Cytotoxic Activity of Complexes Formed as a Result of Interaction between Tumor Necrosis Factor-α and α_2-Macroglobulin

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The conditions under which complexes are formed between tumor necrosis factor-α and α_2-macroglobulin are examined in different molar ratios of ingredients with subsequent isolation of formed complexes using gel-filtration and testing of their cytotoxic activity. It is found that tumornecrosis factor-α binds with the activated (F-form) but not with the native (S-form) α_2-macroglobulin and the optimal molar ratio is about 2:1. The cytotoxic activity of such complexes is twice the baseline cytotoxic activity of free tumor necrosis factor-α.

Key Words: *tumor necrosis factor-α; α_2-macroglobulin; cytotoxic activity*

Two forms of the protease inhibitor α_2-macroglobulin (α_2-MG) [1], the S-form (Slow) and F-form (Fast), have recently been shown to be able to form complexes with interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor-α (TNF-α), defensins, and other biologically active polypeptides [2-7], but the biological activity of the complexes has been not systematically examined, although there are data on a changed activity of these molecules in complexes with α_2-MG [5].

Most of the studies of which we are aware deal only with the fact of α_2-MG binding with biologically active molecules, mainly with the use of radioactively labeled forms, which can result in conformational changes of these molecules and affect the intensity of complex formation with α_2-MG when an iodine label is used.

The aim of the present study was to investigate the interaction of S- and F-forms of α_2-MG with recombinant TNF-α without radioactive label and to compare the cytotoxic activity of complexes with the activity of the initial TNF-α.

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MATERIALS AND METHODS

To isolate α_2-MG we used a new technique we devised of "gentle" freeing of fresh donor blood plasma. The procedure included gel-filtration of whole citrate plasma on a TSK-60 HW column of 2.5×80 cm and rechromatography of concentrated α_2-MG preparation. The last step consisted of ion-exchange chromatography on diethylaminoethyl cellulose. The purity of the preparation was no less than 95% according to polyacrylamide gel electrophoresis and its yield was 50-55%. Since the procedure of α_2-MG sedimentation by ammonium sulfate and polyethyleneglycol was absent, practically all the obtained α_2-MG was in the S-form, as was confirmed by electrophoresis. The amount of α_2-MG in purified preparations was assessed both according to protein (Lowry method) and according to the proportion of trypsin added in excess, which bound with α_2-MG at a molar ratio of 2:1. Bound trypsin was detected by the rate of its catalyzed N-benzoyl-L-arginine-para-

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TABLE 1. Cytotoxic Activity of Pools of High- and Low-Molecular Fractions after Gel-Filtration of Incubation Mixtures of α_2-MG and TNF-α in Different Molar Ratios

Form and quantity of α_2-MG, nM	Quantity of TNF-α, nM (U)	Cytotoxic activity of fraction pool, U	
		high-molecular	low-molecular
S-form, 700	350 (5 000)	0	5 000
	700 (10 000)	0	10 000
F-form, 700	350 (5 000)	1 250	3 750
	700 (10 000)	2 500	7 500

nitroanilide hydrolysis at 37°C after free enzyme was blocked by soybean inhibitor. The F-form of α_2-MG was obtained by treating native α_2-MG with monomethylamine for 60 min at 37°C. The final concentration of monomethylamine was 0.5 M. Under these conditions α_2-MG fully lost its capacity to bind with proteinases. After the processing with monomethylamine, 5% polyacrylamide gel electrophoresis revealed one band moving more quickly than the initial preparation. A standard commercial preparation Refnolin (Fermentas) with an activity of 1.7×10 U/mg was used as recombinant human TNF-α. Gel-filtration was carried out with buffered physiological saline (pH 7.3) on the column with TSK-60 HW gel. The rates and volumes of elution were chosen so that α_2-MG and TNF-α left the column in different fractions in the separated gel-filtration. TNF-α activity was determined by the standard cytotoxicity test. One unit of activity was taken as the dose of TNF-α which caused one-half of the maximal lysis of mouse L929 fibroblasts in the presence of actinomycin D stained with crystal violet [7]. Each experiment was performed at least twice. The data were processed statistically by nonparametric methods.

