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Effects of Proteasome Inhibitor (lactacystin) and Cysteine Protease Inhibitor (E-64-d) on Processes of Mitosis in *Xenopus* Embryonic Cells

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ABSTRACT—At least two different protease pathways have been implicated in the degradation that is required to control the eukaryotic cell cycle; these two pathways center on the activities of ubiquitin/proteasome and cysteine protease. The proteasome inhibitors, lactacystin and AcLLnL and the cysteine protease inhibitor E-64-d were tested for their ability to inhibit the cell cycles of *Xenopus* embryos. Lactacystin, AcLLnL and E-64-d all caused the complete arrest of the cell cycle. To define the specific cell cycle processes that were affected by the two inhibitors, we performed a cytological analysis. Inhibition of the cell cycle by lactacystin and E-64-d occurred during prophase and metaphase. The number of cells that arrested in prophase was 1.4-times higher in the E-64-d-treated group than in the control group and the number of arrested cells in the lactacystin-treated group was 1.4-times higher than in the E-64-d-treated group. The number of cells that arrested in metaphase was 3-to-4-times higher in the E-64-d and lactacystin groups than in the control group. These results indicate that both cysteine protease(s) and proteasomes are involved in the prophase and metaphase stages of cell division.

Key words: cysteine protease, E-64-d, proteasome, lactacystin, *Xenopus* embryo

INTRODUCTION

The degradation of cyclins, p53, cyclin-dependent kinase inhibitor and nuclear oncoproteins is required for multiple processes in mitosis and for the onset of DNA replication (Ciechanover, 1994; King *et al*., 1996; Hoyt, 1997). Regulated degradation by specific protease pathways is critical for the control of various steps in the cell cycle. Protein degradation mainly occurs via the ubiquitin-dependent proteolytic pathway (Glotzer *et al*., 1991; King *et al*., 1995; Sudakin *et al*., 1995). On the other hand, benzyloxy- carbonyl-Leu-Leu-Tyr diazomethyl ketone, which is thought to inhibit calpain, reduces the proliferation of human TE2 and C-33A cells and WI-38 fibroblasts in the S-phase (Mellgren *et al*., 1994; Zhang *et al*., 1997; Mellgren, 1997). N-acetyl Leu-Leu-norleucinal (AcLLnL) inhibits the proliferation of fibroblasts, affecting the progress of the cell cycle from the G1 to the S phase (Sherwood *et al*., 1993; Dietrich *et al*., 1996). AcLLnL is not only selective for proteasomes, but it inhibits both cathepsin L and calpain (Roch *et al*., 1994; Tsubuki *et al*.,1996). (L-3-*trans*-Ethoxycarbonyloxirane-2 Carbonyl)- L-leucine (3-methylbutyl) amide (E-64-d), an inhibitor of cathepsin and calpain, has been reported to arrest the proliferation of carcinoma A431 cells during metaphase (Shoji-Kasai *et al*., 1988). We previously reported that E-64-c inhibits the cell cycle of embryonic *Xenopus* cells (Ishii *et al*., 2000). Consequently, the assumption that proteasomes alone control the cell cycle remains to be verified. To analyze the relation of proteasomes and cysteine proteases to the cell cycle, we studied the effects of a more specific proteasome inhibitor, lactacystin, and the cysteine protease inhibitor, E-64-d, on the process of mitosis in *Xenopus* embryonic cells.

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MATERIALS AND METHODS

Embryos

Eggs of *Xenopus laevis* were obtained by injection of a gonadotropic hormone and de-jellied with 2.5% cysteine. The embryos were allowed to develop to stage 8 at room temperature.

Cell culture

Animal cap pieces were dissected from stage 8 blastulae. Single cells from the inner surface of the pieces were separated off by directing a gentle stream of calcium- and magnesium-free medium (50 mM phosphate buffer containing 35 mM NaCl and 1 mM KCl, pH 7.0), as described by Godsave and Slack (1989). Two or three cells were transferred into a well of a Terasaki plate containing 10

μl of 2 mg/ml γ-globulin in a simple salt solution (NAM/2) and cultured in the absence or presence of protease inhibitors for 20 hr at 25°C. Lactacystin and E-64-d were purchased from PEPTIDE INSTITUTE, INC. (Osaka), and AcLLnL was purchased from SIGMA-ALDRICH (Missouri).

