IMPAIRED TH1-RELATED IMMUNE SYSTEMS IN CANCER PATIENTS

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(Received on Mar. 10, 2000, accepted on June 19, 2000)

Key Words: Cancer patients; immune systems.

Subjects: Cancer patients.

Abbreviations: IL-12 = Interleukin-12; TNF α = tumor necrosis factor-alpha; INF γ = interferon-gamma; Th = helper T cell subjects; NK = natural killer.

Abstract

In order to evaluate the influence of Th1-related cytokines on disease progression, the levels of interleukin-12 (IL-12), interferon-gamma (INF- γ) and tumor necrosis factor-alpha (TNF- α), helper T cell (Th) subsets, and natural killer (NK) cells were examined in 337 Japanese patients with histologically confirmed primary adenocarcinomas of the stomach and colorectal and in 172 control subjects.

Levels of IL-12, INF- γ , TNF- α , and Th1 cells were significantly lower in cancer patients than those in the control group. On the other hand, levels of Th2 cells and NK cells were significantly higher in cancer patients than those in the control group. Th1-related cytokines were suppressed even in early-stage patients. Results of the present study suggest that Th1-related immune parameters were suppressed in cancer patients, even in early-stage patients, and that they were useful as immunological markers for detecting groups of patients at high risk for cancer.

Introduction

It is still difficult to discover early-stage cancers, though preventive medicine has become more important. Conventional methods, including hematological, biochemical, and urinary examinations as well as X-rays, are not sensitive enough to detect cancers at an early stage. The development of better techniques for detecting cancer is desired. Comparative studies of Th1 and Th2 subsets have revealed that INF-gamma (INF- γ) and tumor necrosis factor-alpha (TNF- α) produced by Th1 cells and interleukin-12 (IL-12) by antigen-presenting cells (APC) through cross-talk between these cells is a key factor for the differentiation of Th cells into Th1 cells which are

responsible for cancer immunity (Brunda, Luistro *et al.*, 1993; Cassatella, Meda *et al.*, 1995; Chan, Kobayashi *et al.*, 1995). Both cytokines seem to modulate and optimize the immune responses, including anti-tumor responses, originally initiated in an antigen-specific or non-specific manner (Zou, Yamamoto *et al.*, 1995).

In order to evaluate the influence of Th1-related cytokines on disease progression, the levels of IL-12, INF- γ and TNF- α , Th subsets, and Natural Killer (NK) cells were examined in patients with primary adenocarcinomas of the stomach and the colorectal and in normal healthy subjects.

Patients and Methods

Three-hundred and thirty-seven Japanese patients diagnosed between November 1, 1998 and May 31, 2000 with histologically confirmed primary adenocarcinomas of the stomach and the colorectal were included in the present study. Their ages ranged from 14 to 85 years (mean: 57 years), and 58.8% were men. Their characteristics are shown in Tables I and II. Cancer patients were classified by means of TNM classification [International Union Against Cancer (UICC) (1997)], into the following groups: a T1-group, whose tumors were limited to invasion of the mucosa or the submucosa; early-stage groups; a T2-T4 group, whose tumors invaded proper muscle (T2, T3, and T4); and an advanced-stage group (T-factor). One hundred seventy-two individuals, aged 27-80, in whom no abnormal findings were revealed by blood chemistry or upper- and lower-gastrointestinal examinations, were enrolled as normal healthy controls. Informed consent was obtained from all individuals.

Heparinized peripheral blood cells were diluted with phosphate buffered saline without calcium or magnesium (PBS (-)). Centrifuged on Ficoll-Conray at x 400 G for 20 min, mononuclear cells were then collected. After being washed, mononuclear cells were resuspended in RPMI-1640 (IBL, Co., Fujioka, Gunma, Japan) with 10% FCS (Cancera, Rexdale, Ont.) at a concentration of 1×10^6 cells/ml of lymphocytes. Lymphocytes (2×10^5 /200 μ l) and 20 μ g/ml of phytohemagglutinin P (PHA-p; Difco, Laboratories, Detroit, MI) were added to 96 holes micro titer plates, to yield 220 μ l in final volume.

After incubation at 37° C for 24 hours with 5% CO₂, supernatants were collected for cytokine assay. IL-12 was measured by an enzyme linked immuno solvent

assay (ELISA) kit (R&D Systems, Minneapolis, MN). IFN- γ and TNF- α were measured with ELISA kits (IFN- γ : Biosource, Nivelles, Belgium; TNF- α : JIMRO, Gunma, Japan). All cytokines were measured according to the manufacturers' instructions.

