Age-Associated Changes in Tumor Necrosis Factor α Receptor on Peripheral Blood Monocytes and Bone Marrow Macrophages from ICR Mice and Wistar Rats

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Abstract: We examined the age-associated changes in tumor necrosis factor α (TNF α) receptor on peripheral blood monocytes and bone marrow macrophages obtained from *slc*:Wistar rats and *slc*:ICR mice. The TNF α receptor on monocytes and macrophages in both rats and mice showed high and low affinity sites. The K_d values of those sites gradually increased with age in both types of cells from both species. Binding capacities (receptor number) were low in 52-week-old animals. These results suggest that the TNF α receptor on monocytes and macrophages of these species might decrease with age in both quality and quantity.

Key words: aging, macrophage, monocyte, rodent, tumor necrosis factor α receptor

Introduction

In preclinical studies for drug development, mice and rats are routinely used in hematological examinations, i.e., erythrocyte and leukocyte counts; and the bone marrow is usually examined [1]. Recently, the hematopoietic and cytokine systems have gained importance in research for hematological toxicity. The bone marrow macrophage is strategically positioned to function as a regulatory cell via close-range interaction, e.s., by helping erythroblasts to create erythroblastic islands. And Bessis *et al.* gave rise to the concept that macrophages function as ‘nurse’ cells for erythroblast development [2]. Additionally, macrophages and monocytes produce many hematopoietic regulatory factors, e.s., interleukin-1 (IL-1), interleukin-6 (IL-6), granulo-

cyte colony-stimulating factor (G-CSF), granulocyte and macrophage colony-stimulating factor (GM-CSF), and interferon α [3], as well as tumor necrosis factor α (TNF α , ref. 4), and macrophage colony stimulating-factor (M-CSF, ref. 5). Of these cytokines, M-CSF, GM-CSF, G-CSF, IL-1 and IL-6 potentiate hematopoiesis, whereas TNF α is found to inhibit growth of bone marrow progenitors for granulocytes, monocytes and erythroid cells [3]. On the other hand, TNF α is found to act in a direct manner to strongly potentiate stem cell growth in the presence of IL-3 and GM-CSF [6]. Thus, the role of TNF α in hematopoiesis is multi-functional with complex biological effects, many of which are shared by IL-1 [7]. Earlier we have examined the age-associated cytokine levels (IL-1, IL-6, TNF α , etc.) in bone marrow, and concluded that changes in cytokine

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levels were closely related to the state of hematopoiesis in the bone marrow [8]. And we also examined the levels of IL-1 receptors on bone marrow macrophages in ICR mice and Wistar rats [9]. Based on the results of that study, we suggested that these receptors on bone marrow macrophage might decrease with age in both terms of quality and quantity.

To see if such changes might also occur in other types of receptors, we decided to examine possible age-associated changes in the TNF α receptor on bone marrow macrophages and peripheral blood monocytes from ICR mice and Wistar rats.

Materials and Methods

Test materials: Murine TNF α and rat TNF α were purchased from Kokusan Kagaku Co. Ltd. and Kanto Kagaku Co. Ltd., respectively.

Animals: Male and female Slc: ICR mice (SPF) and male and female Slc: Wistar rats (SPF) of 4, 6, 8, 10, 12, 18, 26 and 52 weeks old of age were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in a barrier system at 23 ± 2 °C and humidity of $55 \pm 15\%$ with twelve hours of light per day (7 a.m. – 7 p.m.), and fed standard pellet food (FR-2, Funabashi Farm Co., Ltd., Chiba, Japan) and tap water *ad libitum*. All animals procedures conformed to the Guide for the Regulation of Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987).

