GENETICS

Comparative Study of the Effects of Recombinant Tumor Necrosis Factor and Synthetic Peptides Corresponding to Its Fragments on Phagocytosis Mediated by Fc- and MF-Receptors

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The effects of human recombinant tumor necrosis factor and its peptides on the macrophage-like cells of continuous cell line P388D1 were studied. One of the peptides, 123-131, was found capable of not only mimicking, but also of blocking the effects of recombinant tumor necrosis factor. The results permit us to tentatively identify the 123-131 site of the factor molecule as being responsible for its activating effect on macrophages.

Key Words: *macrophages; tumor necrosis factor-α; peptides; Fc- and MF-receptors; phagocytosis*

Tumor necrosis factor (TNF-α) is an autocrine regulator of macrophage function during the activation of macrophages [9]. Increased expression of Fc-receptors (FcR) is an indicator of macrophage activation [2]. Another characteristic change of surface phenotype concomitant with macrophage activation is reduced expression of mannose-fructose receptors (MFR).

This study was aimed at comparing the effects of TNF-α and its peptide fragments on FcR- and MFR-mediated phagocytosis. Our goals were to study the relationship between the effects and concentrations of TNF-α proper and its peptide fragments and to assess the cooperative effects of TNF-α and its peptides.

MATERIALS AND METHODS

The murine macrophage-like continuous cell line P388D1 was used in the study, cultured in RPMI-1640 medium (Flow) with 10% fetal calf serum, 2 mmol/ml L-glutamine, and 50 mg/ml gentamicin as a monolayer in Lyton's tubes with cover slips. Sheep red cells sensitized with specific antibodies (1% suspension) were the object of FcR-mediated phagocytosis. For studies of MFR-mediated phagocytosis, inactivated *Saccharomyces cerevisiae* yeast was used.

Human recombinant tumor necrosis factor Refnolin (Ferment) was used as TNF-α.

The peptides were synthesized by the solid-phase method using an automated peptide synthesizer and purified by gel-filtration and high-performance liquid chromatography. The amino acid composition of each peptide was verified using an automated amino acid analyzer. For studying the biological activity, the following peptides were selected, corresponding to amino acid sequence sites of human TNF-α: 1-12, 103-113, 123-131, and 126-136 (see scheme).

In ready preparations, 200 cells per preparation were counted. The level of phagocytosis was assessed by 3 parameters: the phagocytic index, or the percent of monolayer cells participating in phagocytosis; the

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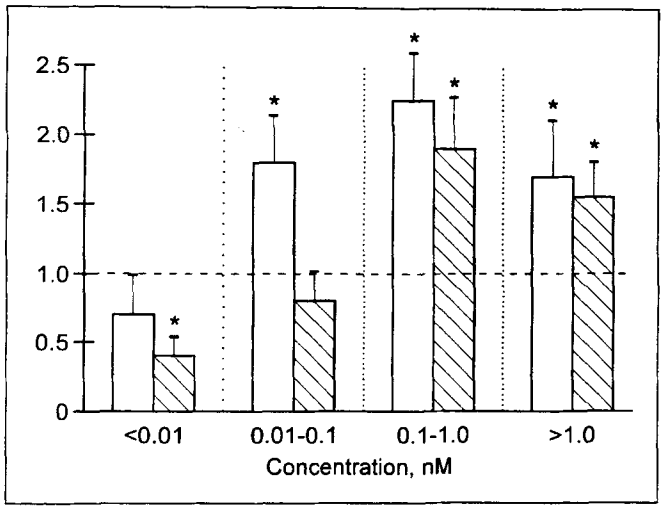

Fig. 1. Effect of TNF-α (light bars) and its peptide 123-131 (cross-hatched bars) in different concentrations on FcR-mediated phagocytosis. Here and in Fig. 2: ordinate: indexes of changes in phagocytosis parameters. *$p<0.05$ *vs.* the control (broken line).