For identification of Th cell subsets, heparinized peripheral blood cells were stimulated with 25 ng/ml of Phorbol 12-Myristate 13 acetate (PMA; Sigma Chemical Co., St. Louis, MO), 1 μ g/ml of Ionomycin (Sigma Chemical Co., St. Louis, MO) and 10 μ g/ml of Brefeldin-A (Sigma Chemical Co., St. Louis, MO) at 37° C with 5% CO₂ for 4 hours. 300 μ l of stimulated blood was added to 20 μ l of peridinin chlorophyll protein (perceпт)-conjugated anti-CD4 antibody (Immunotech, Marseille, France) and incubated at room temperature for 15 min. 4 μ l of x 10 Lysing Solution (BDIS, San Jose, CA) was added and this solution was incubated for fixing cell surface epitopes, lysing red blood cells and pretreatment for permialization. Then 1.5 μ l of permializing solution (BDIS, San Jose, CA) was added and the solution was incubated at room temperature for 10 min. For staining of intracellular cytokines, 20 μ l of fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ antibody (BDIS, San Jose, CA) and phycoerythrin-conjugated anti-IL-4 antibody (BDIS, San Jose, CA) were added and this solution was incubated for 30 minutes at room temperature. FITC-conjugated IgG2a/PE-conjugated IgG1 antibodies (BDIS, San Jose, CA) were used as negative controls. The FACS analysis was performed with a FACScan (BDIS, San Jose, CA).

For the assay of NK cell activity, target cells (K-562) 3×10^6 were incubated with 100 μ Ci of ⁵¹Cr-sodium chromate (Diichi Radioisotope, Tokyo, Japan) at 37° C for 1 hour. After being washed, $0.1 \times 10^5/10 \mu$ l of the target cells were mixed with $1 \times 10^6/200 \mu$ l of the effector cells. After incubation at 37° C for 3.5 hours, the supernatant was collected and its radioactivity measured. NK cell activity was calculated by means of the following formula.

$$\text{NK cell activity (\% lysis)} = (\text{test cpm} - \text{spontaneous cpm}) / (\text{total cpm} - \text{spontaneous cpm})$$

The analyses were performed with a commercially available statistical package (SPSS Ver 10.0). We used Mann-Whitney U and Kruskal-Wallis tests for the comparison. A result was considered significant when the p value was less than 0.05.

Results

Among normal healthy controls, levels of IL-12, INF- γ , TNF- α , and Th1 were significantly higher than those in cancer patients, while levels of Th2 and NK cell activity in normal healthy controls were lower than those in cancer patients (Table III).

Levels of IL-12, INF- γ , TNF- α , and Th1 in normal healthy controls were significantly higher than those in patients with early-stage gastric cancer, while NK cell activity in normal healthy controls was significantly lower than that in cancer patients. Levels of IL-12, INF- γ , and Th1, and NK cell activity in normal healthy controls were also significantly higher and lower, respectively, than those in patients with early-stage colon and rectal cancer. In the advanced-stage gastric and colorectal cancers, similar results were obtained. Levels of NK cell activity in patients with colon cancers differed significantly between those with early-stage and those with late-stage cancers. Between gastric and colorectal cancers there was no significant difference even when early-stage and advanced patients were compared (Table IV).

Discussion

IL-12, INF- γ and TNF- α are known to induce proliferation and differentiation of Th1 cells (Cassatell, Meda, *et al.*, 1995). These Th1-related cytokines are shown to negatively modulate the growth of tumors. In animal models, Th1 cells have been shown to be activated shortly after the implantation of tumor cells and to exert anti-tumor effects, but these activities were suppressed and overwhelmed by expanding Th2 cells as tumor cells grew (Zou, Shimizu *et al.*, 1992).

Our results demonstrated that levels of mitogen-induced Th1-related cytokines (IL-12, INF- γ , TNF- α) and those of Th1 subsets were significantly lower in cancer patients than those in the normal healthy control group, while that of the Th2 subset and NK cell activity was higher in cancer patients compared to those in the control group. Among early- and advanced-stage cancer patients, there was no difference between these levels, except levels of NK cell activity in those with colon cancer.

