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Size Heterogeneity of Murine Tumor Necrosis Factors Induced from Mouse Peritoneal Macrophages

Na Gyoung Baik, Jee Yeong Jeong and Soung Soo Kim*

Department of Biochemistry, College of Science and Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

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Abstract: Three kinds of mouse tumor necrosis factor (TNF), which have molecular weights of 35 kDa, 45 kDa, and 18 kDa on SDS-PAGE, were partially purified from serum-free culture supernatants of mouse peritoneal macrophages induced with lipopolysaccharide. Analysis of the native molecular weights by gel filtration indicated that the 18 kDa and 45 kDa TNFs aggregate into 50 kDa and 100 kDa molecules, respectively, while the 35 kDa TNF is contained in high molecular weight aggregates of approximately 200 kDa. The three kinds of cytotoxic factors all elicited tumor reducing responses.

Key words: Size heterogeneity, murine tumor necrosis factor.

Tumor Necrosis Factor (TNF) exhibits a cytotoxic or cytostatic effect in a broad range of animal and human transformed cell lines *in vitro* without killing normal cells (Carswell *et al.*, 1975; Helson *et al.*, 1975). This factor has also been effective in causing hemorrhagic necrosis of certain animal tumors and some heterotransplanted human tumors (Carswell *et al.*, 1975; Haranaka *et al.*, 1984). Recombinant mouse TNF has a molecular mass of 50 kDa, which dissociates into 17 kDa polypeptides on SDS-PAGE (Wingfield *et al.*, 1987). The native TNF purified from mouse tumor necrosis serum has a molecular weight of 39 kDa by gel filtration and a molecular weight of 16 kDa to 18 kDa by SDS-PAGE (Haranaka *et al.*, 1986). On the other hand, Kull and Cuatrecasas (1981) reported three peaks of cytotoxic activity in mouse tumor necrotic serum, which had molecular weights of 50 kDa, 160 kDa, and 225 kDa. Other investigators have also found that the size of native murine TNF ranges from 40 kDa to 150 kDa (Green *et al.*, 1976; Itoh *et al.*, 1984). The significance of higher molecular mass species with TNF activity has yet to be clarified.

In this report the presence of the three smallest forms of mouse TNF yet identified is reported. These TNFs, which are from BCG-primed mouse peritoneal macrophages induced with endotoxin, aggregate into different molecular species.

Materials and Methods

*To whom correspondence should be addressed.
Tel: (02) 361-2698, Fax: (02) 361-2698

Preparation of peritoneal macrophages

ICR mice were injected intraperitoneally (i.p.) with 4×10^7 viable Bacillus Calmette Güerin (BCG) per mouse in 1 ml of phosphate buffered saline (PBS), pH 7.0, and subsequently injected i.p. with 1 ml of 3% thioglycolate (Difco Lab., Detroit) per mouse 9 days later. Macrophage-rich peritoneal exudates were collected 4 days later by peritoneal lavage with PBS. After washing the cells twice with PBS, pH 7.0, enriched peritoneal macrophages were cultured in 60 mm culture dishes (Becton Dickinson Labware, Lincoln Park) containing RPMI 1640 medium (Sigma Chemical Co., St. Louis) supplemented with 20% fetal bovine serum and 50 μg/ml of gentamycin for 24 h at 37℃ under 5% CO_2. The dishes were rinsed several times with PBS, pH 7.0, to remove nonadherent cells. The adherent cells were examined by Giemsa stain to detect mononuclear phagocytes.

Induction of TNF-like factors

For induction of the TNF-like factors the peritoneal macrophages (2×10^6 cells/plate), which had been grown in RPMI 1640 medium for 24 h, were transferred to serum-free medium HB 101 (Irvine Scientific, Santa Ana) containing 50 μg gentamycin/ml. After adding 20 μg/ml of *E. coli* 055:B5 lipopolysaccharide (LPS, Sigma Chemical Co,. St. Louis), the cells were cultured for 18 h at 37℃ under 5% CO_2.

