods. The suspension was then put on Percoll discontinuous density gradient and centrifuged at $400 \times g$ for 30 min. The fractions enriched in TIL at interface of 50-55% were collected, washed 3 times in fresh medium and counted in a hemacytometer. The TIL-enriched suspension was cultured under various conditions and its antitumor activities were assayed.

Inhibition of rIL-2 functions by anti-rIL-2 monoclonal antibody 100 μl of CTLL-2 cell suspension (2×10^5 cells · ml^{-1}) containing rIL-2200 IU · ml^{-1} was transfered into a 96-well plate, and 100 μl of anti-rIL-2 monclonal antibody in different dilutions were added. The cultures were incubated in 5% CO_2, at 37℃ for 40 h and continued for another 8 h with 1.85×10^4 Bq of [^{3}H]thymidine. The cells were subsequently collected on glass fiber filters with automated sample harvester, and [^{3}H]TdR incorporation (cpm) was determined using liquid scintillation method. Samples were assayed in triplicate.

Cytotoxicity test by ^{51}Cr release assay[8] Well-grown YAC-1 or H22 cells was labeled with $Na_2^{51}CrO_4$ (adding 3.7×10^6 Bq to 2×10^5 cells) for 2 h, and then washed 3 times. Cell concentration was adjusted to 1×10^5 cells · ml^{-1}. According to various ratio of effector to target cells were added to the 96-well plates and incubated for 4 h. The supernatant was collected with conventional method: their cpm values were measured with a γ-counter. Spontaneous release group (control 1) was target cells with medium only, and maximal release group (control 2) was target cells with 2.5% Triton X-100. Every group had 3-4 wells.

Killing rate (%) = (dpm of experiment group − dpm of control 1) × (dpm of control 2 − dpm of control 1)$^{-1}$ × 100%.

RESULTS

Effects of the 2 new type of rIL-2 on NK cell proliferation In the presence of 125-Ser-rIL-2, 125-Ala-rIL-2, or 125-Cys-rIL-2 (the native one), proliferation of NK cells was observed. The 2 new rIL-2 had similar functions as native rIL-2 (Tab 1). All 3 types of rIL-2 kept the NK cell proliferation, and increased the number of NK cell more than 5-fold by d 10. There were no significant differences among them ($P > 0.05$).

Effects of anti-rIL-2 antibody against these 2 new rIL-2 on CTLL-2 proliferation Increased anti-rIL-2 monoclonal antiboby concentration caused a decreased capacity of rIL-2 for maintaining CTLL-2 proliferation. When 1:8 dilution of anti-rIL-2 was added,

Tab 1. Effects of 3 recombinant interleukin-2 (rIL-2) on proliferation of NK-like cells. $n=3$, $\bar{x}\pm s$; $^{*}P>0.05$, **between every 2 rIL-2 groups;** $^{***}P<0.01$ *vs* **control.**

Culture time / d	0	2	4	6	8	10
Control	15.0±0.8	6.6±1.6	5.3±1.2	4.3±0.5	4.6±0.9	3.6±0.5
125-Cys-rIL-2	15.0±0.8	8.0±1.3	13.0±0.8	19.7±2.0	59.0±1.6	79.3±2.0***
125-Ser-rIL-2	15.0±0.8	7.5±0.5	13.8±2.1	27.3±0.5	66.7±2.3	85.3±0.5***
125-Ala-rIL-2	15.0±0.8	7.3±0.5	12.6±2.8	21.3±2.0	62.3±0.5	83.3±0.5***

Tab 2. Inhibition on abilities of different rIL-2 to propagate CTLL-2 cells by anti-rIL-2 monocoional antibody (Mc-Ab). $n=3$, $\bar{x}\pm s$; $^{*}P>0.05$ **between every 2 rIL-2 groups;** $^{***}P<0.01$ *vs* **control.**

Type of rIL-2	$10^{-3}\times$ [^{3}H]Thymidine incorporation of CTLL-2 cells / cpm				
	0	1 : 64	1 : 32	1 : 16	1 : 8
Control	1.7±0.1	1.8±0.7	1.6±1.3	1.7±0.6	1.8±0.7
125-Cys-rIL-2	14.8±3.4***	11.2±0.7	8.4±1.1	4.9±0.7	2.1±0.7
125-Ser-rIL-2	14.6±4.4***	11.2±0.6	8.2±0.8	5.8±1.5	1.9±0.1
125-Ala-rIL-2	15.3±6.7**	12.3±1.0	8.4±0.6	5.0±0.7	2.2±0.9

the ability of r-IL-2 to propagate CTLL-2 cell had almost been completely inhibited(Tab 2). The results from 2 new types of rIL-2 and from native rIL-2 showed no significant differences ($P>0.05$).

