Tumor Necrosis Factor Production and Colon Cancer

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Tumor necrosis factor (TNF) production in B10 mice exhibiting H-2 gene heterogeneity and in C3H/He mice differing in lipopolysaccharide (LPS) responsiveness was investigated following stimulation with OK-432. TNF-producing capacity in these mice was H-2-restricted, while their LPS responsiveness was independent of the gene. TNF production in humans was found to be HLA-B antigen-restricted. An investigation was then made of the effect of endogenous TNF induction with OK-432 on the survival rate of colorectal cancer patients. Patients in the TNF-producing group showed a trend toward having a better prognosis as compared to those in the TNF-nonproducing group. Cancer therapy formulated with consideration of host responsiveness to OK-432 may afford greater therapeutic benefit and may prolong survival.

Key words: Tumor necrosis factor — Colon cancer

Tumor necrosis factor (TNF) was first discovered by Carswell *et al.*¹⁾ in 1975. It possesses a wide variety of physiological activities, notably a potent antitumor action.^{2,3)} The TNF-production gene has been mapped near the H-2-D⁴⁾ and HLA-B loci.⁵⁾ TNF production is thus thought to be genetically determined. Cases with TNF production were grouped into 3 types, i.e., high, mixed and low, according to the results of DNA analysis of human TNF gene.⁶⁾ Cases were also divided clinically into 2 groups, i.e., TNF-producing and TNF-nonproducing, according to the capacity for producing TNF as assessed from TNF levels in the ascites after stimulation with OK-432. Here we have examined the capacity for TNF-production both in experimental animals and in clinical cancer patients. The survival rate of colorectal cancer patients was studied in relation to TNF-producing ability.

MATERIALS AND METHODS

Mouse ascites TNF activity Congenic mice differing in the H-2 gene⁷⁾ (B10, B10D2 and B10A, $n=5 \times 3$) and mice with distinct lipopolysaccharide (LPS) responsiveness⁸⁾ (C3H/HeN, C3H/HeJ) were used.

OK-432 was administered to these animals in three 1 Klinische Einheit (KE) i.p. doses every other day, with an additional 10 KE i.p. dose 2 weeks later. The unit "KE" is used to express the strength of the preparation; 1 KE corresponds to 0.1 mg of dried streptococci. OK-432, a heat- and penicillin-treated lyophilized preparation of the Su strain of *Streptococcus pyogenes* of human origin, was developed by Okamoto *et al.*⁹⁾ and is a product of the Chugai Pharmaceutical Co. (Tokyo). One hour after the final dose, ascitic fluid was collected from each animal.

TNF activity in mouse mononuclear cells Congenic mice differing in the H-2 gene (B10, B10D2 and B10A, $n=5 \times$

3) were splenectomized and mononuclear cells were separated from splenocytes. Mononuclear cells thus collected were cultured with 0.01 KE/ml OK-432 or 10 μ g/ml LPS for 4 h, then the cell culture was centrifuged and the supernatant was subjected to assay.

Assay of mouse TNF TNF in the supernatant was measured by cytotoxicity assay using L-929 cells, which are highly susceptible to TNF. TNF activity was expressed as units (U), one unit being defined as that concentration at which cytotoxic damage is caused to 50% of exposed L cells.

TNF-producing capacity of human peripheral blood mononuclear cells Mononuclear cells, collected from human peripheral blood at a concentration of 1×10^6 /ml were cultured with 0.01 KE/ml OK-432 or 10 μ g/ml LPS for 4 h. The cell culture was then centrifuged and the resultant supernatant was assayed for TNF.

Human TNF was measured by ELISA using anti-human recombinant TNF monoclonal antibody (mAb) and peroxidase as the enzyme marker. Briefly, standard TNF solutions or a test sample were added to antibody plates incorporating anti-human TNF- α mAb as a solid phase to allow reaction to occur. Then, after washing of the plates, peroxidase-labeled anti-human TNF- α mAb was added to the plates to allow the antigen bound to the solid phase to react with this second enzyme-labeled antibody. After washing of the plates once again, substrate for the enzyme was added and the enzyme activity was determined as the absorbance at the wavelength of 492 nm. A standard curve (calibration curve) was constructed by plotting the values of absorbance found for standard antigen solutions. The concentration of TNF- α in the original sample was estimated by relating the observed enzyme activity, i.e., absorbance, to antigen concentration by use of the standard curve.