Cytotoxicity assay

TNF activity was determined by an assay for killing of L929 cells (American Type Culture Collection) as described by Ruff and Gifford (1980). One unit of TNF

activity was defined as the amount required for 50% cell lysis. The protein content was determined by the method of Lowry *et al.* (1951).

Purification of TNF-like factors

Serum-free culture supernatants (1 L) were centrifuged at 15,000 x g for 10 min and the supernatants were concentrated on YM10 ultrafiltration membrane (Amicon, Beverly), then dialyzed against the starting buffer (10 mM sodium phosphate containing 50 mM sodium chloride, pH 7.0) for 24 h. The dialyzed proteins were applied to a DEAE-Sephacel column (2.7× 3.3 cm) previously equilibrated with the starting buffer. The column was washed with the starting buffer, then bound proteins were eluted with a linear NaCl gradient (50 to 500 mM). Fractions with cytotoxic activity were concentrated using a YM10 ultrafiltration membrane and applied to a Sephadex G-150 gel filtration column (1.3×108 cm) pre-equilibrated with the starting buffer. The column was eluted with the same buffer. The active fractions were pooled, concentrated, then dialyzed overnight against 5 mM sodium phosphate buffer, pH 7.0. The samples were subjected to SDS-PAGE according to the procedure of Laemmli (1970). Gels were cut into 3 mm strips, eluted by incubating the slices in PBS (pH 7.0) overnight, and assayed for cytotoxicity.

Molecular weight determination

The native molecular weights of TNF-like factors were determined by Sephadex G-150 column chromatography (1.3×108 cm) eluting with 50 mM sodium phosphate buffer, pH 7.0, at a constant flow rate of 3 ml/h. The column was calibrated with γ-globulin (150 kDa), bovine serum albumin (67 kDa) and myoglobin (18 kDa). The molecular weight in denatured and reducing conditions was determined by SDS-PAGE on 12.5% polyacrylamide gel, as described by Laemmli (1970). Standard proteins used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa)

***In vitro* neutralization and Western blot analysis**

In vitro neutralization of cytotoxicity was performed using hamster anti-mouse TNF monoclonal antibody (Genzyme, Cambridge). TNF samples of 50 μl (100 U/ml) were pre-incubated for 2 h at 37℃ with 50 μl of various concentrations of the antibody, and the inhibition of cytolytic activity by the antibody was quantified by cytotoxic assay. Western blot analysis was performed by the modified procedure (Shin *et al.*, 1991) of Towin *et al.* (1979) using rabbit anti-mouse TNF monoclonal antibody (obtained from Dr. Robert D. Schriber, Washington University, Department of Pathology, St. Louis) as the primary antibody.

Antitumor activity of murine TNF-like factors

Sarcoma 180 tumor cells were carried as an ascites in the peritoneum of female BALB/c mice by weekly passage of 2 to 4×10^6 cells into naive recipients. For the necrosis assay, a test sample was injected intraperitoneally three times a day for 3 days containing 2,000 U of each TNF-like factor. The necrotic effect was observed 10 days later according to the method of Itoh *et al.* (1984). Meth A sarcoma was induced 6 to 8 weeks later after subcutaneous injection of methylcholanthrene (1.5 mg) into ICR mice, and animals were treated with 2,000 U of TNF sample per mouse. The necrotic effect was estimated by measuring the diameters of solid tumors 15 days after inoculation of TNF samples.

Results and Discussion

Purification of TNF-like factors from mouse macrophages

Peritoneal macrophages from BCG primed mice were incubated in a serum-free medium with 20 μg/ml LPS for 18 to 20 h to induce TNF-like factors. This induction procedure consistently led to optimum induction of cytotoxic factors among the various combinations of LPS concentrations tested (data not shown).

