Inhibitory effect of ubiquitin-proteasome pathway on proliferation of esophageal carcinoma cells

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METHODS: Esophageal carcinoma cell strain EC9706 was treated with MG-132 to inhibit its UPP specificity. Cell growth suppression was evaluated with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. DNA synthesis was evaluated by 3H-thymidine (3H-TdR) incorporation. Morphologic changes of cells were observed under microscope. Activity of telomerase was examined by telomeric repeat amplification protocol (TRAP) of PCR-ELISA. Cell cycle and apoptosis were detected by flow cytometry (FCM). DNA fragment analysis was used to confirm the presence of apoptosis. Expression of p27kip1 was detected by immunocytochemical technique.

RESULTS: After exposed to MG-132, the growth and value of 3H-TdR incorporation of EC9706 cells were obviously inhibited. Cells became round, small and exfoliative under microscope. Contrast to cytoplasm staining in control group, the positive signals of p27kip1 and 3,3-diaminobenaidine (DAB) kit were purchased from Sigma Co. Ltd (USA). 3H-thymidine (3H-TdR) was provided by Beijing Atomic Power Research Institute. Telomeric repeat amplification protocol (TRAP) ELISA telomerase detection kit was obtained from intergen Company (USA). Monoclonal mouse antibody of p27kip1, ultra sensitive S-P kit, and 3,3-diaminobenaidine (DAB) kit were purchased from Fuzhou Maixin Biotechnology Co. Ltd. RPMI 1640 medium was obtained from GibcoBRL Company (USA). Low melting-temperature agarose was purchased from Promega Company (USA). DNA-PREP™ LPR and DNA-PREP™ stain were obtained from America Beckman Coulter Company.

CONCLUSION: MG-132 can obviously inhibit proliferation of EC9706 cells and induce apoptosis. The mechanisms include upregulation of p27kip1 expression, G1 arrest and depression of telomerase activity. The results indicate that inhibiting UPP is a novel strategy for esophageal carcinoma therapy.

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of samples was measured three times for each group with three
wells at a wavelength of 550 nm with the enzyme linked
immunosorobant assay meter (PR 2100, SANOFI company,
France). The inhibitory rate (IR) was calculated according to
the formula: IR = [1-(absorbance of MG-132 group-absorbance of
background group)/(absorbance of control group-absorbance of
background group)]×100%.

**Incorporation test of ^3^H-TdR**

As described above, experimental group and control group
were cultured for 3 h with 100 mL/L FCS RPMI 1640, and then
for 12 h with FCS-free RPMI 1640. MG-132 and 1 μCi ^3^H-TdR
were added to each group, which was rinsed with PBS after 24,
48, 72 and 96 h and fixed with methyl alcohol and absolute ethyl
alcohol for 10 min each. Finally, 200 μL of 0.1 mol/L NaOH was
added, then 200 μL of each was taken after blowing, and mixed
in 5 mL scintillation liquid for overnight. On the following day,
the count per minute (CPM) of ^3^H was tested three times for
each group with three wells.

**Morphologic changes of cells**

Morphologic changes of EC9706 cells were observed under
microscope 24, 48, 72 and 96 h after treated with MG-132 (5 μmol/L).

**Telomerase assay**

The cells (10^5^-10^6^) treated with MG-132 (5 μmol/L) for 24, 48, 72
and 96 h were collected, respectively. After addition of 300 μL
telomerase assay lysis buffer (1×CHAPS), the cells were lysed
on ice. The lysate was incubated on ice for 30 min and then
centrifuged at 13,000 g for 25 min at 4 °C. The supernatant (2 μL)
was added to reaction solution containing 10 μL of TRAP
buffer, 2 units of Taq polymerase and 48 μL of DH2Oqs. PCR
was carried out through 33 amplification cycles, each cycle
consisting of denaturation at 94 °C for 30 s, primer annealing at
55 °C for 30 s, and extension at 72 °C for 30 s. The amplified
product was added to block/dilution buffer (250 μL), and
incubated at 37 °C for 30 min, and 5 μL of TRAP reactant was
then added and mixed. After incubated at 37 °C for 60 min, 100 μL
working solution of anti-DNP Ab was added and incubated for
30 min, then 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB)
substrate solution and 100 μL of stop reagent were added. The
absorbance value in each well was read at the wave lengths of
450 nm and 690 nm on an enzyme linked immunosorobant assay
meter. Telomerase activity was considered positive when the
absorbance value of a sample was at least 0.8 units. When
those were lower than 0.2 units, they were regarded as negative.