Cytological analysis

To observe the chromosomes, the embryo cells were soaked in 0.02% KCl solution for several min and then fixed with a solution of acetic acid – ethanol (1:3) for 5 min. The cells were then placed on a glass slide, air-dried, and stained with 10% Giemsa in PBS for 30 min.

Preparation of enzyme samples

To assay the proteasome activities of the *Xenopus* embryos *in vitro*, aliquots of enzyme samples were prepared using the method reported by Tokumoto and Ishikawa (1993). Five hundred embryos in the blastula stage were homogenized in 5 ml of 25 mM Tris-HCl buffer (pH 7.9) containing 10 mM of 2-mercaptoethanol and 20% glycerol. The homogenate was then centrifuged at 36,000 g for 30 min at 4°C

Enzyme assays

Native gel electrophoresis was performed, as described by Mahaffey *et al*. (1993), on a 4.5% resolving gel and a 2.5% stacking gel. Gels were cast in 90 mM Tris-HCl (pH 8.3), 1.6 mM borate, and 0.08 mM EDTA. After electrophoresis, the gels were soaked for 30 min at room temperature in 10 ml of 30 mM Tris-HCl (pH7.8), 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 2 mM ATP, and 50 μM Suc-Leu-Leu-Val-Tyr-MCA (sLLVY-MCA), with or without a protease inhibitor. The fluorescent gels were transilluminated by a UV light and photographed with a Polaroid camera.

RESULTS

The effects of E-64-d, AcLLnL and Lactacystin on cell division in isolated cells were examined *in vitro*. Single cells from mid-blastula-stage *Xenopus* embryos were shown to divide in a non-nutritive medium. Under the standard conditions of the present study, most cells divided between 4 and 10 times. The occurrence of cleavage arrest increased with increasing concentrations of E-64-d (Table 1). A concentration of 400 μ of E-64-d resulted in the arrest of cleavage in 77% of the treated cells, and a concentration of 800 μM resulted in the arrest of cleavage in 96% of the treated cells. A concentration of 1200 μM of E-64-d completely blocked the cell cycle.

Treatment with 100 μM of AcLLnL resulted in cleavage arrest in 19% of the treated cells; AcLLnL concentration of 200 μM, 400 μM and 800 μM resulted in cleavage arrest in 79%, 91% and 96% of the treated cells, respectively (Table

Table 1. Effects of E-64-d treatment on cells isolated from blastula embryos. Isolated cells from blastulae were cultured in a medium containing E-64-d. After 20 hr, blastomere cleavage was observed under a microscope.

Concentration of E-64-d	No. of non-cleavage cell	No. of cleavage cell
Control	11 (5%)	202 (95%)
200 μM	49 (48%)	53 (52%)
400 μM	72 (77%)	22 (23%)
800 μM	154 (96%)	6 (4%)
1200 μM	80 (100%)	0 (0%)

Table 2. Effects of AcLLnL treatment on cells isolated from blastula embryos. Isolated cells from blastulae were cultured in a medium containing AcLLnL. After 20 hr, blastomere cleavage was observed under a microscope.

Concentration of AcLLnL	No. of non-cleavage cell	No. of cleavage cell
Control	3 (4%)	66 (96%)
100 μM	11 (19%)	46 (81%)
200 μM	55 (79%)	15 (21%)
400 μM	74 (91%)	7 (9%)
800 μM	98 (96%)	4 (4%)

Table 3. Effects of lactacystin treatment on cells isolated from blastula embryos. Isolated cells from blastulae were cultured in a medium containing lactacystin. After 20 hr, blastomere cleavage was observed under a microscope.