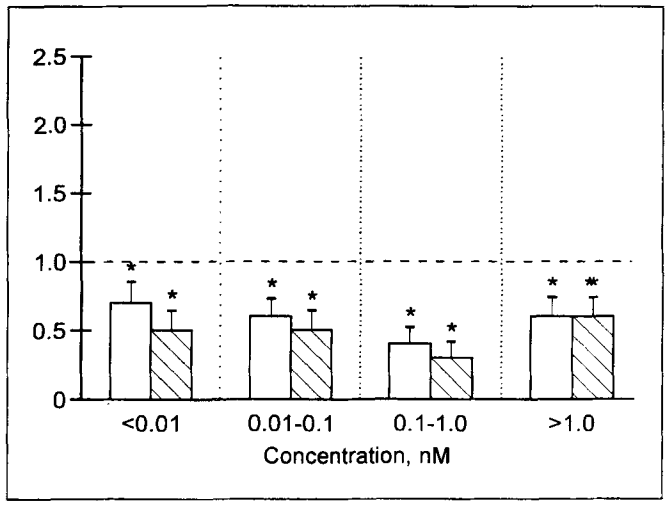

Fig. 2. Effect of TNF-α (light bars) and its peptide 123-131 (cross-hatched bars) in different concentrations on MFR-mediated phagocytosis.

phagocytic number, or the number of absorbed objects of phagocytosis per monolayer cell; and the integral phagocytic index - the product of the phagocytic index and phagocytic number divided by 100. Indexes of changes in phagocytosis parameters were calculated as a function of the parameters of control tests for each experiment individually, taken as 1. The results were statistically processed using methods of variational statistics. The reliability of differences in the mean values was assessed using Student's *t* test.

RESULTS

TNF-α in concentrations of 0.01 to 1.0 nmol stimulated FcR-mediated phagocytosis. If the concentration of TNF-α was reduced to less than 0.01 nM, a tendency for FcR-mediated phagocytosis to be depressed was observed. Further reduction of the concentration below 0.01 nM resulted in a loss of the effect. The dose-dependence of the stimulating action of TNF-α is characterized by a curve with an extremum (Fig. 1).

The most pronounced stimulating effect of TNF-α in FcR-mediated phagocytosis was observed at concentrations ranging from 0.1 to 1.0 nM. In order to elucidate the effects of TNF-α peptides on the level of FcR-mediated phagocytosis, we used them in concentrations equimolar to the above concentrations of TNF-α and in 10 times higher concentrations: from 0.1 to 10.0 nM. Of the four peptides we studied, only peptide 123-131 reliably raised the level of FcR-mediated phagocytosis when used in concentrations equimolar to the stimulating concentrations of TNF-α (Table 1). Peptides 103-113 and 126-136 did not affect the level of phagocytosis, whereas peptide 1-12 reliably stimulated FcR-mediated phagocytosis only in a concentration 10 times higher than the equimolar. Studies of a wide range of concentrations of peptide 123-131 revealed an extreme dose dependence which resembled the analogous curve of dose dependence of the effects of an entire TNF-α molecule (Fig. 1).

Next we investigated the effect of TNF-α on FcR-mediated phagocytosis after preincubation of P388D1 cells with peptides, that is, under conditions of competition for TNF-α receptors. TNF-α was used in concentrations maximally stimulating FcR-mediated phagocytosis. Peptides were used in a wider range of concentrations, including those 10 and 100 times surpassing the concentrations of TNF-α (Table 2). The indexes of changes of the phagocytosis parameters were calculated as a function of the parameters under the effect of TNF-α alone, taken as 1. Incubation of P388D1 cells with peptides 1-12 and 103-113 did not alter their sensitivity to the subsequent FcR-stimulating effect of TNF-α. After incubation of macrophages with peptide 123-131, their sensitivity to the stimulating effect of TNF-α on FcR-mediated phagocytosis was lowered. Higher concentrations of the peptide further weakened the stimulating action of TNF-α. Incubation with pep-

Scheme of Amino Acid Sequences of TNF-α Peptides

Peptide 1-12:	Val – Arg – Ser – Ser – Ser – Arg – Thr – Pro – Ser – Asp – Lys – Pro
Peptide 103-113:	Arg – Glu – Thr – Pro – Glu – Gly – Ala – Glu – Ala – Lys – Pro
Peptide 123-131:	Val – Phe – Gln – Leu – Glu – Lys – Gly – Asp – Arg
Peptide 126-136:	Leu – Glu – Lys – Gly – Asp – Arg – Leu – Ser – Ala – Glu – Ile

