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SIGNIFICANCE OF INCREASED NEUTROPHILS IN PATIENTS WITH ADVANCED COLORECTAL CANCER

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We examined the ratio of neutrophils to lymphocytes (N/L ratio) in the peripheral blood in patients with colorectal cancer. The ability to produce active oxygen and phagocytosis of neutrophils, G-CSF, sIL-2R and IAP (immunosuppressive acidic protein) were also measured. The N/L ratios were significantly higher in the advanced stages of cancer than in normal controls. The ability to produce active oxygen in the terminal stage was 33% lower than in the control group. The G-CSF levels had no relationship with the neutrophil counts. IAP levels increased with cancer stage, and were inversely related to the ability to produce active oxygen. The IAP levels correlated well with the sIL-2R levels and the N/L ratio. These findings suggest that the ability to produce active oxygen, N/L ratio and IAP reflect anticancer mechanisms and that they may be useful when considering treatment or prognosis of patients with advanced stages of cancer.

An increase of leukocytes, mainly neutrophils, not related to infections is noted in patients with advanced cancer. It has been pointed out previously that CSF (colony stimulating factor) is responsible for the increase of neutrophils, but details of its mechanism remain to be elucidated. So far there have been few reports on the possible functions of the increased neutrophils. One theory says that they suppress the function of lymphocytes, thereby causing a decline of the immunological resistance (1).

To study the cause and the signficance of increased neutrophils, we examined the changes of white blood cell (WBC) count and WBC fractions in patients with colorectal cancer, and the relationship between neutrophils and lymphocytes. Moreover, we also measured the ability to produce active oxygen and phagocytosis of the neutrophils, human granulocyte colony stimulating factor (G-CSF), serum soluble interleukin 2 receptor (sIL-2R) levels and serum immunosuppressive acidic protein (IAP).

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Material and Methods

One hundred and seventy-seven patients with colorectal cancer, comprising 102 males and 75 females ages 46–76 years (average 63) without signs of infection or inflammation were included in the study. Forty healthy volunteers served as controls (Table 1).

We examined WBC, neutrophil and lymphocyte counts and the ratio of neutrophils to lymphocytes (N/L ratio) in all the cancer patients and controls. The ability to produce active oxygen and phagocytosis of neutrophils were also measured in 90 of our 177 patients and in 20 of the controls. IAP, G-CSF and sIL-2R were determined in 50 of our 177 patients and in 20 of the controls.

For clinical staging the rules of the Japanese Research Society for staging of cancer of the colon and rectum were used (2).

Peripheral venous blood sampling was performed with the consent of the patients on the first day of hospitalization in our hosptal. Heparin was added to the samples

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Table 1

Characteristics of patients

	Colorectal cancer (stage)						Normal controls	p-value
	I	II	IIIa	IIIb	IV	terminal		
No. of patients	15	29	29	27	38	39	40	
			(177)					
Age (yr)			63 ± 18				59 ± 10	NS
Sex (F : M)			75 : 102				17 : 23	NS

Mean ± SD.

used for determination of the ability to produce active oxygen, and the samples were imediately assayed. The samples for IAP, sIL-2R and G-CSF were allowed to clot. The sera were centrifugated for 10 min at 2 000 rpm and serum samples were stored at −70°C until analysis.

The ability to produce active oxygen and phagocytosis were estimated by the flow cytometry method (FACScan, Becton Dickinson Co. USA) (3). The ability to produce active oxygen: 2,7-dichloro-fluorescein diacetate (Kodak Co.) was added to 200 μl samples of heparinized venous blood, and the samples incubated for 15 min at 37°C. Ethylene-diamine tetraacetic acid (EDTA) and phorbol myristate acetate (PMA, Sigma Chemical Co., USA) were then added, and the samples incubated again for 15 min at 37°C. The samples were then washed with phosphate buffer solution (PBS, pH 7.4), and centrifugated for 10 min at 1 500 rpm. Lysing reagent (Ortho. Co) was then added to the sediment, and the separated neutrophils washed with PBS. After centrifugation measurement was carried out by FACScan. The proportion of neutrophils with the ability to produce active oxygen was calculated, using 5 000 neutrophils. Phagocytosis: After addition of fluorescent monodisperse carboxylated microspheres (excitation 488 nm, ϕ 1μ, Polyscience Inc., Warrington P.A.), the 200 μl samples of heparinized venous blood were incubated for 15 min at 37°C. After adding lysing reagent, they were measured by FACScan.

G-CSF was detected at the pM level by a simple sequential sandwich enzyme immunoassay (4). The G-CSF levels of normal healthy persons were too low to be detected by this assay (< 30 pg/ml).

