Eur J Cancer Clin Oncol, Vol. 21, No. 2, pp. 181–184, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

Superoxide Anion Generation by Polymorphonuclear Leukocytes Enhanced in a Patient with Colony-stimulating Activity-producing Lung Cancer

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Abstract—*We examined superoxide (O_2^-)-generating activity of polymorphonuclear leukocytes (PMN) from a patient with lung cancer in whom there was a marked granulocytosis. There was a high level of colony-stimulating activity (CSA) in both the serum and the supernatant of the culture of the primary tumor. The PMN from the patient produced almost three times more O_2^- than did the PMN from healthy donors and other patients with lung cancers but with no granulocytosis. The binding of wheat germ agglutinin (WGA), used as a stimulus for O_2^- generation, to the surface membrane of cells and the protein content in the cells were about double that noted in the case of healthy donors. Following total extirpation of the tumor, the level of CSA in the serum decreased, as did activity of PMN for O_2^- generation, and the binding of WGA to the cells reverted to normal levels.*

INTRODUCTION

COLONY-STIMULATING activity (CSA) is a carbohydrate-containing polypeptide produced from various cells in humans [1]. CSA works as a hematopoietic regulator and has stimulatory activity against various kinds of cells, including bone marrow cells, macrophages, neutrophils and eosinophils [1–3]. A high activity of CSA has been detected in the serum and urine of patients with nonhematological malignant tumors [4]. In such patients there is a marked granulocytosis in the peripheral blood and no evidence of bacterial infections or diseases related to a granulocytosis. Tumors in those patients produce CSA, which is responsible for granulocytosis, and CSA-producing tumor cell lines have been established [5–7]. *In vitro* effects of CSA on the function of phagocytic cells have been extensively investigated. CSA enhances phagocytic, cytocidal [2] and antibody-dependent cell-mediated cytotoxic activities of macrophages against tumor cells [3]. In addition, CSA increases RNA synthesis [8] and production of prostaglandin E by phagocytic cells [9].

We now report that PMN from a patient with a CSA-producing tumor generated an increased amount of O_2^-, and that the cells had an elevated number of binding sites on the surface membrane for wheat germ agglutinin (WGA) and increased protein content compared with cells from healthy donors.

MATERIALS AND METHODS

Case

A 48-yr-old Japanese man was admitted to Kyushu Cancer Center on 6 January 1983 because of a tumor shadow on X-ray films of the chest. Examination of peripheral blood revealed a marked granulocytosis with 75% segmented, 13% band forms, 10% lymphocytes and 2% monocytes. Bone marrow aspiration showed marked myeloid hyperplasia with no evidence of maturation arrest or infiltration of the tumor cells. Curative operation with right upper lobectomy was

Accepted 25 July 1984.
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performed and the pathological examinations of the tumor revealed a type of giant cell carcinoma (pT2N0M0: stage I). On the second postoperative day the white cell count dropped to approximately half of the preoperative value (from 25,800/μl to 11,800/μl). On the 30th postoperative day bone marrow aspiration showed normocellular marrow and the peripheral blood white cell count was 7800/μl, with normal differentiation.

CSA assay

Tests for CSA activity were done using serum (before and on day 10 after operation) and the supernatant of the primary tumor culture. The sera from healthy donors and human placental conditioned medium (HPCM) were used as a negative and positive control for human colony formation assay respectively. The sera were dialyzed against distilled water for 72 hr before use. The culture supernatant of the primary tumor was prepared as follows: the lung tumor excised from the patient was minced into small fragments with scissors and then trypsinized to obtain single cells. A confluent monolayer of the cells in α-minimum essential medium (α-MEM, Flow Laboratories) and 20% fetal bovine serum (FBS) were cultured for 5 days and the supernatant was collected for CSA assay. The slight modification of the method of Worton *et al.* [10] was used for CSA assay. Briefly, 2×10^5 human nonadherent bone marrow cells in 1 ml of the medium containing α-MEM, 20% FBS and 0.88% methycellulose (4000 cps, Dow Chemical, U.S.A.) were placed in a 35-mm plastic dish and then cultured at 37°C in a humidified atmosphere with 5% CO_2. On day 14 colonies containing more than 20 cells were counted under an inverted microscope. For morphological analysis more than 50 colonies in individual batches were harvested with microhematocrit tubes, smeared on slides and stained with 0.6% orcein in 60% acetic acid or Giemsa.