Due to previous studies of Th1 and Th2 subsets (Gately, Desai *et al.*, 1991; Mosmann, Cherwinski *et al.*, 1986), the mechanism of the positive interaction between IL-12 and INF- γ for Th1 induction has been defined (Chen, Kobayashi *et al.*, 1997; Sen,

TABLE I
Characteristics in cancer patients and normal healthy controls

organ/control	mean age*	age range	male(%)	female(%)	smoker(%)	total case	test**
Stomach	54±13	19-83	52.9	47.1	49.7	155	N.S.
Colon	59±11	27-85	55.2	44.8	42.2	116	N.S.
Rectum	56±12	14-77	78.8	21.2	42.4	66	N.S.
Cancer total	57±12	14-85	52.0	48.0	45.2	337	N.S.
Healthy control	54±11	27-80	58.8	41.2	50.0	172	

(*mean age: mean±S.D., **test: mean age of cancer patients v.s. healthy control)

TABLE II
Characteristics in patients with the adenocarcinoma of the stomach, the colon and the rectum

cancer	stage*	mean age**	age range	male(%)	female(%)	smoker(%)	total case	test***
Stomach cancer	early advanced	55±13 53±14	28-83 19-82	46.2 56.3	53.8 43.7	48.1 50.5	52 103	N.S. N.S.
Colon cancer	early advanced	57±10 60±12	34-73 27-85	65.7 50.6	34.3 49.4	51.4 38.3	35 81	N.S. N.S.
Rectal cancer	early advanced	60±11 55±12	39-77 14-76	73.3 80.4	26.7 19.6	53.3 39.2	15 51	N.S. N.S.

(*stage: early-staged (T1), advanced-stage(T2-T4), **mean age: mean±S.D., ***test: mean age of cancer patients v.s. healthy control)

TABLE III
Immunological parameters in patients with cancer patients and in healthy controls.

parameters	cancer patients			test*	healthy control		
	max	min	95% int.		max	min	95% int.
IL-12 (pg/ml)	122	7.8	10.4-12.4	A	157	7.8	25.6-33.1
IFN-gamma (IU/ml)	94	0.1	10.5-13.5	A	152	1.3	28.8-35.6
TNF-alpha (pg/ml)	5660	155	1488-1701	A	5960	200	2102-2493
Th1 (%)	64.3	6.2	21.1-23.2	A	54.4	5.2	25.9-28.4
Th2 (%)	7.4	0.2	2.73-3.04	B	5.4	0.7	2.36-2.66
NK cell activity (%)	96	3	40.1-44.3	A	57	10	33.1-35.9

(95% int.: 95% confidence intervals, test*: cancer patients v.s. healthy control, A: p<0.01-0.001, B: p<0.001, Mann-Whitney U test)

TABLE IV
Immunological parameters in cancer patients.

cancer	parameters	stage	max	min	95% int.	median	test1	test2
stomach cancer	IL-12	early	31.8	7.8	9.5-12.7	7.8	C	
	(pg/ml)	advanced	122	7.8	9.5-14.6	7.8	C	NS
	IFN-gamma	early	43.9	0.1	8.5-14.5	8.45	C	
	(IU/ml)	advanced	94	0.1	10.2-17.2	8.8	C	NS
	TNF-alpha	early	3340	210	1155-1594	1280	C	
	(pg/ml)	advanced	5660	150	1437-1810	1520	C	NS
	Th1	early	43.1	9.5	19.9-24.6	19.45	B	
	(%)	advanced	51.4	8.2	21.4-25.6	21.4	B	NS
colon cancer	Th2	early	6.4	0.2	2.37-3.22	2.35	NS	
	(%)	advanced	7.4	0.2	2.71-3.37	2.8	B	NS
	NK cell activity	early	82	12	37.8-46.0	40	C	
	(%)	advanced	82	3	39.9-46.3	43	C	NS
colon cancer	IL-12	early	41.1	7.8	9.6-15.0	7.8	C	
	(pg/ml)	advanced	47.9	7.8	9.8-13.4	7.8	C	NS
	IFN-gamma	early	60.2	0.4	7.5-15.5	10	C	
	(IU/ml)	advanced	78.7	0.2	9.8-15.7	10.3	C	NS
	TNF-alpha	early	4340	240	1578-2346	1750	NS	
	(pg/ml)	advanced	4290	240	1314-1766	1220	C	NS
	Th1	early	30.2	7.1	15.9-20.3	19.1	C	
	(%)	advanced	49.2	6.2	19.8-23.9	20.3	C	NS
colon cancer	Th2	early	5.3	0.9	2.20-3.02	2.2	NS	
	(%)	advanced	5.7	0.4	2.58-3.11	2.8	NS	NS
	NK cell activity	early	76	5	40.2-51.8	45	C	
	(%)	advanced	70	7	36.0-42.8	39	B	A
rectal cancer	IL-12	early	29.2	7.8	6.6-14.2	7.8	B	
	(pg/ml)	advanced	32.4	7.8	8.4-11.5	7.8	C	NS
	IFN-gamma	early	32.1	0.6	4.4-15.3	6.3	C	
	(IU/ml)	advanced	32.1	0.4	6.6-11.2	6.3	C	NS
	TNF-alpha	early	3950	280	1012-2298	1370	NS	
	(pg/ml)	advanced	4290	280	1284-1871	1310	C	NS
	Th1	early	64.3	6.9	13.6-28.9	17.2	A	
	(%)	advanced	64.3	6.9	19.7-26.0	19	A	NS
rectal cancer	Th2	early	7.1	1.2	1.97-3.82	2.4	NS	
	(%)	advanced	7.1	1.1	2.49-3.28	2.7	NS	NS
	NK cell activity	early	69	21	37.0-54.2	44	B	
	(%)	advanced	69	5	39.4-48.9	46	C	NS