Typical results of the purification procedure are summarized in Table 1. The TNF activity bound tightly to a DEAE-Sephacel column and was eluted as a single activity peak at approximately 250 mM NaCl when a linear gradient of 50 to 500 mM NaCl was applied

Table 1. Purification of TNF-like factors from mouse macrophages

Sample	Protein (mg)	Total cytotoxicity ($U\times10^{-3}$)	Specific activity ($U/mg\times10^{-3}$)	Purification fold	Purification yield (%)
Crude supernatant	750	960	1.28	1.0	100
Concentrate	675	952	1.41	1.1	99
DEAE-Sephacel	0.54	512	1024	800	53
Sephadex G-150					
Fraction I	0.15	76.8	512	400	8
II	0.04	81.9	2048	1600	8.5
III	0.08	44.8	560.6	438	4.6

Table 2. Neutralization of cytotoxic activity by hamster anti-mouse TNF monoclonal antibody[a]

TNF-like factor	Antibody concentration		
	0.02 μg	0.2 μg	2 μg
34 kDa	60%	82%	100%
45 kDa	60%	96%	100%
18 kDa	54%	92%	100%

[a]% inhibition of cytolytic activity was quantitated by using L929 cells.

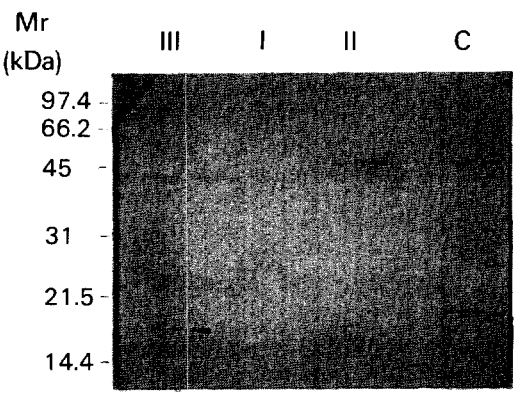

Fig. 4. Western blot analysis of mouse TNFs by using rabbit anti-mouse TNF monoclonal antibody. I: Sephadex G-150 fraction I (5,000 U), II: Sephadex G-150 fraction II (3,000 U), III: Sephadex G-150 fraction III (3,000 U), C: crude TNF (7,000 U).

(Fig. 1). This step resulted in an approximate 800 fold purification of TNF-L929 cytotoxic activity from the crude supernatant (Table 1). Eluate fractions containing cytotoxic activity were pooled, concentrated, and chromatographed on a Sephadex G-150 gel filtration column. As shown in Fig. 2, three peaks of cytotoxic activity which had molecular weights of 200 kDa, 100 kDa, and 50 kDa were obtained from the gel filtration step. The specific activity of the first peak was enriched 400 fold to a final value of 510 U/μg, while the specific activity of the second peak was 2,048 U/μg with a 1600 fold purification over the crude culture supernatant. The third peak was 560.6 U/μg with a 438 fold purification. Purification was reproducible without substantial changes in the sizes of the three activity peaks which appeared in each gel filtration chromatography analysis.

Molecular heterogeneity of murine TNF

The three active peak fractions were pooled, concentrated separately, and concentrated using YM10 ultrafiltration membrane for SDS-PAGE elution and subunit molecular weight analysis. As shown in Fig. 3, the first active peak in the gel filtration step was composed of a 35 kDa cytotoxic factor, and the second active peak

Table 3. Effects of TNF-like factors on sarcoma 180 transplanted intraperitoneally into BALB/c mice

	Dose (U/mouse/day)[a]	Cured mice
Control (PBS)[b]		0/3
Cytotoxic factors		
Fraction I	3000	2/3
II	3000	2/3
III	3000	3/3

[a]Animals were treated with this dose of cytotoxic factors three times on days 1, 2 and 3 and examined on day 10.
[b]Phosphate buffered saline.