Serum IAP is known as a non-specific tumor marker observed in patients with various cancers (5). It is produced in the liver, mainly by macrophages and granulocytes, and shows various immuno-suppressive reactions (6, 7). Serum IAP levels were measured by turbidimetry immunoassay (TIA), using human anti-IAP serum purchased from Sanko Pharm. Co., Japan. An IAP value < 500 μg/ml was considered normal (negative) (8). sIL-2R was measured by enzyme-linked immunosorbent assay using Cellfree interleukin 2 receptor kits (Yamanouchi Pharm. Co., Tokyo, Japan), according to the manufacturer's instructions (9). Briefly, serum samples were reacted with a monoclonal antibody that recognized one epitope of human soluble IL-2R. After 2 hours of incubation, the samples were washed and horseradish peroxidase-conjugated monoclonal antibody directed to a second epitope was added. This bound to the IL-2R captured by the first monoclonal antibody. The color reaction was terminated by the addition of NH_2SO_4 and absorbance was measured at 490 nm.

Data are summarized as means ± SD. The data were analyzed by Dunnett's post-hoc procedure for multiple comparisons (10). For correlation, simple regression was used. A p-value <0.05 was considered statistically significant for all results in this study.

Results

The WBC, lymphocyte and neutrophil counts were compared between the different stages of colorectal cancer and the control group. WBC and neutrophils increased with cancer stage. Conversely, lymphocytes decreased with stage (Table 2). The N/L ratio increased sharply from stage IIIb, and was highest in the terminal stage. The ratios in stage IIIb, stage IV and the terminal stage were statistically different from those in the controls (1.6 ± 0.47 (SD) vs. 3.4 ± 0.84; 1.6 ± 0.47 vs. 4.6 ± 1.2; 1.6 ± 0.47 vs. 6.6 ± 2.3, $p < 0.01$).

The ability of the neutrophils to produce active oxygen began to decrease from stage IIIa, and the mean was 34% lower in the terminal stage than in the control group (59 ± 17 (SD) vs. 92 ± 1%, $p < 0.05$). There was a significant difference between stage IV and the terminal stage (78 ± 11 (SD) vs. 59 ± 17%, $p < 0.05$) (Table 3). Phagocytosis in the terminal stage was 62% compared to 93% in the controls.

The G-CSF levels were not proportional to the number of neutrophils. In most cases (45/50), G-CSF they were below the sensitivity of the assay (< 30 pg/ml). There was no correlation between IAP and G-CSF.

IAP had a tendency to increase with the advancement of the cancer, and was inversely proportional to the ability to produce active oxygen (Fig. 1). There was also a correla-

Table 2

WBC count, neutrophils, lymphocytes and N/L ratio in normal subjects and in patients with colorectal cancer

	Normal control	Colorectal cancer (stage)					
		I	II	IIIa	IIIb	IV	terminal
No. of patients	40	15	29	29	27	38	39
WBC (10^9/l)	55.5 ± 9.8	63.2 ± 13.2	61.0 ± 11.0	72.5 ± 12.4*	74.7 ± 8.7*	86.4 ± 10.4**	84.1 ± 8.5**
Neutrophils (10^9/l)	27.3 ± 5.3	32.1 ± 8.1	37.7 ± 9.0*	42.5 ± 9.5*	48.7 ± 7.2*	61.3 ± 10.1**	64.1 ± 7.2**
Lymphocytes (10^9/l)	18.5 ± 6.4	19.2 ± 4.2	15.6 ± 4.0	18.5 ± 6.4	13.9 ± 3.9*	13.6 ± 2.8**	10.4 ± 2.6**
N/L ratio	1.6 ± 0.8	1.6 ± 0.4	2.5 ± 0.9	2.5 ± 0.9	3.4 ± 0.8*	4.7 ± 1.2**	6.6 ± 2.3**

Mean ± SD. N/L : lymphocyte/neutrophils *$p < 0.05$ **$p < 0.01$ compared with control

Table 3

The ability to produce active oxygen and phagocytosis of neutrophils

	Normal controls	Colorectal cancer (stage)					
		I	II	IIIa	IIIb	IV	terminal
No. of patients	20	5	20	8	12	20	25
The ability to produce active oxygen (%)	92 ± 1	88 ± 3	82 ± 9	78 ± 11	75 ± 12	78 ± 10	59 ± 17**
phagocytosis (%)	93 ± 2	83 ± 6	79 ± 10	74 ± 5	75 ± 16	76 ± 12	61 ± 24*

Mean ± SD. *$p < 0.05$ **$p < 0.01$ compared with control

tion between IAP and N/L ratio ($r = 0.705$, $p < 0.001$) (Fig. 2).

sIL-2R levels were almost unchanged until stage IIIb, but in stage IV, they rose sharply to 1 234 ± 340 U/ml (mean ± SD) (Table 4). The values for the controls were statistically different from those for stage IV and the terminal stage (481 ± 36 vs. 1 234 ± 198 U/ml, 481 ± 36 vs. 886 ± 113 U/ml, $p < 0.05$). Moreover, the sIL-2R levels correlated with the IAP (Fig. 3). There was a correlation between sIL-2R and N/L ratio ($r = 0.61$, $p < 0.001$) (Fig. 4).