(95% int.; 95% confidence intervals, test 1; vs. healthy control, test 2; early staged vs. advance-staged, A: p<0.05-0.01, B: p<0.01-0.001, C: p<0.001, Kruskal-Wallis test)

and Lengyel 1992; Trinchieri, 1993; and Tripp, Kanagawa *et al.*, 1995). APCs produce IL-12, which stimulates Th1 cells (Gately, Wolitzky *et al.*, 1992; Locksley, 1993; and Romagnani 1994) to proliferate and differentiate into effector cells, including cytotoxic T cells (Gately, Desai *et al.*, 1991), and delayed-type hypersensitivity-committed T cells (Trinchieri 1994). In the presence of IL-12, Th1 cells produce IFN- γ , with which the Th1 cell itself proliferates and in turn, IFN- γ augments IL-12 production by APCs. On the other hand, Th2 cells, which are mainly involved in allergic reactions and Ig production, are dependent on APC-derived IL-1, instead of IL-12, and they produce IL-4 with which Th2 cells proliferate. Th1 cells are negatively regulated by IL-10 (D'Andrea, Aste-Amezaga *et al.*, 1993; and Young, Gartner *et al.*, 1999) and IL-4, which are produced by Th2 cells by the mechanism of blocking macrophage-derived IL-12 production. Thus, Th1 and Th2 negatively regulate each other and are mutually inhibitory.

After implantation of tumor cells in a mouse model, Th1 cells were activated to show anti-tumor responses in the presence of Th1-related cytokines. Later in the process of tumor progression, the Th1 system was down-regulated and overwhelmed by the Th2 system, which allowed tumor cells to grow (Corry, Reiner *et al.*, 1994; Kishikawa, Song *et al.*, 1997; and Nastala, Edington *et al.*, 1994). Therefore, IFN- γ and IL-12 (Brunda, Luistro *et al.*, 1993; Mu, Zou *et al.*, 1995; and Zou, Shimizu *et al.*, 1992) also seem to participate in T cell-mediated anti-tumor immunity. In this study we demonstrated that levels of Th1-related cytokines were lower in cancer patients than those in the control group, even in the early-stage patients, which appeared compatible with the above-mentioned findings in mice. These cytokines were already down-regulated at an early stage, there being no difference between early-stage and advanced-stage patients.

IL-12 was originally found to be a cytokine that stimulates NK cells (Kobayashi, Fitz *et al.*, 1989; Liebermann, Siegal *et al.*, 1991; and Yago, Tukuda *et al.*, 1998), and NK cells were found to be required for IL-12 induced anti-tumor responses in mice (Pham-Nguyen, Yang *et al.*, 1999). In the present study, NK cell function was not impaired in cancers. The result of these studies and our data suggest that even in early-stage cancer, the Th1 system is already impaired. However, this is quite different from the results of animal model reports, in which the Th1 system was activated shortly after the implantation of tumors (Siders, Wright *et al.*, 1998).

In this study, we employed *in vitro* mitogenic stimulation to evaluate cytokine production and Th subset induction. In this investigation we were clearly determining the function not only of tumor-specific T cell clone(s) but also of other T cells. Therefore, the fact that we detected abnormal Th1 functions in patients with cancer suggests that detected abnormalities were not limited to the tumor-specific Th1 subset and its precursor, but also spread to other T cell repertoires that would be involved in other specific cancers.

The Th1-related immune system may have been impaired in patients whose tumor cells were small in size and this may have affected the immune system, being down-regulated by a Th1-related function. Therefore, we conclude that Th1-related immune parameters might be useful immunological markers for detecting groups at high risk for cancer among subjects undergoing medical examinations.

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