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Inhibitory effect of oryzacystatins and a truncation mutant on the replication of poliovirus in infected Vero cells

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Poliovirus, a picornavirus family member, requires the processing of its poly-protein by its own cysteine proteinase for replication. Oryzacystatin-I and oryzacystatin-II, proteinaceous cysteine proteinase inhibitors (cystatins) of rice seed origin, were found to inhibit the replication of poliovirus effectively in infected Vero cells in vitro. Truncated oryzacystatin-I, which lacks the NH_2-terminal 25 amino acid residues of the intact protein, is an even more effective inhibitor, eliciting its effect at concentrations of less than 0.25 nmol/ml. The low molecular weight cysteine proteinase inhibitors, E-64, E-64C and loxistatin, showed no anti-viral effect at any concentration investigated.

Oryzacystatin; Poliovirus; Rice seed; Cysteine proteinase inhibitor; Cystatin superfamily; Anti-viral effect

1. INTRODUCTION

The replication of members of the picornavirus family, including poliovirus, requires the processing of the viral poly-proteins in the cytoplasm of infected cells [1,2]. The capsid proteins of poliovirus consist of VP1–VP4, along with VPg as a minor component; they are formed by processing the poly-protein at nine glutamine–glycine bonds, two tyrosine–glycine bonds and one asparagine–serine bond [3]. Various proteinases are involved in this processing [4]; in particular, a cysteine proteinase is involved in processing at the glutamine–glycine bonds [5]. If internalized, therefore, cystatins, a family of proteinaceous cysteine proteinase inhibitors, might be excepted to inhibit the replication of picornaviruses in infected cells. In fact, egg white cystatin has been shown to exhibit this type of inhibitory action [6]. Recently, cystatin C, which exists in human blood, tears and bone marrow, has also been reported to have an antiviral effect against herpesvirus [7].

Oryzacystatins, which occur in rice seeds, are the first well-defined cystatins of plant origin [8,9]. Oryzacystatins comprise two known molecular species, oryzacystatin-I (OC-I) [10] and oryzacystatin-II (OC-II) [11], which inhibit different cysteine proteinases, respectively papain and cathepsin H [11]. Recently, we reported that truncated OC-I, which lacks 21 amino acid residues from the NH_2-terminus, still retains its papain-inhibitory activity [12].

In this study, we investigated the anti-polioviral effects of oryzacystatin and its truncation mutant in comparison with egg white cystatin and low molecular weight cysteine proteinase inhibitors. Here we report the results with special emphasis on the effect of truncated OC-I (tOC-I), a molecular species that may have a practical use as an antiviral agent.

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2. MATERIALS AND METHODS

2.1. *Viruses and cells*

Attenuated poliovirus (type 1), vesicular stomatitis virus (VSV, New Jersey strain), and influenza virus (Kumamoto Y567(H2N2) strain), were obtained from stocks at Kumamoto University Medical School. Vero, L929 and a dog kidney cell line (MDCK) were used as host cells. The cells were cultured in Eagle's minimal essential medium (MEM, Gibco Ltd.) containing 10% fetal calf serum, and 100 μg/ml each of penicillin and streptomycin.

2.2. *Preparation of cystatins*

OC-I and OC-II were expressed by incorporating their cDNA clones into *E. coli* [11]. The expression products were purified by sequential chromatographies on DEAE-Sephadex, Mono Q (FPLC system) and ODS columns (HPLC). Truncated OC-I (tOC-I), which lacks 25 amino acid residues from the NH_2-terminal, was prepared similarly [12]. Egg white cystatin was purchased from Takara Shuzo Co., Kyoto, Japan. The low molecular weight cysteine proteinase inhibitors, E-64 [13], E-64-c [14], and loxistatin [15], were obtained from Taisyo Seiyaku Co.