Concentration of Lactacystin	No. of non-cleavage cell	No. of cleavage cell
Control	8 (8%)	88 (92%)
10 μM	5 (9%)	61 (91%)
50 μM	46 (53%)	41 (47%)
100 μM	80 (99%)	1 (1%)
200 μM	83 (98%)	2 (2%)

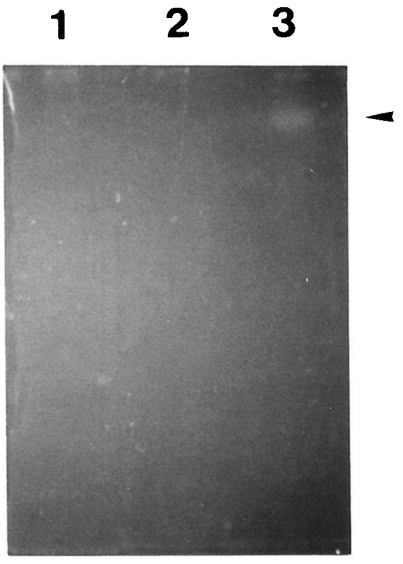

Fig. 1. Effects of lactacystin and AcLLnL on proteasomes in embryonic cells at stage 8. Extract samples of embryos at stage 8 were electrophoresed on a native 4.5% gel and the gels were treated with a 50 μM sLLVY-MCA solution with or without a protease inhibitor. The fluorescent proteolytic product was visualized by illumination with UV light. Lane 1, 400 μM AcLLnL; lane 2, 100 μM lactacystin; lane 3, no protease inhibitor. The arrow indicates the proteasome band, which co-migrates with the same protease from the reticulocyte lysate.

2). Control blastomers were treated with an equivalent volume of solution in which the protease inhibitor was dissolved, which affected 4% of the treated cells by arresting the cell cycle. However, 96% of the treated cells developed normally. The effects of lactacystin on the division of these cells were also studied (Table 3). A concentration of 50 μM of lactacystin resulted in the inhibition of cleavage in 53% of the individual cells, and a concentration of 100 μM of lactacystin inhibited cleavage in 99% of the cells.

We measured the proteasome activity after gel electrophoresis (Fig 1). The fluorescent band of lane 3 showed hydrolytic activity by the proteasome in embryonic cells at stage 8 toward sLLVY-MCA in the presence of ATP. The protease activity was inhibited by lactacystin (Lane 2) and AcLLnL (Lane 1). We have already reported that 400 μg/ml of E-64 inhibited the activity of calcium-activated protease in embryonic cells, but E-64 did not inhibit the proteasome activity, even at the same concentration (Ishii *et al*., 2000).

To analyze the effects of protease inhibitors on specific stages of the cell cycle, we observed the behavior of the