Isolation of monocytes from blood and macrophages from bone marrow: Monocytes and bone marrow macrophages were isolated by the adhesion method [10, 11]. On the day after arrival, each animal was anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 25 mg/kg, and peripheral blood was collected from a jugular vein with a heparinized syringe. After the animals had been killed by bleeding, each femur bone was removed, and the bone marrow was suspended in fetal bovine serum (FBS). The number of nucleated cells in the bone marrow was counted. The nucleated cells in bone marrow or blood were suspended in sodium phosphate buffer (pH 7.4) and centrifuged at $3,000 \times g$ for 5 min at 4 °C. Next the pellets were mixed with an equal volume of Hanks' balanced salt solution, and layered onto a Ficoll-Hypaque solution (Pharmacia Biotech). Thereafter, these solutions were centrifuged at $1,600 \times g$ for 30 min at 15 °C, and the

mononuclear cells were removed from the middle layer. These cells were then washed with Hanks' balanced salt solution twice, suspended in culture medium (AIM-V, Gibco Laboratories Co. Ltd.), added to a plastic plate (Primaria, Falcon) previously coated with PBS containing 10% fetal bovine serum (FBS, Dainippon Pharmaceutical Co., Ltd; Japan), and incubated for 1 hr at 37 °C under a 5%CO₂/95% air atmosphere. After the plate had been washed with PBS to remove the non-adhering cells, the same medium supplemented with

Fig. 1. Effects of incubation time on ligand binding to tumor necrosis factor α receptor on bone marrow macrophages of 10-week-old ICR male mice.

Fig. 2. Scatchard plot of tumor necrosis factor α receptor on bone marrow macrophages of 10-week-old ICR male mice.

0.5% EDTA was added to the plate to obtain the adherent cells, i.e., monocyte or macrophage. These cells were washed three times with the same medium, and the final suspension was adjusted to a concentration of 1×10^6 cells/ml.

Measurement of TNF α receptors on monocyte or macrophage: The europium-labeled TNF α binding assay was performed to assess TNF α receptor. We preliminarily examined the binding tests for incubation temperature and duration, and binding states using bone marrow macrophages of 10-week-old ICR male mice. It was performed that incubation temperature was carried out at 4 or 37 °C, and incubation duration was done for 10, 30, 60 or 120 min. On the basis of the results in the binding test, 100 μ l of monocyte or bone

marrow macrophage suspension (1×10^5 cells) was incubated at 37 °C for 60 min with shaking with 50 μ l of europium-labeled murine or rat TNF α (1,590 counts/mg protein or 1,890 counts/mg protein) solution, 50 μ l of free TNF α solution, and 50 μ l of 0.05 M Tris-HCl buffer (pH 7.4) in each of several microtubes. Immediately after incubation, the reaction was terminated by cooling in an ice bath and addition of 1 ml of PBS containing 0.1% bovine serum albumin. The cell suspension was filtered through a membrane filter (0.45- μ m pore size, HA type; ADVATEC, Tokyo, Japan). The membrane filter, bearing the cells, was washed with cold PBS and immersed in 0.2 ml of the enhancement solution and shaken at 4 °C for 5 min. The cell suspension resulting from the agitation was centrifuged at 400

Fig. 3. K_d value of tumor necrosis factor α receptor on bone marrow macrophages of ICR mice. Data are the mean $\pm$ S.D. of three animals.

Fig. 4. Binding capacity of tumor necrosis factor α receptor on bone marrow macrophages of ICR mice. Data are the mean $\pm$ S.D. of three animals.

$\times$ g for 2 min at 4 °C. The fluorescence of europium in the supernatant was measured with a DELFIA™ fluorometer (Pharmacia Biotech). The density (B_{max}) and dissociation constant (K_d value) of TNF α receptor on monocyte or macrophage were calculated from the binding equation for receptor binding and Scatchard analysis [12].

Results

1. Binding tests (Figs. 1 and 2)

In the binding tests using bone marrow macrophages of 10-week-old ICR male mice, the maximum binding was observed at 37 °C for 60 min incubation.

And from the Scatchard plot, low and high affinity binding sites were observed on macrophages and another cells obtained from different age or sex animals (data not shown).

2. Mouse bone marrow macrophages (Figs. 3 and 4)

The K_d values of low- and high-affinity sites were low in 4-week-old mice and high in 52-week-old ones. These were increased with age. The change in high-affinity sites was remarkable. The binding capacity of low-affinity sites was almost constant except 4-week-old, but that of high-affinity ones was notably high in 4-week-old animals but decreased with age, especially low in the 52-week-old ones. No difference between

Fig. 5. K_d value of tumor necrosis factor α receptor on bone marrow macrophages of Wistar rats. Data are the mean $\pm$ S.D. of three animals.

