ONCOLOGY

Biological Properties of Human Mutant Tumor Necrosis Factor-α

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Antitumor activities of tumor necrosis factor-α and its mutant analog R31Q are studied in HEp-2 and U-937 cell cultures. Cytotoxic activity of R31Q virtually does not differ from that of the initial form of tumor necrosis factor-α and its cytostatic effect was even slightly higher. The nonspecific toxicity of R31Q toward human diploid fibroblasts L-68 is lower. Comparative study of the drug pharmacokinetics *in vivo* showed a better preservation and higher concentration in the blood of the mutant analog of tumor necrosis factor-α.

Key Words: *mutant tumor necrosis factor; antitumor activity; pharmacokinetics*

Tumor necrosis factor-α (TNF-α) is a broad-spectrum immunomodulator causing hemorrhagic necrosis of some tumors. It plays an important role in the development of anti-infection immunity and is a prospective pharmacological preparation. On the other hand, TNF-α is the major mediator of inflammation; it exerts serious untoward effects, such as fever, hypotension, hepatotoxicity, lung edema, intravascular thrombosis and hemorrhages, etc. These toxic effects limit the use of TNF-α in the treatment of cancer patients [6,9]. One approach to solving this problem is the preparation by protein engineering of mutant forms of TNF-α with a lower toxicity for the host and high cytotoxicity toward tumor cells [4,11].

A new structural analog of human TNF-α with Arg31 substituted for Gln has been obtained (R31Q). Its resistance to proteolysis has been demonstrated *in vitro* [1]. We proceeded with studies of its biological properties, investigating the stability of the drug *in vivo* (pharmacokinetics) and its antitumor activity in transformed human cell lines.

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MATERIALS AND METHODS

Electrophoretically homogenous recombinant human TNF-α (rTNF-α) and its analog obtained by oligonucleotide-directed mutagenesis with substitution of Arg31 residue to Gln (R31Q) were used. Recombinant TNF-α was isolated from *E. coli* strain SG 20050/rTNF 311Δ by the method developed at the Vektor Research Center. The preparation was characterized by all parameters required for gene-engineering immunobiological preparations. It is a polypeptide identical to natural human TNF-α with amino acids 5-157. Mutagenic analog of TNF-α R31Q was isolated as described elsewhere [2].

Human diploid fibroblasts L-68, mouse fibroblasts L-929, human laryngeal carcinoma cells HEp-2, and human monoblastoid leukemic cells U-937 from Vektor Institute of Cell Cultures were used.

Specific cytotoxic activity was assessed routinely on mouse fibroblasts L-929 in the presence of

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actinomycin D (Sigma) in 96-well plates [9]. Reference sample with 3.5×10^4 U act per ampoule (8.0×10^4 IU with regard to International Reference Human rTNF-α, No. 87/650) was the reference activity sample.

HEp-2 target cells were inoculated in 96-well plates in a dose of 1.5×10^4 cells per well in Eagle's MEM with 5% fetal calf serum, 0.06% L-glutamine, and 100 mg/ml gentamycin, and incubated at 37°C in an atmosphere with 5% CO_2 for 2 h. Successive dilutions of TNF in concentrations from 0.1 ng to 10 mg in the maintenance medium with 100 μg/ml cycloheximide (Sigma) were added in each well. The plates were incubated for 24 h, after which medium was discarded and cells stained for 30 min with 0.5% crystal violet (Serva) in 20% ethyl alcohol (50 μl/well). Then the plates were washed 4-5 times with distilled water, 100 μl eluting solution (250 ml ethyl alcohol, 0.5 ml acetic acid, water to 500 ml), and light absorbance was measured in a Multiscan vertical spectrophotometer at a wavelength of 540 nm. Cytotoxic effect was assessed by comparing the resultant values with light absorbance of control samples (without TNF).

For assessing cytostatic activity, human monoblastoid leukemic cells U-937 were inoculated in 96-well plates in a dose of $(5.3\text{-}5.5)\times10^4$ cells/well. TNF in doses 0.1 ng to 10 μg in 100 μl maintenance RPMI-1640 with 10% fetal calf serum, 0.06% glutamine, and 100 μg/ml gentamycin was added to each well. Cells were incubated at 37°C in an atmosphere with 5% CO_2 for 3 days. Cell proliferation was assessed by comparing cell counts in experiment and control.

For toxicity studies, human diploid fibroblasts L-68 were inoculated in 96-well plates in a dose of 1.5×10^4 cells/well in Eagle's MEM medium with 5% fetal calf serum, 0.06 % L-glutamine, and 100 μg/ml gentamycin, and incubated at 37°C for 24 h in an atmosphere with 5% CO_2. After a monolayer had formed, the growth medium was discarded, successive double dilutions of TNF in the maintenance medium with 2% fetal calf serum were added, and the plates were incubated for 72 h at 37°C. The reaction was read under a light microscope (×100).