TABLE 1. Effects of TNF-α Peptides in Different Concentrations on FcR-Mediated Phagocytosis (n=10, $M\pm m$)

Concentrations of TNF-α peptides, nM	Phagocytic index	Phagocytic number	Integral phagocytic index
Peptide 1-12			
0.1-1.0	1.03±0.09	1.03±0.20	1.07±0.26
1.0-10.0	1.23±0.02*	1.30±0.18	1.61±0.22*
Peptide 103-113			
0.1-1.0	1.06±0.08	0.97±0.07	1.04±0.14
1.0-10.0	1.14±0.10	1.05±0.16	1.21±0.27
Peptide 123-131			
0.1-1.0	1.41±0.21*	1.43±0.09*	2.02±0.37*
1.0-10.0	1.50±0.27*	1.79±0.16*	2.71±0.72*
Peptide 126-136			
0.1-1.0	1.23±0.20	1.10±0.12	1.36±0.37
1.0-10.0	1.02±0.14	0.92±0.18	0.94±0.25

Note. Here and in Table 3: *p<0.05 *vs.* the control.

tide 126-136 suppressed the stimulating effect of TNF-α, but only if the peptide concentration was high.

TNF-α caused an appreciable decrease of MFR-mediated phagocytosis of the yeast. Similarly as with FcR-mediated phagocytosis, TNF-α caused a dose-dependent effect on MFR-mediated phagocytosis, with the effect maximally expressed in the same range of concentrations: from 0.1 to 1.0 nM (Fig. 2).

TNF-α peptides representing different sites of the cytokine molecule differently affected the level of MFR-mediated phagocytosis. Not only peptide 123-131, but also peptide 126-136 and, to a lesser degree, peptide 1-12 were capable of inhibiting yeast phagocytosis (Table 3). Peptide 123-131 reliably suppressed MFR-mediated phagocytosis in all concentrations used. The inhibitory effect of the peptide in a concentration equimolar to that of TNF-α was even more expressed than that of a whole TNF-α molecule (Fig. 2).

Preincubation of P388D1 cells with peptides 1-12 and 103-113 did not affect their sensitivity to the subsequent MFR-inhibitory action of TNF-α (Table 4). Incubation of macrophages with peptide 123-131 attenuated the inhibitory effect of TNF-α on yeast phagocytosis, the capacity of this peptide to block the effect of the cytokine being more expressed when it was used in higher concentrations. Incubation of macrophages with peptide 126-136 had an ambiguous influence on the MFR-inhibitory effect of TNF-α: in low concentrations this peptide reliably boosted the MFR-inhibiting effect of the cytokine, whereas in higher concentrations it reliably blocked its action.

The experimental data on the activating effect of TNF-α on mononuclear phagocytes are in line with published reports. Previously we found an increase of the bactericidal activity of macrophages under the effect of human recombinant TNF-α. An increase of FcR expression on the surface of cells of the U937 monocyte-like line under the influence of a number of cytokines, including TNF-α, has been reported [3]. There are data on an inverse regulation of MFR expression on macrophages under the effect of cytokines, specifically, γ-interferon [8]. We were able to show that recombinant TNFαa has a dose-dependent activating effect on the macrophage-like P388D1 cells, manifested

TABLE 2. Effect of TNF-α on FcR-Mediated Phagocytosis after Preincubation of Cells with Peptides (n=10, $M\pm m$)

Concentrations of TNF-α peptides, nM		Phagocytic index	Phagocytic number	Integral phagocytic index
Peptide 1-12				
0.1-1.0	0.01-0.1	1.00±0.12	0.99±0.05	1.00±0.16
1.0-10.0	0.1-1.0	0.96±0.10	0.93±0.06	0.89±0.08
Peptide 103-113				
0.1-1.0	0.01-0.1	0.96±0.10	0.98±0.04	0.94±0.09
1.0-10.0	0.1-1.0	1.04±0.07	1.10±0.17	1.16±0.24
Peptide 123-131				
0.1-1.0	0.01-0.1	0.80±0.14*	0.87±0.07*	0.71±0.16*
10.0-100.0	0.01-0.1	0.82±0.19	0.78±0.08*	0.66±0.07*
1.0-10.0	0.1-1.0	0.92±0.15	0.77±0.09*	0.71±0.17
>100.0	0.1-1.0	0.91±0.09*	0.66±0.10*	0.60±0.19*
Peptide 126-136				
0.1-1.0	0.01-1.0	1.11±0.28	1.16±0.30	1.30±0.52
1.0-10.0	0.1-1.0	0.84±0.07*	0.73±0.14*	0.62±0.16*

Note. Here and in Table 4: *p<0.05 of the level of the effect of TNF-α alone in the corresponding concentrations.