Human HLA typing Typing for human HLA antigens

was performed by microcytotoxicity assay with a Terasaki HLA typing tray in accordance with the method recommended at the Eighth International Workshop.

Method for inducing TNF at operation At the time of closing operative wounds after surgery for colorectal cancer, a Tenkoff catheter was inserted into the pouch of Douglas and clamped. Mitomycin C (MMC) (10 mg) and OK-432 (10 KE) in 300 ml of physiological saline were introduced into the peritoneal cavity and the abdominal incision was closed. Two, 4, 6, 12 and 24 h later ascitic fluid was collected via the Tenkoff catheter and TNF activity was measured. Cases were classified into 2 groups, i.e., TNF-producing and non-TNF-producing.

RESULTS

TNF activity in mouse ascitic fluid TNF levels in ascitic fluid averaged 9.0 ± 2.0 U/ml for B10 mice, 1.4 ± 0.9 U/ml for B10D2 mice and 25.2 ± 15.5 U/ml for B10A mice (Fig. 1). The corresponding values for C3H/HeN and C3H/HeJ mice were 12.8 ± 2.4 U/ml and 13.4 ± 5.0 U/ml, respectively (Fig. 2).

TNF-producing capacity of mouse mononuclear cells Mean TNF levels in mononuclear cells were 1.08 ± 0.53 U/ml, 0.19 ± 0.02 U/ml and 0.52 ± 0.31 U/ml, respectively, for B10, B10D2 and B10A mice (Fig. 3).

TNF-producing capacity of human peripheral blood mononuclear cells Under stimulation with OK-432, TNF levels of peripheral blood mononuclear cells averaged 2268 ± 220 pg/ml for individuals possessing HLA-B5 antigen and 1168 ± 104 pg/ml for HLA-B35 individuals, with the difference being statistically significant ($P < 0.05$) (Fig. 4).

Under stimulation with LPS, on the other hand, the corresponding TNF concentration was 358 ± 52 pg/ml

for HLA-B5 antigen-positive individuals as against 172 ± 33 pg/ml for B35-positives, although this difference failed to achieve statistical significance.

TNF induction attempted at operation There were 23 patients in whom postoperative follow-up measurement of TNF was feasible, with TNF being successfully induced in 12 of them. Mean TNF levels thus found were 396 ± 215 U/ml at 2 h, 498 ± 256 U/ml at 4 h, 261 ± 172 U/ml at 6 h, 8 ± 5 U/ml at 12 h and 0 U/ml at 24 h postoperatively (Fig. 5). TNF production peaked at 2 h in 1 patient, at 4 h in 10 patients, at 6 h in 2 patients and at 12 or 24 h in none.

Fig. 2. TNF production from ascites in C3H/He mice treated with OK-432.

Fig. 1. TNF production from ascites in three B10 strains of congenic mice treated with OK-432.

Fig. 3. TNF production from monocytes in three B10 strains of congenic mice.

Fig. 4. TNF production from human monocytes. □ OK-432 (0.01 KE/ml), ■ LPS (10 μ g/ml).

Fig. 5. Time course of TNF production in patients given OK-432 therapy on operation.

HLA-B antigens and TNF production Among patients possessing HLA-B antigens of high TNF-production type, 2 were in the TNF-producing group and none were

Table I. Background of Colorectal Cancer Patients Treated by Radical Operation

	TNF-producing group	TNF-nonproducing group	χ^2 test
Sex			
male	7	1	ns
female	2	5	
Age			
below 40	1	1	ns
below 70	5	3	
over 70	3	2	
Location			
R	5	1	ns
S	1	2	
D	0	1	
T	1	1	
A	2	0	
C	0	1	
Type			
Type 1	3	0	ns
Type 2	5	5	
Type 3	1	1	
Type 4	0	0	
Stage			
II	3	4	ns
III	2	2	
IV	4	0	
Histology			
well	7	6	ns
moderate	2	0	
poorly	0	0

Fig. 6. Survival rate of colon cancer patients given OK-432 therapy on operation.

in the TNF-nonproducing group. Of patients with HLA-B antigens of mixed TNF-production type, 6 were classified into the TNF-producing group and 1 into the TNF-nonproducing group. Of those with HLA-B antigens of low TNF-production type, 1 was in the TNF-

producing group and 7 were in the TNF-nonproducing group.

