Studies on structural features of human tumor necrosis factor*

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Abstract The microstructure of human tumor necrosis factor alpha (TNF-α) and its mutant (TNF-b) has been investigated by utilizing positron annihilation lifetime spectroscopy, radioiodination of human TNF and L929 cells assay. The experimental results show that the long lifetime (τ_2) and corresponding intensity (I_2) of lower ortho-positronium annihilation in TNF-α are longer and less than those in the TNF-b, respectively. It suggests that the TNF-b is smaller in free volume and higher in density than the TNF-α. The TNF-b may maintain a more favorable conformation for binding to TNF receptors, thus increasing its biological activity. It is then concluded that the increases in the cytotoxicity and in the density for the TNF-b result from the decreases in the free volume in the TNF-b.

Keywords Positron annihilation technique (PAT), Receptor binding assay, Tumor necrosis factor (TNF), Cytotoxicity

1 Introduction

Human tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine which is a 17 KD protein having cytotoxic, cytostatic, immunomodulatory as well as other activities, and is also involved in septic shock. The role of TNF-α as an anticancer therapeutic agent has been investigated. The clinical trails have shown that local injection of TNF-α into tumor loci and systemic administration may lead tumor regression in some cases, however, the severe toxic complications of TNF-α limit the utility of the currently available agent.

A TNF-α mutant, designated as TNF-b[1], has two basic amino acid residues (Lys-Arg) substituted for 3-8 amino acid residues (Ser-Ser-Ser-Arg-Thr-Pro). The cytotoxic activity of TNF-b against mouse cell L929 is four fold higher than that of TNF-α.

Positron annihilation lifetime spectroscopy (PALS) is an useful tool for the study of TNF. This technique is sensitive to change in free volume caused by TNF-b[2], so the measurable parameters of importance are lifetime τ_2 and corresponding intensity I_2. The I_2 represents percentage of annihilation events due to the lower o-Ps annihilation, it relates to the free volume number, the τ_2 is lifetime of the lower o-Ps annihilation, it relates to the free volume size. A simple quantum-mechanical calculation for a square well leads to the approximate result[3]:

$$\tau_2 = \text{const} \times V^{0.87} \quad (1)$$

where V is the volume of the potential well, it may be thought of as the free volume approximately and the constant is determined by unit of the well depth and the τ_2, where τ_2 is the probability for two-quantum annihilation by pickoff. Thus, the microstructure of the TNF-b is probed by the change of the intensity I_2 and lifetime τ_2 in lower o-Ps annihilation.

2 Experimental

Materials: The solution of the samples for positron annihilation studies were prepared by lyophilizing, no any TNFs involved in the solution as a control. The powder of each sample was filled into the mylar film sample holder having a volume of 1cm×1cm × 0.2 cm.

The positron annihilation lifetime measurements were done by a fast-fast coincidence circuitry positron lifetime spectrometer modified from the fast-slow delayed coincidence

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positron lifetime spectrometer (CANBERRA) with a resolution of 290ps. A ^{22}Na positron source of 740 kBq was sandwiched between two pieces of the TNF samples. This assembly was then placed between two detectors. The total counts of each lifetime spectrum were upto 5×10^5 with good statistics. The lifetime spectrum was resolved into two components as follows:

$$N(t) = N_1\exp(-r_1t) + N_2\exp(-r_2t) + B \quad (2)$$

where r_1 and r_2 are the annihilation rate constants (reciprocal values of the lifetime) for the two modes of annihilation, N_1 and N_2 are characteristic constants for each component and B is the background resulting from random coincidences. A short lifetime component (lifetime τ_1 and corresponding intensity I_1) related to the rapid free annihilation of a positron and para-positronium (p-Ps) selfannihilation, and the long lifetime component (lifetime τ_2 and corresponding intensity I_2) were obtained by a POSIFIT-EXTENDED program.

Human TNF-α was iodinated with ^{125}I by the iodogen method. Briefly, 20μg hTNF-α and 74 MBq $Na^{125}I$ in 100μl PBS were incubated with a glass bead coated with 0.47μg iodine for 1 min.[4] The labeled TNF-α was separated from free iodine by filtration on a Sephadex G 25 column equilibrated with PBS and saturated with 0.2% BSA. The [^{125}I]-TNF-α had a specific activity of 0.4 MBq/μg. The majority(80%) of the initial bioactivity was recovered, as determined by the L929 cytotoxicity assay.

Binding assays were performed by using the 24-well method. Briefly , the target cells were seeded in 24-well tissue culture plates at 2.5×10^5 cells/well and incubated overnight at 37°C, 5% CO_2. For the competitive radiolabelled ligand assay, a serial dilutions (100 to 0.1 n mol/L) of unlabelled TNFs and 0.6 n mol/L [^{125}I]-TNF-α were added to triplicate wells. The two remaining wells contained only labeled hTNF-α (total binding), and a 500-fold excess of unlabelled hTNF-α (background), respectively. The all reactions were done in 0.5 ml cell culture medium at 4°C for 2 h. Unbound [^{125}I]-TNF was removed by washing with cold PBS and the radioactivity bound to the cells was quantified in a gamma counter. The relative receptor binding inhibition activity (RBI) of TNF mutant is defined as the following: RBI of TNF-b=(ID_{50}TNF/ID_{50}TNF-b)$\times$100%, where ID_{50}, half-inhibition dose, is defined as the amount of a protein to inhibit 50% of the [^{125}I]-TNF binding in the receptor binding inhibition assay.