Fig. 6. Binding capacity of tumor necrosis factor α receptor on bone marrow macrophages of Wistar rats. Data are the mean $\pm$ S.D. of three animals.

male and female was recognized.

3. Rat bone marrow macrophages (Figs. 5 and 6)

The K_d values of low- and high-affinity sites were low in 4-week-old rats and increased with age. The binding capacity of low-affinity sites slightly decreased with age, and that of high-affinity ones showed a more pronounced age-dependent decrease.

4. Mouse peripheral blood monocytes (Figs. 7 and 8)

The K_d value of low-affinity sites on monocytes was low for 4-week-old mice but much higher for 52-week-old ones. That of high-affinity sites was markedly increased for the 52-week-old animal. The binding ca-

capacity of low-affinity sites remained constant with age, whereas that of high-affinity ones dropped in 52-week-old mice.

5. Rat peripheral blood monocytes (Figs. 9 and 10)

The K_d value of the high-affinity sites showed a greater increase with age than that of the low-affinity ones and remarkably high for 52-week-old ones. The binding capacity of both low- and high-affinity sites decreased with advancing age, which drop in number more evident for the high-affinity sites.

Discussion

TNF α is produced by activated monocytes and mac-

Fig. 7. K_d value of tumor necrosis factor α receptor on monocytes of ICR mice. Data are the mean $\pm$ S.D. of three animals.

Fig. 8. Binding capacity of tumor necrosis factor α receptor on monocytes of ICR mice. Data are the mean $\pm$ S.D. of three animals.

rophages, and causes hemorrhage necrosis of certain tumors as well as inhibition of hematopoiesis.

Of a few report regarding with TNF α receptor on monocytes, Imamura *et al.* described that human monocytes possess two receptor sites, i.e., low- and high-affinity ones [13]. We also recognized these two sites on monocytes and macrophages. And Winzen *et al.* indicated the appearance of 55,000 dalton TNF α receptor during differentiation of macrophages [14]. Although several researchers have described TNF α receptors on monocytes and macrophages, none studied the relationship between age and TNF α receptor level, especially in bone marrow macrophages of mice or rats.

In our previous study, we found that the TNF α content in the bone marrow of rats gradually increased

with age, being particularly high in 52-week-old animals [8]. We also found a positive correlation between myelosuppression in aging and TNF α level in bone marrow. In this present study, we showed that the binding capacities of TNF α receptors on bone marrow macrophages from 52-week-old rats and mice were low, which suggests that the receptors were possibly decreased in binding affinity at both receptor sites as a consequence of the increase in ligand, i.e., TNF α content. Although the relationship between TNF α content in bone marrow and characteristics of TNF α receptor on bone marrow macrophages remains unclear, it was speculated that hematopoiesis via TNF α function might be regulated in two distinct ways; one is by its content in bone marrow and the other is by its receptor level.

Fig. 9. Kd value of tumor necrosis factor α receptor on monocytes of Wistar rats. Data are the mean $\pm$ S.D. of three animals.

Fig. 10. Binding capacity of tumor necrosis factor α receptor on monocytes of Wistar rats. Data are the mean $\pm$ S.D. of three animals.

Interestingly, the findings for peripheral blood monocytes were very similar to those for the bone marrow macrophages. Although we did not examine the TNF α level in the blood, the TNF α receptors of peripheral blood monocytes may be similarly regulated by increased this factor in the blood.

On the other hand, the reason for decreases in Kd values of TNF α receptors of both animals at 4 weeks remains unclear.

We reported earlier that the IL-1 receptor on bone marrow macrophages of mice and rats decreased with age [9]. Based on these findings and our present ones, a decrease in cytokine receptor number with age might be a general phenomenon, but additional studies are necessary to confirm this hypothesis.

In conclusion, these results suggest that TNF α receptors on monocytes and macrophages of these species might decrease with age in terms of both quality and quantity. Additionally, our observations are useful as background data for research on the TNF α receptor levels of macrophages and monocytes to define the age-related effects of TNF α and its receptor on hematopoiesis in the bone marrow.

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