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In Vitro Antitumor Activity of Carboxypeptidase G_3 from *Pseudomonas* sp. M-27

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Carboxypeptidase G_3 from *Pseudomonas* sp. M-27 hydrolyzed predominately *N*-acyl glutamic acid. We found that carboxypeptidase G_3, capable of liberating the carboxyl-terminal glutamate from folic acid analogue, showed *in vitro* antitumor activities against human carcinoma KB and PC-3 cells among ten kinds of human carcinoma cell lines. It can be expected to be useful as an anticancer agent against limited cell lines.

Introduction

In 1948, Ferber *et al.*[1] demonstrated the induction of brief remissions in children with acute leukemia using the folate analogue aminopterin. Since then, methotrexate has been widely used as a folic acid antagonist in the chemotherapy of cancer, notably leukemias and choriocarcinoma. Its therapeutic action is based on inhibition of dihydrofolate reductase. However, resistance to methotrexate has been associated with increased levels of the target enzyme, dihydrofolate reductase, and with impaired cellular uptake of the drug. In a search for new antifolate chemotherapeutic agents, several enzymes which hydrolyze the peptide linkage of the C-terminal glutamate residue of folic acid, carboxypeptidase G,[2] G_1[3] and G_2[4] have been found. The action of folic acid-degrading enzyme led to investigation of its usefulness as an antineoplastic agent, and its antitumor activity was demonstrated against several murine tumors *in vitro*[5] and *in vivo*.[6]

Previously during purification of polymyxin acylase from *Pseudomonas* sp. M-27 strain which produces it, we found a new enzyme, carboxypeptidase G_3, that hydrolyzed only *N*-fatty acyl glutamic acid or aspartic acid, but not polymyxins.[7] The enzyme resembles carboxypeptidase G_1 or G_2 in its glutamate-releasing activity. However, it acts not only on the L-from but also on the D-form of acidic amino acids and shows affinity for the long-chain fatty acyl group but not the benzoyl group. In this paper, we describe the *in vitro* antitumor activity of carboxypeptidase G_3 against murine and human tumor cells and its substrate specificity for folic acid and its analogues.

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Materials and Methods

Preparation of carboxypeptidase G_3

Carboxypeptidase G_3 was purified to homogeneity from an acetone-dried cell powder of *Pseudomonas* sp. M-27 by the procedure described in our preceding paper.[7] This enzyme had a single peak with a molecular weight of 60,000 on a calibrated Superdex column and consisted of four subunits of identical molecular weights (Mr:15,000). Its isoelectric point was estimated to be 7.2. The enzyme hydrolyzed predominately acidic peptides and *N*-acyl amino acids with glutamic acid or aspartic acid in the C-terminus. The observed V_{max} and K_m for *N*-octanoyl-DL-glutamic acid were 33μmol/min/mg and 3.5mM, respectively. This

enzyme was not strongly affected by thiol enzyme inhibitors (*p*-chloromercuribenzoate, iodoacetic acid) or serine protease inhibitors (diisopropylphosphoro fluoridate, phenylmethane sulfonylfluoride), but was inhibited by metal chelators.

Enzyme assay Carboxypeptidase or acyl amino acid acylase activity was assayed from the ninhydrin color spot of liberated amino acid on the TLC plate as follows: the reaction mixture (100μl) containing 1μmol of folic acid and its analogue, 1mM $ZnCl_2$ and the enzyme solution (25μl) in 20mM Tris buffer (pH8.0) was incubated at 37℃ for several hours. An 1-μl portion of the reaction mixture was developed by TLC on a silica gel plate [solvent system : upper layer of *n*-BuOH-AcOH-H_2O (4:1:3) containing 1/20 pyridine by volume]. The amount of amino acid liberated was measured by the ninhydrin method with Chromoscan 200/201 (Joyce-Loebl, England). During enzyme purification, this assay method using *N*-octanoyl-DL-glutamic acid was used. The amount of liberated amino acid in the mixture was measured colorimetrically by the ninhydrin method.

In *vitro* antitumor activity of carboxypeptidase G_3

The terazolium-based semiautomated colorimetric assay (MTT assay) developed by Carmichael *et al.*[8] was modified and used for the *in vitro* assay. Briefly, 2000 cells in 180μl of RPMI-1640 medium (Nissui Pharmaceutical Co., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Lab., Grand Island, NY, USA), penicillin G (100 units/ml) and streptomycin (100μg/ml) were seeded in a 96-well flat bottom microtest plate (InterMed, Roskilde, Denmark) and 20μl each of drug solutions of graded concentrations was simultaneously added to each well triplicate samples. The plate was incubated for 3 d at 37℃ in a humidified atmosphere of 5%CO_2. MTT reagent [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (sigma Chemical Co., St Louis, MO. USA) was prepared at a concentration of 2mg/ml in Dulbecco's phosphate buffered saline without calcium and magnesium and strored in a refrigerator. On the third day, MTT reagent (25μl) was added to each well. After another 4-h incubation at 37℃, the microplate was centrifuged at 1500 rpm for 10 min and the medium was removed by aspiration. To solubilize the resulting MTT-formazan, 0.2ml of dimethylsulfoxide was added to each well followed thorough mixing with a mechanical plate mixer. Absorbance at 540 nm (OD_{540}) was measured with an ImmunoReader NJ-2000 (InterMed Japan, Tokyo, Japan). The percentage of cell growth inhibition was calculated by the following formula : % of cell growth inhibition = $(1-T/C) \times 100$, where C is the mean OD_{540} of the control group and T is that of the treated group. IC_{50} value was derermined graphically from the dose-response curve with at least 3 drug concentration points.