Preparation of peripheral PMN

Heparinized peripheral blood was obtained from the patient, healthy donors and other patients with lung cancers but without granulocytosis. PMN were isolated from the whole blood by dextran sedimentation, following by Ficoll-Hypaque density gradient centrifugation. Contaminating erythrocytes were lysed by 0.83% NH_4Cl. The cells thus prepared were more than 95% viable, as detected by trypan blue exclusion, and contamination of the cells other than PMN was less than 2%, as determined by Giemsa staining.

Determination of released O_2^-

O_2^- determination was performed as described previously [11]. Briefly, 1 ml of reaction mixture containing 100 μM ferricytochrome C (Sigma Chemical Co., St. Louis, MO) and 1×10^6 cells in HEPES buffer (Wako Chemical Co., Osaka, Japan) were preincubated for 10 min at 37°C in a shaking plastic cuvette of a spectrophotometer. Cytochalasin-E (Cyt-E, 5 μg/ml; Sigma Chemical Co., St. Louis, MO) and WGA (40 μg/ml; P-L Biochemicals, Inc., Milwaukee, WI) were then added to the reaction mixture and the rate of superoxide dismutase-inhibitable reduction of ferricytochrome C was measured continuously by recording the absorption increase at 550–540 nm (molar absorption coefficient, 19×10^3) with a Hitachi 556 double-beam spectrophotometer. To prevent sedimentation of the cells a cell mixer was attached to the cuvette, as described by Kakinuma *et al.* [12].

Measurement of the binding of ^{125}I-labeled WGA to PMN

WGA was radioiodinated with ^{125}I by the chloramine-T method. A total of 1×10^6 PMN suspended in 1 ml of HEPES buffer were incubated with Cyt-E and ^{125}I-labeled WGA (10 μg, 298,000 cpm/ml) for 30 min at 4°C. Specificity of [^{125}I]-WGA binding to the cells was ascertained by showing that the [^{125}I]-WGA binding was blocked more than 90% by an addition of *N*-acetylglucosamine. After washing the cells twice with HEPES buffer the cell-bound radioactivity was counted in a Packard gamma scintillation counter. Protein content in 10^6 PMN was measured by the method of Lowry *et al.* [13].

RESULTS

Assay of CSA activity

CSA activity in the patient's serum and the supernatant of the primary culture of the tumor obtained from the patient was assayed. As shown in Table 1, the CSA activity in the serum obtained before operation was markedly increased compared with that in sera of healthy controls. The morphological types of the cells in the colony, as induced by CSA in the patient's serum, were predominantly PMN. The CSA activity in the serum obtained on day 10 after complete removal of the tumor by operation was far less compared with that in the serum before operation, though still higher than that in sera from healthy controls. The CSA activity was also detected in the supernatant of the primary tumor culture and it was almost as high as that in HPCM, which was used as the positive control. The morphological types of the cells in the colony were 69% PMN and

Table 1. CSA in the patient's serum and the supernatant of the primary tumor culture

Source of CSA		Colonies per 2×10^5 cells*	Morphological type of colonies (%) PMN	Macrophage	Mixed
Serum (10%):†	healthy controls ($n = 2$)	4.0 ± 2.8‡			
	patient:				
	before operation	102.0 ± 19.4	87	13	0
	after operation§	33.0 ± 1.4	15	85	0
Human placental conditioned medium (10%)		298.0 ± 19.8	66	32	2
Supernatant of the primary tumor culture (20%)		284.0 ± 53.7	69	29	2

*Human non-adherent bone marrow cells.
†Percentage concentration of the test sample.
‡Mean ± S.D. of the data obtained by assay using two individual bone marrow cells.
§Serum on day 10 after operation.