TABLE 3. Effects of TNF-α Peptides in Different Concentrations on MFR-Mediated Phagocytosis (n=8, $M\pm m$)

Concentrations of TNF-α peptides, nM	Phagocytic index	Phagocytic number	Integral phagocytic index
Peptide 1-12			
0.1-1.0	0.96±0.10	0.94±0.07	0.91±0.16
1.0-10.0	0.80±0.06*	0.84±0.05*	0.67±0.09*
Peptide 103-113			
0.1-1.0	1.00±0.06	0.94±0.03	0.94±0.05
1.0-10.0	0.98±0.08	0.91±0.06	0.88±0.09
Peptide 123-131			
0.1-1.0	0.56±0.08*	0.54±0.04*	0.30±0.02*
1.0-10.0	0.84±0.11*	0.92±0.08	0.77±0.18*
Peptide 126-136			
0.1-1.0	0.87±0.05	0.76±0.03*	0.66±0.07*
1.0-10.0	0.68±0.06*	0.72±0.05*	0.49±0.08*

TABLE 4. Effect of TNF-α on MFR-Mediated Phagocytosis after Preincubation of Cells with Peptides (n=8, $M\pm m$)

Concentrations of TNF-α peptides, nM		Phagocytic index	Phagocytic number	Integral phagocytic index
Peptide 1-12				
0.1-1.0	0.01-0.1	0.97±0.07	0.89±0.08	0.86±0.15
1.0-10.0	0.1-1.0	0.96±0.05	0.96±0.05	0.92±0.09
Peptide 103-113				
0.1-1.0	0.01-0.1	0.95±0.11	0.87±0.12	0.83±0.21
1.0-10.0	0.1-1.0	0.98±0.04	0.94±0.08	0.93±0.11
Peptide 123-131				
0.1-1.0	0.01-0.1	1.27±0.09*	1.11±0.05	1.40±0.10*
1.0-10.0	0.1-1.0	1.35±0.18*	1.45±0.11*	1.97±0.36*
Peptide 126-136				
0.1-1.0	0.01-0.1	0.82±0.15	0.76±0.07*	0.61±0.17*
1.0-10.0	0.1-1.0	1.16±0.09	1.25±0.07*	1.45±0.06*

in direct regulation of FcR and inverse regulation of MFR, judging from changes in the intensity of FcR- and MFR-mediated phagocytosis. The biological effects of TNF-α are known to be mediated through its interaction with the specific receptors TNF-R 55 kD and TNF-R 75 kD. Just where the active receptor-binding center is located in the TNF-α molecule is still to be discovered. However, some scientists have offered hypotheses regarding the localization of the active sites of the TNF-α molecule. The active center of TNF-a most probably includes the following molecular sequences: 1-10, 35-66, 107-114, and 110-133 [4-7]. TNF peptide 123-131 proved to be the most active of the four peptides we tested. It completely reproduced the activating effects of the whole TNF-α molecule when used in concentrations equimolar to TNF-α. This is noteworthy, because we observed the previously described mimicry effects of short peptides corresponding to cytokine fragments only at much higher concentrations of peptides [1,6]. The TNF peptide 123-131, for example, exhibited the highest activity in experiments with competitive peptide inhibition of TNF-α binding to specific receptors needed for manifestation of its activating effect on macrophages. Hence, in our experiments peptide 123-131 demonstrated a capacity not only to mimic the direct and inverse regulating effects of the entire TNF-α molecule, but also to block the possibility of realizing the same effects of TNF-α, evidently due to binding with TNF-R. A similar, but less expressed activity was revealed for peptide 126-136, which is similar in composition.

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