Table 4. Effects of TNF-like factors on solid sarcoma 180

	Cured mice	Tumor size (mean, mm)	Dose (U/mouse/day)
Control	0/3	14.2	
45 K	2/3	8.1	2000
35 K	2/3	7.3	2000
18 K	2/3	6.7	2000

*Animals were treated with this dose of cytotoxic factors once a day for 8 days, and examined on day 15.

represented a cytotoxic factor of 45 kDa on SDS-PAGE (Fig. 3A, B). The third active peak contained an 18 kDa cytotoxic factor (Fig. 3C). Since the samples were prepared for SDS-PAGE by boiling with SDS as well as with the reducing agent β-mercaptoethanol, it was concluded that the 35 and 45 kDa cytotoxic factors were not multimeric forms of the 18 kDa cytotoxic factor. Kull and Cuatrecasas (1984), during their studies on necrosin produced by a J774.1 murine macrophage-like cell line, repeatedly observed detectable amounts of cytotoxic activity with an apparent molecular weight of 45 kDa on SDS-PAGE, although no further characterization of properties was reported. On the other hand, the 35 kDa cytotoxic factor, which has not been analyzed before, appears to aggregate to nonsulfhydryl-linked higher molecular forms (Fig. 2). This form of cytotoxin may be analogous to previously reported higher molecular forms found in crude murine tumor necrotic serum (Kull and Cuatrecasas, 1981) or RAW 264.7 supernatant (Kull and Cuatrecasas, 1983).

Immunological relationships among different molecular forms of TNF

In neutralization experiments using hamster anti-mouse TNF monoclonal antibody, the cytotoxic activity of the three kinds of TNF (18 kDa, 35 kDa, and 45 kDa) was completely inhibited when 2 μg of the antibody was added (Table 2). Therefore, these three kinds

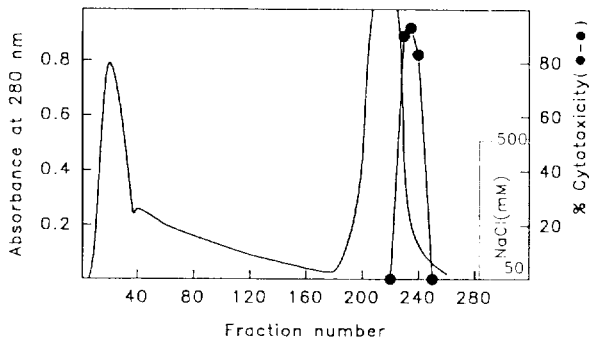

Fig. 1. DEAE-Sephacel ion exchange chromatography of TNF-like factors. The column (2.7×3.3 cm) was equilibrated in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM sodium chloride and eluted with 120 ml of a linear (50~500 mM) sodium chloride gradient. Two ml fractions were collected.

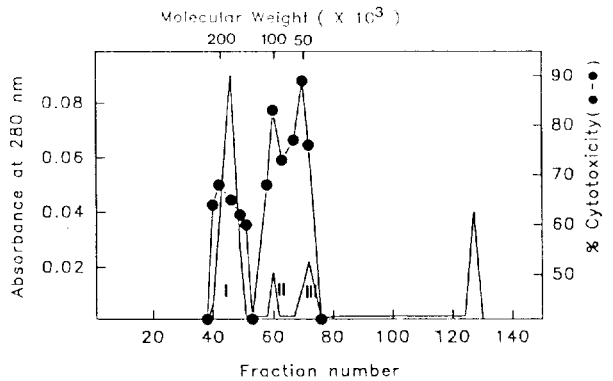

Fig. 2. Sephadex G-150 gel filtration of TNF-like factors. Active fractions (2 ml/fraction) obtained from DEAE-Sephacel ion exchange chromatography were pooled, concentrated and applied to a Sephadex G-150 gel filtration column; TNF-like factor I was pooled from fraction 40~50, TNF-like factor II was pooled from fraction 57~62, and TNF-like factor III was pooled from fraction 66~76. The column was calibrated with γ-globulin (150 kDa), bovine serum albumin (67 kDa) and myoglobin (18 kDa).