Discussion

Neutrophils play an important role in cytoprotection. Recently, the association between neutrophils and various diseases was elucidated, and it has been reported that the cytoprotection by neutrophils may damage the host in a pathologic environment (11). In a tumor-bearing host, neutrophils specifically damage cancer cells or non-specifically damage cancer cells through an antibody. On the

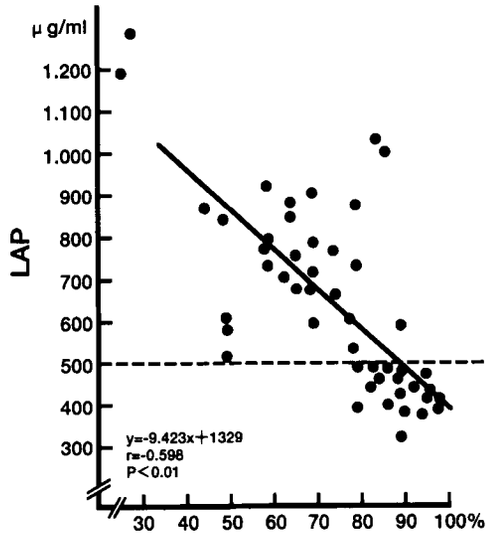

Fig. 1. Radiation between IAP and the ability of the neutrophils to produce active oxygen ($r = -0.59$, $p < 0.01$)

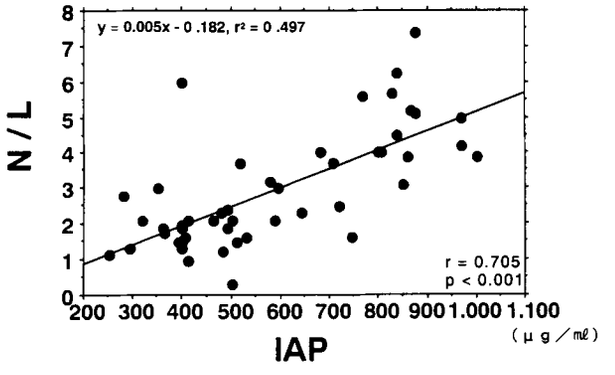

Fig. 2. Correlation between IAP and N/L ratio ($r = 0.705$, $p < 0.001$)

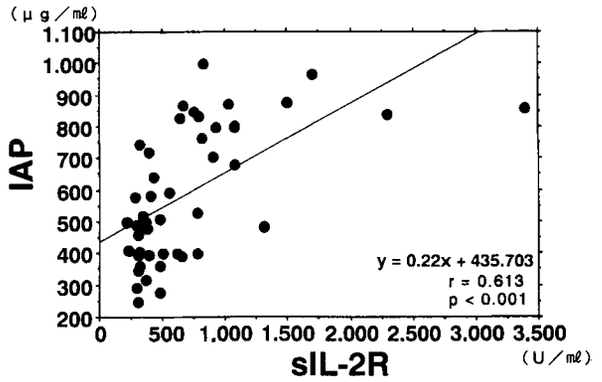

Fig. 3. Correlation between IAP and sIL-2R ($r = 0.613$, $p < 0.001$)

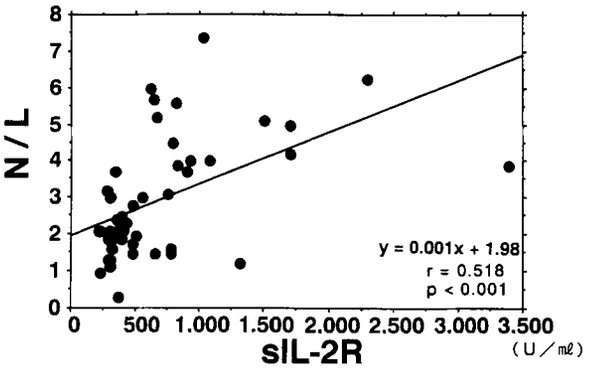

Fig. 4. Correlation between sIL-2R and N/L ratio ($r = 0.518$, $p < 0.001$)

Table 4

SIL-2R and IAP in normal subjects and in patients with colorectal cancer

	Normal controls	Colorectal cancer (stage)					
		I	II	IIIa	IIIb	IV	terminal
No. of patients	20	4	10	8	11	12	5
sIL-2R (U/ml)	481 ± 36	461 ± 17	476 ± 35	505 ± 21	512 ± 29	1234 ± 198*	886 ± 113*
IAP (μg/ml)	403 ± 49	404 ± 80	476 ± 38	548 ± 110	621 ± 112	802 ± 98*	762 ± 76*

Mean ± SD. *$p < 0.05$ compared with control

other hand, it has been reported from experimental studies that neutrophils may promote transformation, growth, progress and metastasis of cancer, and have a suppressive effect on antitumor immunology (12).

