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SUPEROXIDE ANION-MEDIATED TUMORICIDAL FUNCTION OF NEUTROPHILS IN THE FLUID OF A METASTASIZED SUBMANDIBULAR LYMPH NODE : A CASE OF GINGIVAL SQUAMOUS CELL CARCINOMA

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The purpose of this investigation was to demonstrate host immune responses occuring in the fluid of a metastasized submandibular lymph node as a result of tumor cell cytotoxicity induced by superoxide anion (O_2^-) generated from neutrophils, and also, whether bleomycin (BLM) ointment injection to the lymph node augments such tumoricidal activity. The fluid of a metastatic submandibular lymph node was used as material. The patient was a 60-year-old female with gingival squamous cell carcinoma of the lower jaw. Neutrophil O_2^- generation was measured in terms of cytochrome C reduction. O_2^- generation levels of neutrophils in the fluid of a metastasized lymph node of the patient were higher as compared to those of peripheral blood neutrophils. Neutrophil O_2^- generation levels were slightly elevated one week after BLM ointment injection into the metastasized lymph node as compared to those seen in the period of pretreatment. In the fluid specimens from this lymph node, we observed a rosette formation comprised of a large central tumor cell surrounded by 4 to 12 neutrophils. In experimental study, in vitro cytotoxicity of neutrophils against scc-25 was demonstrated, as was rosette formation of scc-25 cells and neutrophils. These results suggest that tumor cell cytotoxicity occurs in the fluid of a metastasized lymph node as one of the host defence mechanisms, and further that cytotoxic activity may be enhanced by BLM ointment injection.

Key words : Superoxide anion-mediated tumoricidal function / Neutrophils / Metastasized lymph node / Gingival squamous cell carcinoma

It has been reported that O_2^- generation from neutrophils is closely associated with not only phagocytosis but also the ability to kill tumor cells (1-7). In addition, O_2^- plays an important role in the process of cytotoxicity against tumor cells, collaborating with proinflammatory cytokines including tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-8 (IL-8), interferon-γ (IFN-γ), and granulocyte colony stimulating factor (G- CSF) (6-8). We had an opportunity to treat one patient with gingival squamous cell carcinoma (scc) metastasizing to her submandibular lymph node and we obtained neutrophils from the lymph node-derived fluid. In this study, we first measured changes in the O_2^- producing ability of neutrophils in the fluid of the metastasized lymph node during cancer therapies of carboplatine, vincristine, and fluorouracil, and then, subsequently, using bleomycin (BLM) ointment. We also perfomed histopathological examinations on smeared specimens from the fluid of the metastasized lymph node and we experimentally investigated cytotoxicity of neutrophils against scc-25 derived from human well-differentiated squamous cell carcinoma of the tongue.

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MATERIALS AND METHODS

MATERIALS

The fluid of a metastatic submandibular lymph node obtained from one gingival scc patient was used as material. The patient was a 60-year-old female with a primary intraoral tumor of 25×18×10 mm in size, showing diffuse swellings to the bilateral submandibular region. Our axial CT scanning revealed fluid-filled lymph-node-like shadows as a mass 40mm in diameter in the left submandibular region and 30mm in diameter in the right submandibular region (Fig.1). The patient was diagnosed as a well-differentiated scc in stage III (T2N2CM0). Initially, two courses of intravenous chemotherapy using carboplatine (450 mg), vincristine (1.2mg) and fluorouracil (1000mg) were administered as pretreatment. BLM ointment of 4.8mg was subsequently injected into the right metastasized lymph node after the fluid was withdrawn. This lymph node was filled with a serous light brown liquid containing a large number of tumor cells and leukocytes. After completion of the above therapies, the patient was given 62 Gy-irradiation to the primary lesion including the bilateral metastasized submandibular lymph nodes.