TNF cytotoxicity assay was done with L929 cells assay.[5]

3 Results and discussion

Fig.1 gives the positron annihilation lifetime spectra for the control sample, TNF-α and TNF-b. The parameters of above-mentioned spectra are summarized in Table 1. The biological characterization is shown in Fig.2.

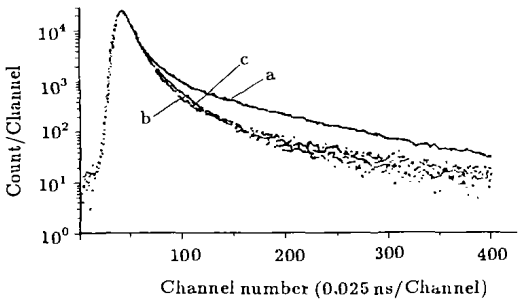

Fig.1 Positron annihilation lifetime spectra for the control sample (a), TNF-α (b), and TNF-b(c)

The difference in the positron annihilation lifetime spectra for the control sample, TNF-α and TNF-b is very obvious (Fig.1). It indicated that the variation of their long lifetime (τ_2) took place. It can be seen that the free volume of TNF-α is more than that of TNF-b; and the density of TNF-α is less than of TNF-b. It seems that difference of conformation between TNF-α and TNF-b is rather large; and the variation in conformation of a TNF has a pronounced effect on its biological activity.

TNF-α mediates its activities by binding to specific receptors on the surface of cells.[6,7] Two distinct TNF receptors of 55 KD (TNF-R55) and 75KD(TNF-R75) have been identified.[8] The TNF-R55 mediated cytotoxic

response and TNF-R75 mediates proliferation response.

Table 1 Parameters of positron annihilation lifetime spectra for the samples

Samples	τ_1 /ns	τ_2 /ns	I_2/%
Control sample	0.175±0.004	0.512±0.005	37.35±0.36
TNF-α	0.168±0.006	0.442±0.006	29.73±0.50
TNF-b	0.163±0.007	0.372±0.003	44.48±0.64

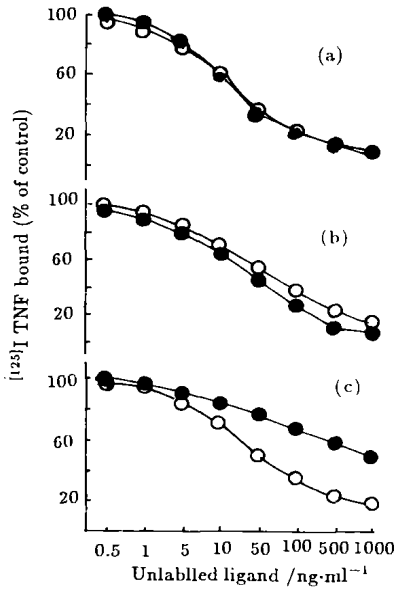

Fig.2 Competition of non-labeled hTNF-α and its mutants with ^{125}I labelled hTNF-α for binding to (a)L929cells, (b)Hep-2 cells and (c) U937 cells. Target cells were incubated with 0.6 n mol/L [^{125}I]-hTNF-α and graded amounts of non-labelled hTNF-α (o), TNF-b (•) at 4°C for 2 h.

In cytotoxicity assay with L929 cells, TNF-b has shown 4.5-fold higher activity than TNF-α. The specific activities for TNF-b and TNF-α are 9×10^7 unit/mg and 2×10^7 unit/mg, respectively. In order to determine whether the altered biological activities of TNF-b are caused by altered binding to the target cells, competitive radiolabelled ligand binding assays were performed, which showed that the effect of TNF-b almost unaltered compared to TNF-α when [^{125}I]-TNF was used as a competitive inhibitor for binding to L929 cells. These results suggested that the relative cytotoxicities of TNFs to L929 cells might correlate with their affinity for the receptors. We also tried on HEP-2 (only carries hTNF-R55) and U937 (expresses mainly hTNF-R75) cell lines in this experiment and found that the concentrations of TNF-b required to 50% of binding to HEP-2 cells and U937 cells were about 2-fold lower and 40-fold higher than those of TNF, respectively (Fig.2).

References

1 Guo Dong-Lin, Shen Bao-He, Dong Xue-Yin *et al.* Biochem Biophys Res Commun, 1995; 207:927
2 Edward D Handel, Georoe Graf. Biophys J, 1980; 62:697
3 Wilson R K, Johnson P O, Skeem S R. Phys Rev, 1963; 129:2091
4 Markwell M A K. Anal Biochem, 1982; 125:427
5 Aggarwal A B, Kohr W J, Hass P E *et al.* J Biol Chem, 1985; 260:2345
6 Englmann H, Novick D, Wallach D. J Biol Chem, 1990; 265:1531
7 Brockhaus M, Schoenfeld H J, Schlaeger E J *et al.* Proc Nati Acad Sci USA, 1990; 8(7):3127.
8 Hohmann H P, Remy R, Brockhaus M *et al.* J Biol Chem, 1989; 364:14927