2.3. *Antiviral effect*

Cells were cultured until they formed a monolayer, infected with viruses at MOI 0.1, and then incubated for 30 min at 37°C. The virus suspensions were then removed and the cells were incubated for another 18 h at 37°C in medium containing various concentration of cystatins or inhibitors. Inhibition of the viral cytopathic effect (CPE) by cystatins was then observed with a microscope. The supernatant of each culture medium was subjected to a plaque forming assay. Cells were then treated with a given amount of the supernatant used for the CPE inhibitory effect. After incubation for 30 min at 37°C, the cells

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Table I

Antiviral activity of various proteinaceous inhibitors

Inhibitors	Polio virus (virus yield, PFU/ml)	VSV
Oryzacystatin-I	1.0×10^{4}	2.3×10^{8}
Oryzacystatin-II	1.0×10^{5}	2.0×10^{8}
Truncated oryzacystatin-I	1.2×10^{4}	2.4×10^{8}
Egg white cystatin	1.0×10^{4}	2.0×10^{8}
Soy bean trypsin inhibitor	4.1×10^{7}	3.3×10^{8}
E-64	5.4×10^{7}	3.1×10^{8}
E-64C	3.3×10^{7}	3.0×10^{8}
Loxistatin	3.5×10^{7}	3.0×10^{8}
Pepstatin	3.4×10^{7}	2.9×10^{8}
None (control)	3.5×10^{7}	3.0×10^{8}

The concentration of each inhibitor is 8 nmol/ml in medium

were covered with Eagle's MEM containing 10% FCS and 0.9% agar and incubated for 48 h at 37°C until plaques formed.

3. RESULTS

Table I shows the inhibition of the viral CPE by various cysteine proteinase inhibitors. OC-I and OC-II are apparently potent antiviral agents against poliovirus at a concentration of 8 nmol/ml, similar to the inhibitory effect of egg white cystatin reported previously [6]. The truncated oryzacystatin-I mutant, tOC-I, is also effective. Furthermore, OC-I, OC-II and tOC-I also have antiviral activities against VSV, although the effects are far weaker than observed with poliovirus. The effect of oryzacystatins on influenza virus infection was examined using MDCK cells, but the results were not so remarkable as in the case of poliovirus (data not shown). On the other hand, E-64 had no effect. We also tested the effects of E-64C and loxistatin, which are designed to penetrate the cell membrane more effectively than E-64. E-64C and loxistatin, however, also showed no antiviral effect on any viruses investigated, even at concentrations of 8,000 nmol/ml or more. Fig. 1 illustrates the morphology of virus-infected cells in the presence or absence of cystatins. Cells cultured in the absence of cystatins are damaged by the infecting virus (CPE), whereas those cultured in the presence of a sufficient amount of cystatin remain viable and vivid in appearance.

Table II

Anti-polioviral activities of various cystatins

Cystatins	Concentrations (nmol/ml)	Virus yield (PFU/ml)
Oryzacystatin-I	8	1.0×10^{4}
	4	1.2×10^{6}
	2	1.0×10^{7}
	1	3.0×10^{7}
Oryzacystatin-II	8	1.0×10^{5}
	4	5.0×10^{6}
	2	6.5×10^{6}
	1	2.3×10^{7}
Truncated oryzacystatin-I	2	1.2×10^{5}
	1	9.0×10^{6}
	0.5	1.6×10^{6}
	0.25	2.8×10^{7}
Egg white cystatin	8	1.0×10^{4}
E-64	8	5.4×10^{7}
None (control)	–	3.5×10^{7}