MEASUREMENT OF O_2^- GENERATION

Neutrophil O_2^- generation was measured in terms of cytochrome C reduction, according to the method of Korchak and Weissmann (11). Neutrophils were separated from the lymph node fluid by the sedimentation method using Mono-Poly Resolving Medium (Flow Laboratories, ICN Biomedicals, Irvine, Scotland). Neutrophils collected by centrifugation at 300g for 30 minutes at room temperature were suspended in Hank's balanced salt solution (Nissui Pharmaceutical Co, Tokyo,Japan) until adjusted to pH 7.40 using phosphate buffer. Duplicate reaction mixtures (2ml) consisting of 2×10^6 of neutrophils were preincubated with cytochalasin B (5.0μg/ml in 0.1% dimethyl sulfoxide)(Sigma Chemical Co, St. Louis, USA), and

75 μM of horse heart ferricytochrome C (type III, Sigma Chemical Co.) in a circulating water bath for 10 min at 37℃. Reference cuvettes also contained 10 μg of superoxide dismutase (Sigma). 4 μl each of either N-formyl-methionyl-leucyl-phenylalanin (fMLP) (50 μM;Sigma) or phorbol 12-myristate 13-acetate (PMA) (400ng/ml;Sigma) (without cytochalasin B) were added at zero time. During incubation, cytochrome C reduction was monitored continuously in terms of increase in an optical density at 550nm in a spectrophotometer (Hitachi U-3210, Tokyo, Japan). The amount of generated O_2^- was expressed as nmoles of cytochrome C reduced per min calculated by the formula, Coefficient=21.1×10^4 $M^{-1}cm^{-1}$.

CYTOLOGICAL EXAMINATION OF THE METASTASIZED LYMPH NODE FLUID

Smear samples of the fluid from the metastasized lymph node were stained with peroxidase antiperoxidase complex (PAP) and Giemsa solution and subjected to microscopic examination.

CYTOTOXICITY OF NEUTROPHILS AGAINST SCC-25

Different concentrations of neutrophils which were isolated from a healthy volunteer were added with tumor cells to microtiter wells to give effector : target cell ratios 400 :1, 200:1, 100:1 and 50:1. Cytotoxicity was assayed 3~4 hours later by trypan blue exclusion test. SCC-25 used as target cells were derived from human well-differentiated squamous cell carcinoma of the tongue.

RESULTS

As shown in Table 1, O_2^- generation levels of neutrophils in the fluid of the metastasized lymph node of the patient with gingival scc, in response to triggering with fMLP and /or PMA, were higher as compared to those of peripheral blood neutrophils. Specifically, O_2^- generation values in intra-lymph node neutrophils were 4.36 and 5.07 when stimulated with fMLP and PMA, respectively, whereas they were 2.83 and 4.33, respectively, in the peripheral blood neutrophils. The neutrophil O_2^- generation levels were slightly elevated one week after BLM ointment injection into the metastasized lymph node as compared to those seen in the period of pretreatment with carboplatine, vincristine and fluorouracil. Finally, O_2^- generation levels decreased below 1.0 nmol/min/10^6 cells after completion of irradiation therapy (Fig.2). Application of superoxide dismutase completely suppressed O_2^- generation of neutrophils. In the fluid specimens from the metastasized lymph node, we observed a rosette formation comprised of a large central tumor cell surrounded by 4 to 12 neutrophils. Fig.3 shows the representative rosette consisting of a tumor cell and a large number of neutrophils as found in the lymph node fluid. Rosette formation was noted in 92 of 162 (56.8%) observed tumor cells in six high-power fields (×200). No microorganisms were detected in the fluid specimen. In experimental study, cytotoxicity of neutrophils against scc-25 is shown in Table 2. The percent of cytotoxicity was 17.0±2.1, 10.5± 1.9, 2.0±1.6, and 2.0±1.1 in effector : target ratios of 400:1(A), 200:1(B), 100:1(C), and 50:1(D), respectively. There were significant differences between A and B, B and C, A and D, and B and D. We also noted rosette formation in 60 of 105 (57.1%) observed scc-25 cells in microtiter wells when the effector : target ratio was B.