In vivo pharmacokinetics was studied in Chinchilla rabbits weighing 2.5-3.0 kg. TNF-α was injected intravenously in a dose of 3.5×10^6 U/animal. After 15, 45 min, and then every hour till 8 h, 0.5 ml blood from the marginal vein was collected with a sterile needle into sterile tubes; serum was separated by centrifugation. The level of TNF-α in sera collected in terms of up to 4 h after injections was assessed by cytotoxic activity in biological test with L-929 cells as described above and for the terms after 4 h by enzyme immunoassay using Protein Kontur kits (St. Petersburg). Sera of prepared before injection of the preparation were used as the control.

RESULTS

Biological effects of TNF-α are mediated by two highly affine receptors R55 and R75. The presence of two receptors for the same protein implies that they modulate different biological properties of this cytokine. Mutagenic modification of TNF-α which affects the reaction with receptors, is a promising approach to the detection of important functional sites and can lead to obtaining new forms of protein

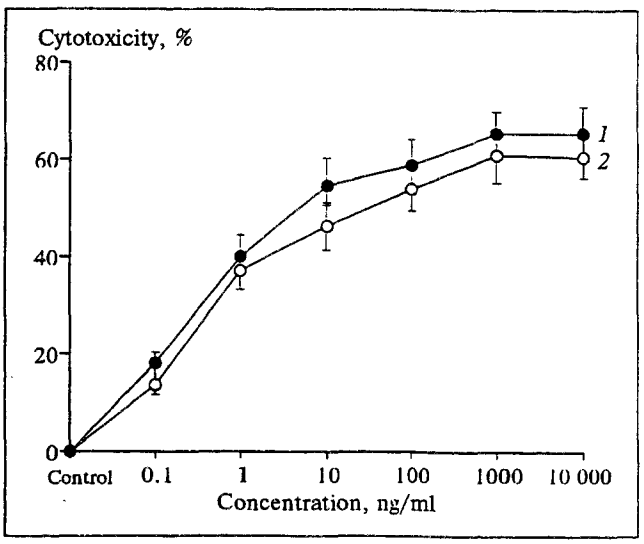

Fig. 1. Cytotoxic activity of tumor necrosis factor-α (*1*) and its analog R31Q (*2*) in HEp-2 cell culture in the presence of 50 mg/ml cycloheximide.

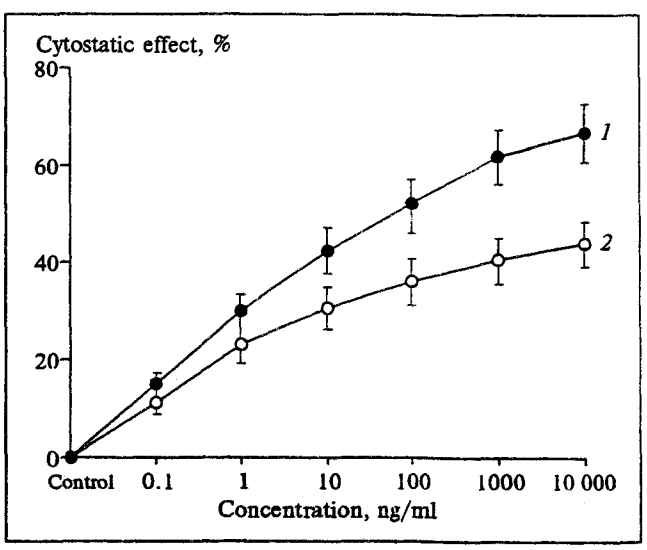

Fig. 2. Cytostatic activity of tumor necrosis factor-α (*1*) and its analog R31Q (*2*) in U-937 cell culture.