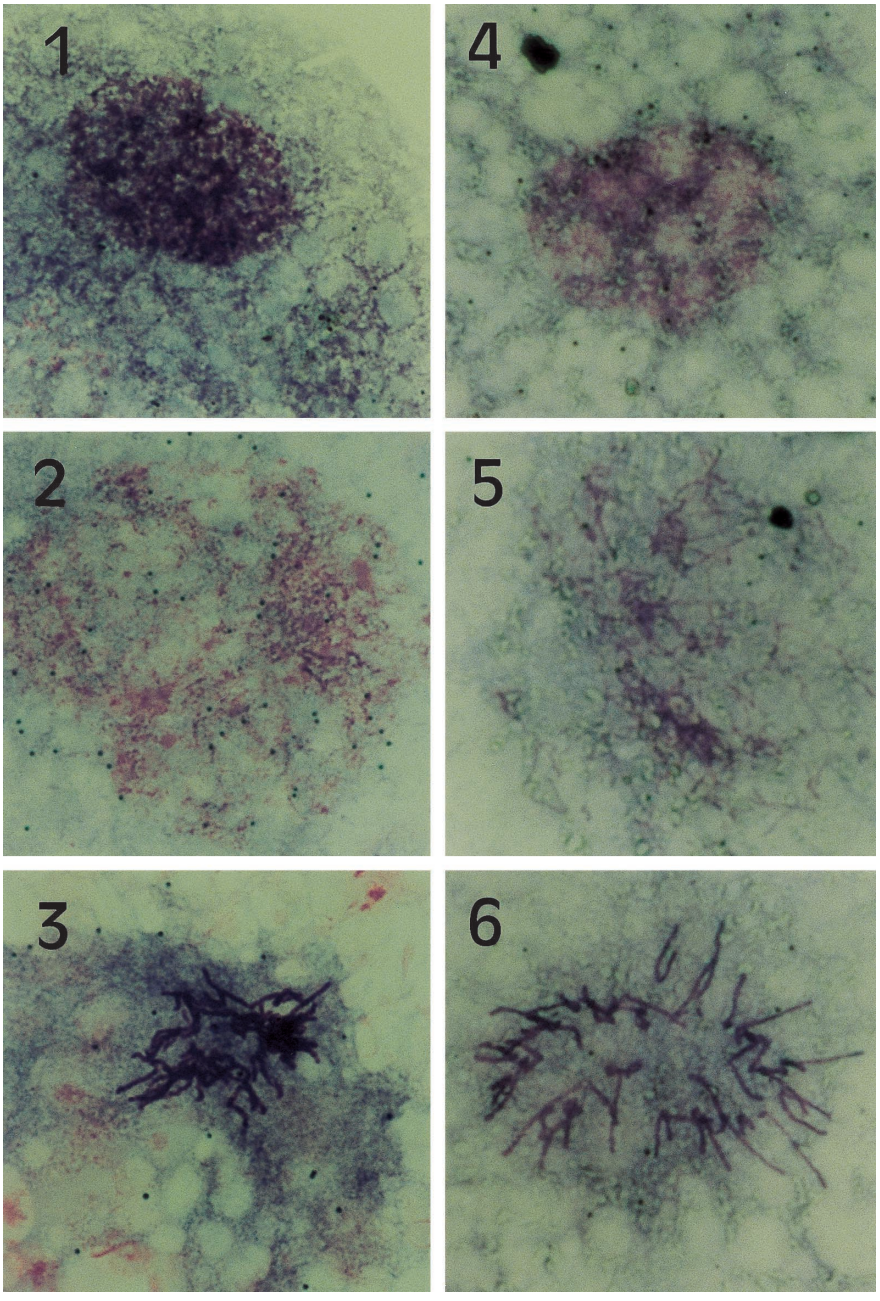

Fig. 2. Nuclei and chromosomes of cells arrested in prophase and metaphase by E-64-d and lactacystin. Embryonic cells were treated with 800 μM E-64-d or 100 μM lactacystin for 1 hr, soaked in a hypotonic solution, and stained with Giemsa. 1 and 2, early prophase and metaprophase in arrested cells following treatment with E-64-d; 3, metaphase in arrested cells following treatment with E-64-d; 4 and 5, early prophase and metaprophase in cells arrested with lactacystin; 6, metaphase in a cell arrested with lactacystin.

Table 4. Number of cells arrested in prophase and metaphase in control preparations without protease inhibitors, colchicine, lactacystin or E-64-d. Isolated cells were treated with a control preparation, 0.5 μg/ml colchicine, 800 μM E-64-d and 100 μM lactacystin for 1 hr. Embryonic cells were treated as described in Fig. 2. The number of cell in prophase includes cells in early prophase and metaprophase.

Treatment	No. of Prophase	No. of Metaphase	Total number
Control	125 (26%)	6 (1.2%)	474
	129 (36%)	4 (1.1%)	355
	152 (29%)	5 (0.94%)	528
Colchicine	144 (30%)	82 (17%)	481
	80 (30%)	51 (19%)	269
E-64-d	188 (43%)	12 (2.8%)	437
	168 (41%)	12 (2.9%)	410
Lactacystin	302 (50%)	22 (3.6%)	610
	342 (67%)	23 (4.5%)	512
	248 (60%)	16 (3.9%)	411