Results and Discussion

Table 1 shows the activity of carboxypeptidase G_3 for folic acid and its analogues. Folic acid was exclusively hydrolyzed by this enzyme, but for methotrexate the glutamate-releasing activity was very weak as compared with those of carboxypeptidase G_1 (725μmol/min/mg) and G_2 (215μmol/min/mg). To investigate the antitumor activity of carboxypeptidase G_3, murine leukemia cells and a variety of human neoplastic cells were used. As shown in Table 2, appreciable antitumor effects were observed for human carcinoma KB and PC-3 cells specifically, but the other cells were very slightly affected.

Earlier, in a search for new antifolate chemotherapeutic agents, McCullough and Chabner[3,9] isolated and purified an enzyme from *Pseudomonas stutzeri*, carboxypeptidase G_1, which hydrolyzed the peptide linkage of the C-terminal glutamate residue of folic acid analogue. They observed that carboxypeptidase G_1 inhibited the growth of L5178Y and L1210 murine leukemia and indicated that the antineoplastic activity was a consequence of folic acid depletion.[5,6] Chabner *et al.*[10] reported that carboxypeptidase G_1 has no selective inhibitory effect on thymidylate synthesis, as does methotrexate, but probably depresses DNA synthesis through its effects on purine synthesis or amino acid metabolism. Valerino *et al.*[11] reported

Table 1. Substrate Specificity of Carboxypeptidase G_3 for Folic Acid and Its Analogues.

Substrate	Activity (μmol/min/mg)
Folic acid	1.4
Dihydrofolic acid	0.6
Methotrexate	0.4
Aminopterin	0.2
Leucovorin	0.02
5-Methyltetrahydrofolic acid	0.04

The enzymatic reaction conditions are described in the text.

Table 2. Inhibitory Effects of Carboxypeptidase G_3 on the Growth of Various Murine and Human Tumor Cell Lines *in vitro*.

Cell line	Origin	IC_{50}(μg/ml)
L5178Y	Murine leukemia	>25
KB	Human epidermoid carcinoma	15
NPC-TY861	Human epipharynx squamous carcinoma	>25
PC-3	Human lung adenocarcinoma	7.4
PC-8	Human lung adenocarcinoma	>25
PC-13	Human lung large-cell carcinoma	>25
QG-56	Human lung squamous cell carcinoma	>25
KATO-III	Human stomach adenocarcinoma	>25
ST-KM	Human stomach adenocarcinoma	>25
NUGC-4	Human stomach adenocarcinoma	>25
OST	Human osteosarcoma	>25

that carboxypeptidase G from *Pseudomonas* can be used in rescue therapy following high-dose methotrexate regimes, because of its ability to hydrolyze the glutamate moiety from methotrexate to form 4-amino-N^{10}-methylpteroate, which is a much less active inhibitor of dihydrofolate reductase. Sherwood *et al.*[4] found that carboxypeptidase G_2 from *Peudomonas* sp. RS-16 had similar K_m values to G_1 for folate (4.0μM) and methotrexate (8.0μM), but less affinity for leucovorin (120μM). This may be of significance if the enzyme is used in rescue resimes together with leucovorin in cases where high-dose methotrexate therapy leads to toxicity. Carboxypeptidase G_2 also exhibited antitumor activity in its own right against Walker carcinoma in rats. Carboxypeptidase G_3 has similar properties such as carboxyl-terminal glutamate-releasing activity and requirement of Zn^{2+}, but the optical specificity of the enzyme is markedly different from that of the known enzyme, G, G_1 and G_2, that is, carboxypeptidase C_3 acts not only on the L-form but also on the D-form of glutamic acid. Carboxypeptidase G_3, capable of liberating the carboxyl-terminal glutamate from folic acid analogue, inhibited very slightly the growth of murine leukemia, but showed remarkable antitumor activities against human carcinoma KB and PC-3 cells among ten kinds of human carcinoma cell lines. The mechanism of the antitumor activity of carboxypeptidase G_3 seems to

be based on the degradation of folic acid. Accordingly, carboxypeptidase G_3 can be expected to be useful as an anticancer agent against limited cell lines, which can regulate the level of folic acid *in vivo*, and be also used in rescue therapy following high-dose methotrexate regimes. Thus carboxypeptidase G_3 is promising as an enhancer of antitumor activity when used with other active reagents, and also may be of significance for chemotherapy to target specific human neoplastic cells, such as KB and PC-3.

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