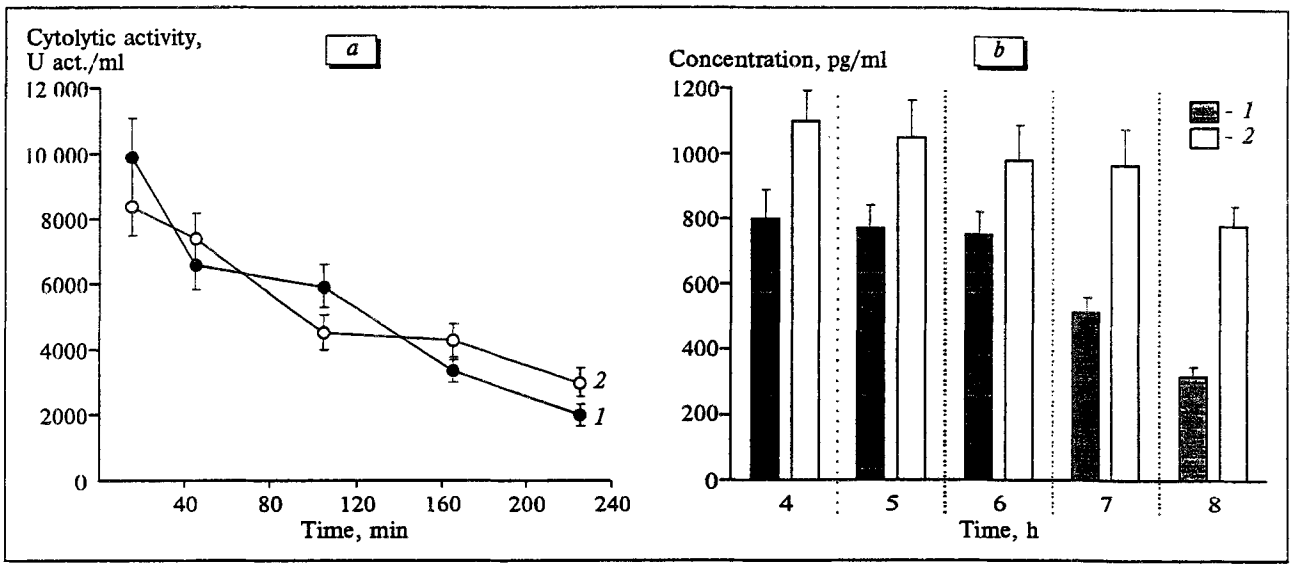

Fig. 3. Time course of concentrations of tumor necrosis factor-α (*1*) and its analog R31Q (*2*) in rabbit serum: *a*) cytolytic activity of blood serum after injection of tumor necrosis factor-α; *b*) concentration of immunoreactive tumor necrosis factor-α and its analog R31Q.

with improved properties [3,4,11]. One prospective region for mutagenesis is situated in the first loop of TNF-α molecule (amino acid residues 31-33), which participates in the binding to cell receptor R55 [7,10] and contains a site available for proteolysis [1].

In our experiments the specific cytotoxic activity of TNF-α and its mutagenic analog R31Q for murine fibroblasts L-929 was 3.5×10^7 and 2.5×10^7 U/mg protein, respectively. Therefore, as we expected, substitution of Arg31 for Gln, an amino acid occupying this position in mouse and rabbit TNF [5], virtually did not affect cytotoxic activity of the preparation, in contrast to substitution of Arg32 for Trp, Arg32 for His, and Ala33 for Ser in this site of the molecule [3,11], which led to a decrease in cytotoxic activity by two orders of magnitude. Thus, binding of R31Q mutant to mouse R55 receptor mediating human TNF-a cytotoxic activity in L-929 cells was not changed.

Study of the effect of mutant protein on human laryngeal carcinoma cells HEp-2 with R55 receptor [11] showed virtually the same cytotoxic activity of R31Q mutant and initial TNF-α (Fig. 1), indicating complete affinity of the mutant protein for human R55 and being in line with previous findings [4].

Mutant TNF inhibits in a dose-dependent manner the proliferation of human monoblastoid leukemic cells U-937, containing both minor (R55) and major (R75) receptors, this inhibition being slightly higher than that by the initial TNF-α (Fig. 2). These data point to changes in the interactions between R31Q and the major TNF receptor that stimulates the activity of the minor receptor and mediates the development of total-system's toxicity [4]. The mutation in this region of TNF-α decreases the binding to R75 receptor *in vitro* [4,11].

Direct nonspecific toxic effect of TNF was studied *in vitro*. It manifests itself by degenerative changes in diploid human fibroblast L-68 culture. Degenerative changes of cells were observed upon the addition of relatively high doses of the agent to the maintenance medium: 0.146±0.07 mg/ml for initial TNF-α and 0.50±0.12 mg/ml for R31Q. A lower (3 times) toxicity of mutant R31Q protein toward normal human cells can be explained by its changed interactions with TNF major receptor.

Substitution of Arg31 for Gln in TNF-α was shown to eliminate one available site for proteolysis with trypsin-like proteases in a protein molecule [1]. Results of comparative study of the pharmacokinetics of TNF-α and R31Q are shown in Fig. 3. Fifteen minutes after injection of the drugs the level of TNF-α is 9.8×10^3 U act./ml and of R31Q 8.2×10^3 U/act./ml. By the end of the fourth hour, 20.4% TNF-α and 36.5% R31Q of this level were still present in the blood. Time course of the blood cytolytic activity after injection of mutant TNF during this period differed little from the kinetics observed after injection of TNF-α. Changes in the rate of degradation and elimination of the drugs manifested themselves later at concentrations approaching the normal (Fig. 3, *b*). During subsequent 4 h, 70% of TNF-α mutant analog but only 37% of initial TNF were present in the blood. Hence, proteolysis stability resulted in a longer elimination of R31Q mutant from the blood of animals.

The results permit us to hope that TNF analog with Arg31 substituted for Gln becomes the basis for

a long-acting antitumor drug with a lower effective dose than that of natural TNF-α and a lower toxicity.

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