Table 1. Superoxide anion generation from neutrophils in the fluid of metastasized lymph node and in the peripheral blood at pretreatment period

	O_2^- generation (nmol/min/10^6cells)		
Stimulant	Intra-lymph node(L)	Peripheral blood (P)	L/P
fMLP	4.36	2.83	1.54
PMA	5.07	4.33	1.17

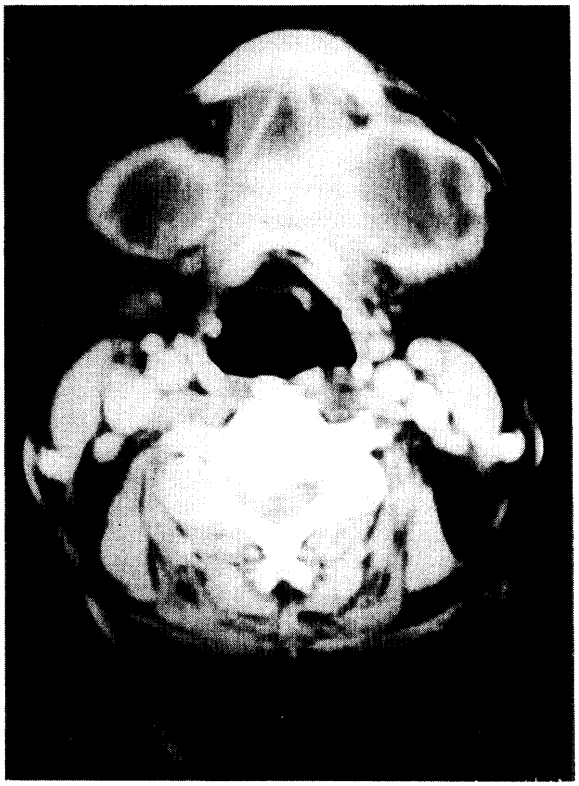

Fig. 1. Axial CT scanning showing lymph-node-like shadow filled with fluid at bilateral submandibular regions.

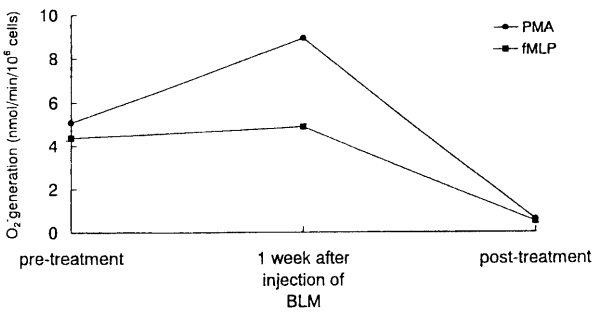

Fig. 2. O_2^- generation levels from neutrophils in the fluid of metastasized lymph node before and after injection of BLM ointment.

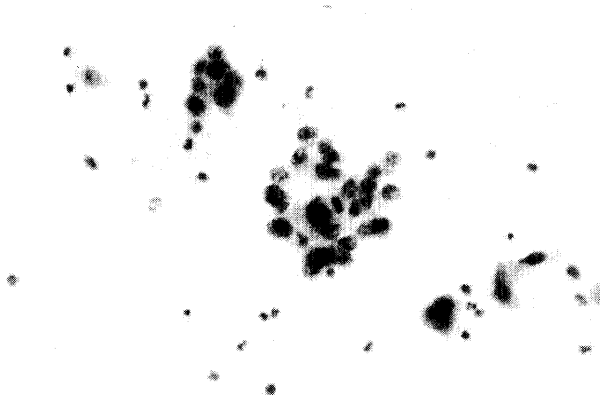

Fig. 3. Clear rosettes are shown. Tumor cells are surrounded by four to twelve neutrophils(×400,Giemsa).