Species, poliovirus type 1

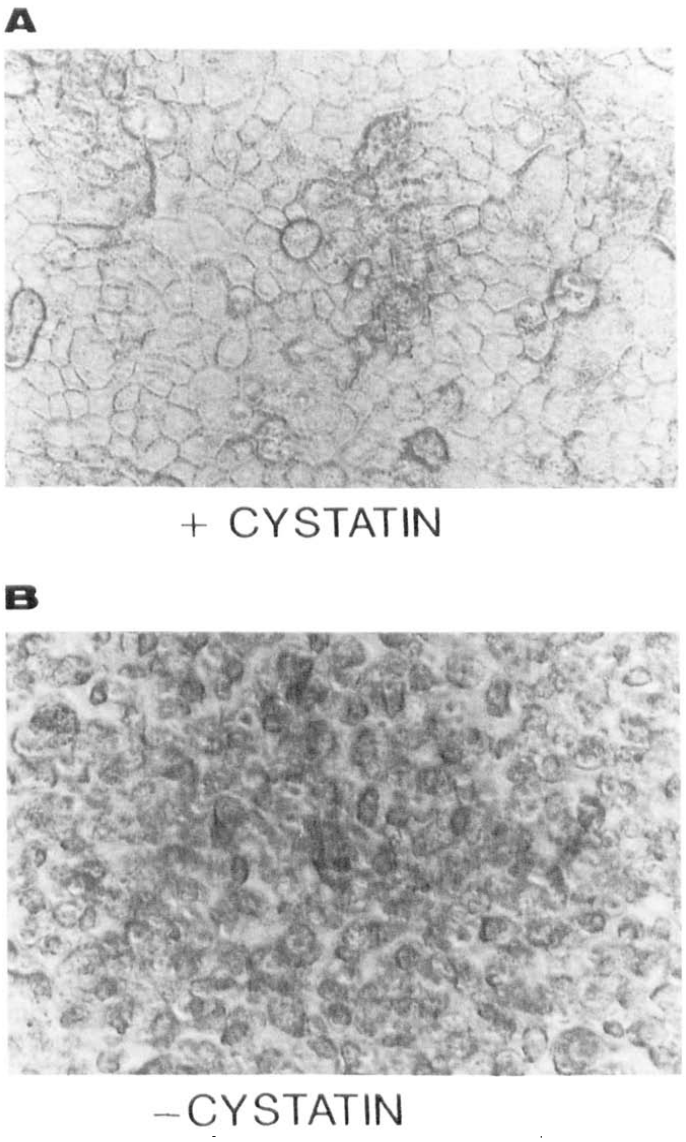

Fig. 1. Cell morphology following infection by poliovirus. (A) Cells infected with poliovirus and cultured in medium containing oryzacystatins at 8 nmol/ml. (B) Infected cells cultured in medium without oryzacystatins. The cell-degenerating cytopathic effect (CPE) is apparent in B. Magnification ×100

However, when cells are cultured first in medium containing cystatin at a concentration of 8 nmol/ml and the cystatin is removed prior to subsequent viral infection, no protection against viral damage is observed (data not shown). Thus, the antiviral effect requires the

presence of cystatins during the later stages of viral infection.

Table II shows the effects of cystatins on growth inhibition by poliovirus based on the plaque forming unit (PFU) assay. When OC-I, OC-II or egg white cystatin is added to the culture medium at a concentration greater then 4 nmol/ml, the values (PFU/ml) are reduced by approximately two orders of magnitude or more compared with the control. The minimum effective concentration of cystatin is at a concentration of about 1.0 nmol/ml. tOC-I is a better inhibitor than native OC-I, since even at a concentration of 0.25 nmol/ml its significant inhibitory activity remains.

4. DISCUSSION

In this report the antiviral activity of oryzacystatins against infectious poliovirus is demonstrated. The mechanism for this effect is assumed to involve the inhibition of the viral cysteine proteinase which take part in processing the poly-protein. Contrary to expectation, the low molecular weight cysteine proteinase inhibitors, E-64, E-64-C and loxistatin, have no effect on viral replication. There are at least two ways to explain this result. First, E-64 and its analogues might be unable to inhibit viral proteinases even though they effectively inhibit typical cysteine proteinases such as papain and cathepsin H. Even though it contains cysteine and histidine residues in its active site, cysteine proteinase of poliovirus might be a prototype of either a serine or cysteine proteinase, in which case it would resemble chymotrypsin rather than papain [16,17]. Compared to E-64, cystatins might have a wider inhibitory spectrum allowing them to inhibit viral cysteine proteinases. The second possibility is that E-64 and its analogues might penetrate the cell membrane only slightly and thus have no chance of interacting with viral cysteine proteinases. This possibility is, however, unlikely, because E-64 and its derivatives are used in the treatment of muscular dystrophy in which intracellular cathepsins are inhibited. Cystatins are thought to be unable to penetrate cell membranes by themselves; but they could be internalized into the cytoplasm of virus-infected cells if changes in the cell membrane structure occur upon viral infection. This type of phenomenon has been observed in T lymphocyte cells infected by HIV virus [18]. Also, many small and large proteins are known to be internalized into cells by endocytosis.

Oryzacystatins are synthesized in rice seeds during the early stages of seed maturation. Considering the meaning for the existence of oryzacystatins in rice seeds, it seems possible that they might play an important role in protecting rice seeds from attack by various plant viruses, all of which are known to be RNA viruses. In vivo experiments are necessary to obtain more detailed information.

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