Table 2. O_2^- generation of peripheral polymorphonuclear leukocytes

Cell source of PMN	O_2^- generation of PMN (nmol/min/10^6 PMN) Before operation	Day 10 after operation
Healthy controls*	3.1 ± 1.6†	4.4 ± 1.5
Other patients with lung cancer but without granulocytosis‡	3.5 ± 1.8	3.9 ± 1.6
Patient	10.3 ± 0.3§	3.1 ± 0.2

*PMN of healthy controls were obtained from three men whose ages roughly matched that of the patient.
†Mean ± S.D.
‡PMN were obtained from four patient who were in stages I and III of lung cancer.
§$P < 0.05$ as compared to healthy controls and other patients with lung cancer, calculated from Student's t test.

29% macrophages, indicating that the nature of the CSA was of the granulocyte-macrophage type.

O_2^--generating activities of peripheral PMN

The O_2^--generating activities of peripheral PMN from healthy controls, patients with lung cancers without granulocytosis or the patient with CSA-producing lung tumor were assayed (Table 2). The PMN from the patient produced three times more O_2^- than did the cells obtained from healthy controls or patients with lung cancers without granulocytosis. This enhancement of O_2^- generation by PMN from the patient disappeared on day 10 after removal of the tumor.

Binding of [^{125}I]-WGA to PMN and protein content in the cells

To investigate the possibility that the enhanced O_2^- generation of the PMN from the patient might be related to an increased number of binding sites for WGA used as a trigger for O_2^- generation, the binding of [^{125}I]-WGA to the surface membrane of the PMN from the patient or healthy controls was tested (Table 3). We found that cells from the patient bound about double the [^{125}I]-WGA than those from healthy donors. On the tenth postoperative day this enhancement of the binding was no longer observed, as was the case with O_2^- generation.

The protein content in the PMN from the patient was almost double that of cells from healthy donors (Table 3).

DISCUSSION

In a patient surgically treated for a malignant lung tumor we found that (1) CSA in the serum of the patient was markedly elevated; (2) it decreased on day 10 after removal of the tumor; (3) the supernatant of the primary tumor culture contained a high level of CSA; (4) on the second postoperative day the white cell count in the peripheral blood decreased to about half the value seen preoperatively. Those results strongly suggest that the granulocytosis was caused by CSA produced by the tumor.

Vadas *et al.* [3, 14] reported that O_2^- generation of eosinophils was enhanced by exposure of the cells to CSA. Therefore we tested the O_2^--generating activity of the patient's peripheral PMN and found that the cells produced three times more O_2^- than did control cells collected from healthy donors or patients with lung cancers without granulocytosis. We also found that protein content in the cells and the binding of WGA to the cells were increased almost double compared to control values. However, the cells were normal in size, as determined electron micrographically (data not shown). Those results imply that protein content in the cells and the

Table 3. Binding of ^{125}I-labeled WGA to PMN and protein content in cells.

Cell source of PMN	Before operation: Binding of [^{125}I]-WGA (cpm bound to 10^6 PMN)	Before operation: Protein content (μg protein/10^6 PMN)	10th day after operation: Binding of [^{125}I]-WGA	10th day after operation: Protein content
Healthy controls*	19508 ± 4800†	182 ± 49	17331 ± 5800	216 ± 62
Patient	35700 ± 1640‡	338 ± 35§	19830 ± 1320	235 ± 21

*The same controls as Table 1.
†Mean ± S.D.
‡$P < 0.02$ as compared to healthy controls, calculated from Student's t test.
§$P < 0.05$ as compared to healthy controls, calculated from Student's t test.

binding sites for WGA per square of the cell from the patient were richer than those of the control cells. The observation by Burgess and Metcalf [1] that CSA enhances synthesis of protein in phagocytic cells lends support to our idea. The precise mechanism for the enhanced O_2^- generation of the PMN from the patient is unclear; however, there may be a relationship between the enhanced O_2^- generation and the increase of protein content and/or the binding sites for WGA per cell as all values returned to normal levels after excision of the tumor.

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