Background factors in colorectal cancer Of colorectal cancer patients treated by radical operation, 15 had stage II or more advanced disease. No striking differences were observed between 9 patients in the TNF-producing group and 6 patients in the TNF-nonproducing group in sex and age distribution, or with respect to the location, macroscopic type, stage and histologic type of cancer.

Survival rate of colorectal cancer patients The 5-year survival rate was 78% for the TNF-producing group as against 50% for the TNF-nonproducing group (Fig. 6).

DISCUSSION

Mouse TNF gene was reported to be located in the neighborhood of the H-2D gene.⁴⁾ Mice of the strain B10 differing in H-2, i.e., B10, B10D2 and B10A mice, were also recognized to be different from one another in their capacity for TNF production. From the observed TNF levels in ascitic fluid and mononuclear cells it was assumed that B10A and B10 mice are responsive to OK-432, while B10D2 mice are hyporeactive to OK-432.

LPS responsiveness in mice was reported to be genetically determined.⁸⁾ No striking difference was observed between LPS-responsive C3H/HeN mice and non-LPS-responsive C3H/HeJ mice in TNF concentration in ascitic fluid. It was therefore suggested that TNF-producing capacity is determined by the mouse H-2 gene, but is unrelated to LPS responsiveness.

Human TNF gene reportedly is located in close vicinity to the HLA-B region.⁵⁾ DNA analysis of the human TNF gene revealed heterogeneity of the molecule with the existence of 2 distinct forms of 5.5 kb and 10.5 kb in size.¹⁰⁾ Thus, there are 3 combinations of alleles, i.e., 5.5/5.5 kb, 5.5/10.5 kb and 10.5/10.5 kb, which were arbitrarily designated high TNF-production type, mixed TNF-production type and low TNF-production type, respectively. HLA-B antigens of the high TNF-production type are B44, B46, B51 and B62, those of the mixed TNF-production type are B13, B52, B60 and B61 and those of the low TNF-production type are B7, B35, B38, B48, B54, B58 and B59.⁶⁾ Higher human TNF levels were noted in patients possessing HLA-B5 antigen, which is of high TNF-production type, than in those with HLA-B35

antigen, which is of low TNF-production type, a finding suggesting that the capacity for producing TNF is governed by HLA antigen genes. Follow-up measurement of TNF production showed that it peaked 4 h after stimulation with OK-432, and according to this peak level of TNF concentration one can classify individuals into TNF-producing and nonproducing groups. A good association was noted to exist between TNF production after TNF induction at operation and HLA-B antigen. This was interpreted as suggesting that TNF production in humans is determined by HLA-B antigen.

Colorectal cancer is not very susceptible to currently available chemotherapy and immunotherapy, and virtually no enhancement of survival rate can be expected from the use of any therapeutic modality in patients with advanced disease.^{11, 12)} In the present clinical study colorectal cancer patients were classified into TNF-producing and nonproducing groups according to the ascites level of TNF and a comparison was made of 5-year survival rate between the 2 groups of patients. The results indicated that patients in the TNF-producing group had a higher 5-year survival rate than those of the TNF-nonproducing group, although the difference was not statistically significant. OK-432, when administered in cases where it successfully induces TNF production, could be of therapeutic benefit.

CONCLUSION

Basic and clinical studies were made of the capacity for TNF production. In addition, we examined the 5-year survival rate of colorectal cancer patients receiving therapeutic TNF induction. The results can be summarized as follows:

- 1) In the mouse, capacity for TNF production is determined by the H-2 gene, but is unrelated to LPS responsiveness.
- 2) Capacity for producing TNF in humans is determined by HLA-B genes.
- 3) Human TNF production peaked 4 h after stimulation with OK-432.
- 4) Colorectal cancer patients in the TNF-producing group had a better prognosis than those of the nonproducing group.

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