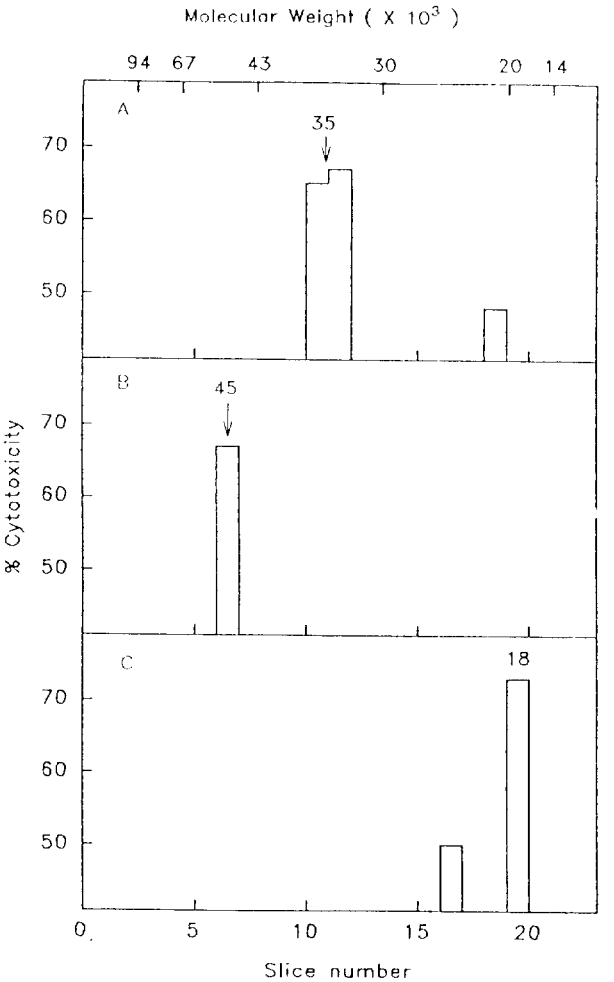

Fig. 3. Profile of cytotoxicity after SDS-PAGE. Fifteen percent polyacrylamide gels (0.7 mm-thick) were run on TNF-like factor I (A), TNF-like factor II (B) and III (C). The gel were sliced into 3 mm pieces, eluted with 0.5 ml of PBS and assayed for cytotoxicity. The standard proteins used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbmin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

of TNF are immunologically similar proteins, or share sirnilar epitopes. Western blot analysis using rabbit anti-mouse TNF monoclonal antibody demonstrated the presence of the 45 and 18 kDa TNFs in the crude extracts (Fig. 4, Lane C). Furthermore, the 45 and 18 kDa TNFs were confirmed in Sephadex G-150 fraction I (Fig. 4, Lane II) and Sephadex G-150 fraction III (Fig. 4, Lane III), respectively. On the other hand, the 34 kDa TNF was not detected in this experiment (Fig. 4, Lane I). Since the 34 kDa TNF was also neutralized, like other molecular forms of TNFs by hamster anti-mouse TNF monoclonal antibody, it may be possible that the amount of protein and not activity required for detection in Western blot analysis could be different depending upon the TNF samples used. Dot blot analysis of the SDS-PAGE eluted 34 kDa TNF, which is twice the amount used in western blot analysis, showed a positive spot (data not shown).

Antitumor activity of the three molecular forms of TNF

The antitumor effects of the three forms of TNF-like factor on transplanted tumors were tested on Sarcoma 180 ascites tumors and a methylcholanthrene induced solid tumor. As shown in Table 3 and 4, the three kinds of TNF-like factors exhibited tumor reducing responses, suggesting that the designation of TNF for these proteins is valid.

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