Table 2. Cytotoxicity of neutrophils against SCC-25

No.	Effector : target ratio	Percent cytotoxicity (%) a)
A	400:1	17.0±2.1
B	200:1	10.5±1.9
C	100:1	2.0±1.6
D	50:1	2.0±1.1

a) The mean± S. D. (n=4)
There were significant differences between A and B, B and C, A and D, and B and D : ($p<0.01$)

DISCUSSION

There have been many reports of evidence for O_2^--mediated and neutrophil-mediated cytotoxicity against cancer cells (1-7). Hafeman and Lucas (1) revealed that O_2^- production is essential for tumor cell cytotoxicity. Garrard *et al.* (4) also showed neutrophils were cytotoxic for tumor cells. However, most of those studies were conducted *in vitro*, and there is no report as far as we know in which measurement was made of the O_2^- generation by neutrophils in metastasized cancer tissues. This is the first report on this matter. The present investigation indicates that the O_2^- generation level of fMLP- or PMA- stimulated neutrophils in the fluid of the metastasized lymph node was significantly higher than that observed for peripheral blood neutrophils during the pretreatment period. This suggests the possibility that potent tumor cell cytotoxicity mediated by O_2^- generated from neutrophils may occur in the metastasized lymph node of tumor tissue. Not only cytokines, including TNF, IL-1, IL-8, IFN-γ, and G-CSF, but also O_2^- are reported to play important roles in the expression of the tumor cell cytotoxicity in the host immune system (8-10). In such types of tumor cell elimination, cytotoxic T, NK, LAK cells, and phagocytes such as macrophages and neutrophils play crucial roles as effector cells in the killing of tumor cells (4,8,9,11). Yamauchi *et al.* (9) showed that recombinant human TNF (rhTNF) induces increased hydroxyl radical production and that this plays an important role in the mechanism of tumor cell killing by rhTNF. In the present investigation, we demonstrated the participation of neutrophils in the cytotoxic reactions seen in the tumor tissue. Additionally, cytotoxicity of neutrophils against scc-25 was observed in vitro. These results support the theory that neutrophils play important roles as effector cells in tumor cell cytotoxicity.

Both in histological examination and in experimental study, rosette formations comprising tumor cells and neutrophils were observed in approximately half of the tumor cells in the fluid smears prepared from the metastasized lymph node during the pretreatment period as well as in the microtiter wells. Relative to this, Abelson and Stossel (12) previously reported neutrophil/ tumor-cell rosettes in ascitic fluid from a patient with lymphoma. Also, Gale and Zighelboim (13) demonstrated that polymorphonuclear leukocytes were capable of mediating immunologically specific cytolysis of antibody-dependent cellular cytotoxicity in such type of rosettes. Therefore, the rosette formations observed in the present investigation may be regarded as morphological evidence of neutrophil-mediated cytotoxicity against cancer cells.

O_2^- plays an important role in the group of quinone carcinostatics, such as doxorubicin hydrochloride (14-16). In the present case, measurement of the O_2^-generation levels of neutrophils one week after BLM ointment injection to the metastasized lymph node revealed elevated levels of O_2^- production as compared to those seen during the pretreatment period. These results suggest that tumor cell cytotoxicity occurs in the fluid of a metastasized lymph node and that cytotoxic activity may be enhanced by BLM ointment injection. Sangeetha *et al.* (14) reported these anticancer drugs augment free radical generation and lipid peroxidation in vitro. Furthermore, Kokura *et al.* (15) indicated that the production of oxygen-derived free radicals in the cancer cell plays an important role in the antitumor effect of adriamycin, and Berlin *et al.* (16) revealed that DNA breakage in cancer cells created by derived adriamycin-free radicals is mediated by molecular hydroxyl-free radicals. In view of these investigations, our findings of increased cytotoxicity due to neutrophil-mediated O_2^- generation may be useful for development of new anticancer drugs and protocols for cancer therapy.

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