nuclei and chromosomes in *Xenopus* embryo cells 1 hr after the addition of protease inhibitors. Fig. 2 shows the prophase (Figs. 2–1, 2, 4, 5) and metaphase (Figs. 2–3, 6) stages of cell division. Following treatment with colchicine, 30% of the cells arrested in prophase and about 18% arrested in metaphase (Table 4). About 30% of the total number of control cells arrested in prophase, while about 42% of the E-64-d-treated cells and about 59% of the lactacystin-treated cells arrested in prophase (Table 4). Thus, the number of cells that arrested in prophase was 1.4 times greater in the E-64-d group than in the control group and 1.4 times greater in the lactacystin group than in the E-64-d group. About 1% of the control cells arrested in metaphase, while 3% of the E-64-d-treated cells and 4% of the lactacystin-treated cells arrested in metaphase (Table 4). Thus, E-64-d and lactacystin arrested 3 to 4 times as many cells in metaphase, compared with the controls.

DISCUSSION

The cell division cycle in eukaryotes contains three major control or transition points: the conversion of quiescent cells to a state of active proliferation, the initiation of DNA synthesis in the growing cell, and the induction of mitosis in cells with newly replicated genomes (Glotzer *et al*., 1991; King *et al*., 1995; Sudakin *et al*, 1995). Proteolysis of cell cycle-related proteins by proteasomes occurs at least twice during mitosis. Proteasomes are activated during prophase and metaphase and are related to both the entry into the mitotic phase and the transition from metaphase to anaphase (Kawahara *et al*., 1992; Georgi *et al*., 2002). Mitotic cyclins A and B are synthesized for the entry into mitosis, and kinase p34 cdk2-cyclin (MPF) induces the various ultrastructual changes required for cell division: namely, the breakdown of the nuclear envelope, chromatin condensation, and the construction of the mitotic spindle (Minshull *et al*., 1989). If the continued presence of cyclin is required to activate MPF, then the destruction of cyclin A and B during the stages of mitosis would lead to the inactivation of MPF.

To analyze how proteasomes and cysteine proteases are related to the stages of mitosis, we used two protease inhibitors and assayed the G2/M transition by visual examination of the nuclear morphology. Prophase nuclei were distinguished from interphase nuclei by the condensation of DNA to form mitotic chromosomes and the breakdown of the nuclear envelope (Fig. 2). Metaphase was distinguished from the other stages by the appearance of condensed chromosomes (Fig. 2). Inhibition by the cysteine protease inhibitor, E-64-d, and the proteasome inhibitor, lactacystin, increased the number of cells that arrested in prophase and metaphase, compared with the controls. These results showed that proteasomes and cysteine proteases are related to prophase and metaphase.

DNA condensation and the breakdown of the nuclear envelope depend on the degradation of Weel by ubiquitine/proteasome through a Cdc 34-dependent reaction (Michael and Newport 1998). Degradation of cyclin A always precedes that of cyclin B and occurs just after nuclear envelope breakdown; furthermore, non-degradable cyclin A arrests cells with condensed chromosomes (Holloway *et al*., 1993). Both cyclin A and B are degraded in mitosis by ubiquitin-mediated proteolysis (Krek 1998; Peters 1999). However, when the cyclin A-type destruction box was substituted for the normal one in cyclin B1 or B2, the resulting constructs could not be digested by proteasomes (Klotzbucher *et al*., 1996). Thus, while ubiquitination is probably necessary for cyclin destruction, it is not solely responsible. Other proteases are likely to be involved in the degradation of cyclin A

The initiation of anaphase is known to depend on the onset of sister chromatide separation, by the ubiquitination of the anaphase inhibitor; this reaction activates the anaphase-promoting complex, a cell cycle-regulated ubiquitin protein ligase that targets proteins for destruction by the proteasome (Nasmyth *et al*., 2000). The injection of calpain II during late metaphase promoted the precocious disassembly of the mitotic spindle and the onset of anaphase (Schollmeyer 1988), and E-64 can arrest carcinoma cells during metaphase (Shoji-Kasai *et al*., 1988). In this study, we showed that cysteine proteases are not only related to the onset of anaphase, but also to the progression of prophase.

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