Thus far, however, the roles of neutrophils in the tumor-bearing host are still unclear. Neutrophils increase and lymphocytes decrease with the advancement of cancer (13), changes that may mirror the status of a living body (14). These findings are in agreement with our study, in which the N/L ratio correlated well with cancer stage. Owada et al. (15) reported that the helper T cells decreased whereas the suppressor T cells increased in cancer patients. Moreover, it has been said that IAP acts to increase suppressor T cells, and that lymphoblastogenesis decreases with increase of IAP. The N/L ratio correlated well with IAP in our study. It seems that the functions of neutrophils and lymphocytes are affected by IAP due to an inverted relation between the ability to produce active oxygen of neutrophils and IAP.

Not only change in the number of neutrophils in patients with cancer, but there are morphological changes such as toxic granules and Doehles inclusion-bodies (16). The appearance of toxic granules seem to demonstrate that neutrophils are stimulated by cytokines, etc., and that the functions of those neutrophils deteriorate. In our study the ability of the neutrophils to produce active oxygen began to deteriorate from stage IIIa and it was 40% lower in the terminal stage than in the control group. Phagocytosis also tended to fall with stage but not as sharply as the neutrophils. Korec et al. (17) reported that a decrease of cytostatic activity of neutrophils was seen in 84% of patients with colon, breast, and lung cancer. Measurement of the ability to produce active oxygen by use of the flow cytometry method gives the proportion of neutrophils that has active oxygen producing function. Therefore, the decrease of the ability to produce active oxygen with the advancement of cancer implies that most of the increased neutrophils have no function. We interpreted the decrease of the function of neutrophils as a result of an increase of immature neutrophils. It has been stated that numerous neutrophils accumulate in the cancer region by cytokines, such as tumor necrosis factor (TNF) and interferon (IFN), and that they have antitumor activity (18). As a cause for the decreased ability to produce active oxygen, one may speculate whether the increase of immature neutrophils results from over-production of neutrophils produced by an increase of cyto-protection with the advancement of cancer, increasing the number of immature neutrophils. These conditions may cause decreased ability to produce active oxygen, and promote a compensatory increase of the number of neutrophils.

G-CSF has been identified as a factor that increases the number and activity of neutrophils (19). It has been reported that G-CSF levels increase in proportion to number

of neutrophils in the terminal stage of cancer (20). However, in our study, there was no correlation between the G-CSF levels and increase of neutrophils. The normal G-CSF level is considered to be about 10 μg/ml, and it is possible that there is slight variation with the sensitivity of the assay. However, the ability of neutrophils to produce active oxygen decreased rather than increased.

In our study the sIL-2R levels increased sharply in stage IV patients. The histopathological differences between stage III and stage IV concern both depth of invasion and lymph node metastasis. Murakami et al. (9) reported that sIL-2R was high in patients with lymph node metastasis. Moreover, it has been reported that IAP suppress helper T cells and that sIL-2R correlates well with IAP and N/L ratio. These findings suggest that IL-2R may suppress the functions of T cells and neutrophils. Rubin et al. (21) have also speculated that IL-2R protects the progress of immunoreaction via reduction of activated T cell receptors.

In cancer bearing hosts, where the activities of neutrophils and lymphocytes decrease with the advancement of cancer stage, there may be a strong antigen stimulation and an overproduction of activated lymphocytes and antibody. These activated lymphocytes may begin to injure the host and IAP or IL-2R increase as protection against the excess immunoreaction, and suppressor T cells may increase.

Recently, mass producing of neutrophils has become possible by the use of G-CSF. Cancer treatment that anticipates anticancer action of neutrophils is under investigation (18). However, Welch et al. (22) have reported a study which indicates that an increase of neutrophils may contribute to metastasis of cancer. Some studies report that a decrease of neutrophils may indicate a better prognosis for stage IV and terminal stage patients (23). There might be a possibility that an increase of non-functional neutrophils cannot suppress invasion and lymph node metastasis of cancer.

In conclusion, our study showed that the ability of neutrophils to produce active oxygen and the N/L ratio were related to the stage in patients with colorectal cancer. Decrease of cytostatic activity of neutrophils was correlated with IAP and sIL-2R levels. We think that N/L ratio, IAP and sIL-2R could be important factors when considering treatment and prognosis in the advanced stages of colorectal cancer.

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