RESULTS

S- and F-forms of α_2-MG were incubated with TNF-α for 1 h at 37°C and then the incubation mixture was subjected to gel-filtration. High-molecular fractions corresponding to the peak of the α_2-MG yield were combined and examined for cytotoxic activity. The remaining low-molecular fractions were also combined and their cytotoxic activity was measured. It was found that TNF-α binds partially with the F-form but not with the S-form of α_2-MG (Table 1). Most of the free TNF-α leaves the column with the low-molecular pool.

A decrease of the cytotoxic activity of the secondary high-molecular fractions to 300 U and synchronous elevation of the cytotoxic activity of the secondary low-molecular complexes from 0 to 900 U was noted after rechromatography of the high-molecular fractions which contain complexes of TNF-α with the F-form of α_2-MG with an initial cytotoxic activity of 1250 U. Therefore, it may be assumed that the formed TNF-α/α_2-MG complexes are not stable and are partially destroyed during gel-filtration.

To ascertain the quantitative ratios of ingredients in complex formation the F-form of α_2-MG was incubated with TNF-α in different ratios. The volume of incubation mixture was the same in all cases. After gel-filtration the high-molecular fractions were combined and their cytotoxic activity was determined. Incubation of the same amount of TNF-α with ascending quantities of α_2-MG results in a progressive increase of the cytotoxic activity of high-molecular substances until the molar ratio of α_2-MG and TNF-α attains 1:2 (Table 2). It may be that in this ratio of ingredients α_2-MG binds all the TNF-α in the reaction mixture. A markedly lower cytotoxic activity of high-molecular fractions after gel-filtration as compared to the activity of added TNF-α may be due to the breakdown of the majority of the α_2-MG/TNF-α complexes in gel-filtration and to the

TABLE 2. Cytotoxic Activity of Pools of High-Molecular Fractions of Incubation Mixtures of the F-Form of α_2-MG with TNF-α in Different Molar Ratios

Quantity of TNF-α, nM	Cytotoxic activity (U) for different amounts of α_2-MG, nM						
	44	88	175	350	700	1 400	2 800
350	312	625	1 250	1 250	1 250	1 250	1 250
700	312	625	1 250	2 500	2 500	2 500	2 500
1 400	312	625	1 250	2 500	5 000	5 000	5 000
2 800	312	625	1 250	2 500	5 000	10 000	10 000

exit of free TNF-α with the low-molecular fractions. From the analysis of the quantitative ratios of ingredients it was concluded that one molecule of the F-form of α_2-MG can bind with two molecules of TNF-α.

To determine the cytotoxic activity of forming complexes, the F-form of α_2-MG was incubated with TNF-α at a molar ratio of 1:2 and then the incubation mixture was examined in the cytotoxicity test without preliminary gel-filtration. It was found that TNF-α in a complex with the F-form of α_2-MG possesses twice the cytotoxic activity as the initial TNF-α (Table 3).

Thus, TNF-α reversibly and noncovalently binds with the F- but not the S-form of α_2-MG and its cytotoxic activity is preserved. The findings show that one molecule of α_2-MG combines with two molecules of TNF-a and the cytotoxic activity of such complexes is twice as high as that of baseline TNF-α. The results demonstrate that TNF-α, like other cytokines, can form large molecular complexes by associating with plasma proteins, notably α_2-MG. The fact that the cytotoxic activity of such complexes is twice as high as that of free TNF-α molecules underscores their physiological and pathological significance.

TABLE 3. Cytotoxic Activity of Incubation Mixture of the F-Form of α_2-MG with TNF-α in 1:2 Molar Ratio

Quantitative ratios α_2-MG/TNF-α, nM	Cytotoxic activity, U	
	of initial TNF-α	of incubation mixture of α_2-MG with TNF-α
175/350	5 000	10 000
350/700	10 000	20 000

REFERENCES

1. A. J. Barret, M. A. Brown, and C. A. Sayers, *Biochem. J.*, **181**, 401 (1979).
2. W. Borth and T. A. Luger, *J. Biol. Chem.*, **264**, 5818 (1989).
3. W. Borth and M. Teodorescu, *Immunology*, **57**, 367 (1986).
4. K. James, *Immunol. Today*, **11**, 163 (1990).
5. A. Panyutich and T. Ganz, *Am. J. Respir. Cell Mol. Biol.*, **5**, 101 (1991).
6. H. Ronne, H. Anude, *et al.*, *Biochem. Biophys. Res. Commun.*, **87**, 330 (1979).
7. A. Woolenberg *et al.*, *Am. J. Pathol.*, **138**, 265 (1991).