Effects of 2 new generation of rIL-2 on NK cell activity All 3 kinds of rIL-2 enhanced NK cell activity. Freshly isolated NK activity was 32.2±2.7%, while cultured in the presence of 125-Cys-rIL-2, 125-Ser-rIL-2, and 125-Ala-rIL-2 respectively for 8 d, it increased 77±4%, 77±4%, and 74±5%, respectively(Tab 3). The 2 new type generations rIL-2 and native rIL-2 were in accordance ($P>0.05$).

Tab 3. Effects of rIL-2 on cytotoxicity of NK-like cells against YAC-1 cells (E : T = 50 : 1). $^{*}P>0.05$ **between every 2 rIL-2 groups;** $^{***}P<0.01$ *vs* **freshly isolated and control groups.**

Types of rIL-2	Cytotoxicity (^{51}Cr release rate / %)	
	Freshly isolated	Cultured for 8 d
Control	32.2±2.7	13.7±1.5
125-Cys-rIL-2	32.2±2.7	77.3±4.2***
125-Ser-rIL-2	32.2±2.7	77.1±4.2***
125-Ala-rIL-2	32.2±2.7	74.2±5.0***

Augmentation of antitumor activity of TIL from H22 liver carcinoma by new types of rIL-2 Freshly isolated TIL showed a very low cytotoxicity against the autologous hepatic carcinoma cells (3.3±0.4%, E / T = 15 / 1), but after cultured with rIL-2 (500 IU · ml^{-1}), the cytotoxicity increased significantly (80±5%, 77±4%, and 80±4%, respectively). Different types of rIL-2 enhanced the antitumor activity of TIL to the same extent ($P>0.05$).

DISCUSSION

For the studies of structure-functions and the production of better rIL-2 for clinical use, many mutants and derivaties were produced and many good results were obtained in Shanghai Institute of Biochemistry, Chinese Academy of Sciences. In the present experiments, we have showed that the new type recombinant interleukin 2 (rIL-2) produced by gene mutation has the same bioactivites as

native rIL-2. That is to say, mutation hadn't affected the abilities of rIL-2 to induce cytotoxicity of NK cells and TILs against tumor cells which up to day are the most important effectors in adoptive immunotherapy for malignancies [9, 10]. Considering the advantage of the new type recombinant rIL-2 which includs good solubility, good stability and easy to refold (data not shown), we suggest that the new type rIL-2 would be more suitable than native rIL-2 in the adoptive immunotherapy of cancer.

The similar inhibitory activity of Anti-rIL-2 antibody on both native rIL-2 and new type rIL-2 demostrates that mutation doesn't change the antigen determinant either. It perhaps help elucidate that new type rIL-2 wouldn't cause more side-effects such as immuno-complex reaction than native rIL-2 when used in treatment of cancer.

We are currently preparing for clinical trial to investigate the application of the new type rIL-2 in adoptive immunotherapy of maligancies *in vivo*.

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新型重组白细胞介素 2 抗瘤活性的研究[1]

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提要 用蛋白工程方法对天然型重组白细胞介素(rIL-2)进行改造，研制的两种新型 rIL-2，125-Ser-rIL-2 和 125-Ala-rIL-2 均能维持 NK 细胞及 CTLL-2 细胞的增殖或长期传代，这种作用可被抗 rIL-2 的单克隆抗体破坏。新型 rIL-2 还能增强 NK 细胞的活性，并显著提高肝癌浸润性淋巴细胞(TIL)的抗瘤活性。这说明新型 rIL-2 的生物学活性与天然型 rIL-2 基本一致，可应用于肿瘤的免疫治疗。

关键词 白细胞介素 2；蛋白质工程；天然杀伤细胞；肿瘤浸润性淋巴细胞；培养的肿瘤细胞

抗肿瘤治疗