**Flow cytometry detection**

After cell cycle was synchronic, the cells of experiment group
were treated with MG-132 (5 μmol/L) for 48 h and 96 h. The
collected cells were added with DNA-PREP™LPR and DNA-
PREP™stain, respectively, after they were washed with PBS
and centrifuged. Cell cycle and apoptosis were detected by
flow cytometry (Epics XL, Beckman Coulter Company, USA)
and SYSTEM II™ software was used to dispose the data.

**DNA ladder demonstration**

As described above, the cells (7×10^5^/sample, both attached and
detached cells) were lysed with hypotonic lysis buffer (10 mmol/L edetic acid, 5 g/L Triton X-100, Tris-HCl (pH 7.4) for
15 min on ice and precipitated with 25 g/L polyethylene glycol
and 1 mol/L NaCI for 15 min at 4 °C. After centrifugation at
16,000 g for 10 min at room temperature, the supernatant was
treated with proteinase K (0.3 g/L) at 37 °C for 1 h and precipitated
with isopropanol. After centrifugation, each pellet was dissolved in
10 μL of Tris-EDTA (pH 7.6) and electrophoresed on a 17 g/L
agarose gel containing ethidium bromide. DNA ladder pattern
was identified under ultraviolet light.

**Immunocytochemical staining**

EC9706 cells cultured with MG-132 (5 μmol/L) for 48 h were
fixed with dimethyl ketone at 4 °C. The cells carrying the detected
antigen were stained following SP immunocytochemical staining
method using anti-p27kip1 as primary antibody. PBS was
substituted for primary antibody as negative control.

**Statistics**

The data were expressed as mean±SD. The difference between
each group was analyzed by t-test. P<0.05 was considered
statistically significant.

**RESULTS**

**Inhibitory effect of MG-132 on EC9706 cell growth**

The growth of EC9706 cells treated with 0.5-20 μmol/L of
MG-132 was significantly inhibited compared with that of
control group. While the cells exposed to MG-132 for 24 h
did MG-132 show significant effect. When the dose of MG-132
exceeded 5 μmol/L, only slight increases in IR of the cells were
observed (Figure 1).

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**Table 1** Evaluation of DNA synthesis by ^3^H-TdR incorporation (mean±SD)

| Group            | 24 h       | 48 h       | 72 h       | 96 h       |
|------------------|------------|------------|------------|------------|
| Control MG-132 (μmol/L) | 4 295.52±136.32 | 5 236.17±221.36 | 5 642.92±105.41 | 5 863.43±206.58 |
| 0.5              | 3 764.68±97.37 | 2 879.83±86.25 | 1 918.73±76.49 | 1 759.29±89.23 |
| 1.0              | 3 526.14±101.42 | 2 643.29±79.38 | 1 547.25±68.94 | 1 366.18±52.49 |
| 2.5              | 3 402.34±93.44 | 2 567.76±68.21 | 1 260.37±51.27 | 910.25±45.37 |
| 5.0              | 3 324.78±65.43 | 2 411.56±69.34 | 840.79±41.17 | 517.83±41.26 |
| 10.0             | 3 301.29±59.28 | 2 360.40±49.28 | 820.56±39.76 | 498.71±40.14 |
| 15.0             | 3 294.12±67.33 | 2 324.25±47.30 | 810.17±45.61 | 485.26±37.56 |
| 20.0             | 3 280.54±62.46 | 2 320.60±46.83 | 804.63±51.34 | 476.90±38.41 |

*P<0.05, *P<0.01 vs control.

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**Figure 1** MTT assay of EC9706 cells after exposed to MG-132.
**Morphologic changes of cells**
EC9706 cells became round, small and exfoliative after exposed to MG-132 under microscope (Figure 2).

**Inhibition of telomerase activity**
After treated with MG-132 (5 µmol/L) for 24, 48, 72 and 96 h, respectively, EC9706 cells showed a gradual decrease in values of A compared with the control group (P<0.01). Furthermore, telomerase activity was negative (Table 2).

Table 2 Effect of MG-132 on telomerase activity of EC9706 cells (mean±SD)

| Groups   | 24 h       | 48 h       | 72 h       | 96 h       |
|----------|------------|------------|------------|------------|
| Control  | 1.871±0.061| 2.234±0.092| 2.907±0.113| 3.025±0.120|
| MG-132   | 0.154±0.008| 0.085±0.006| 0.072±0.004| 0.067±0.003|

\( ^{b} P<0.01 \) vs control group.

**Inhibition of DNA synthesis**
The values of \(^{3}H\)-TdR incorporation of MG-132 group were decreased compared with the control group (Table 1).

**Changes of cell cycle and apoptosis**
The percentage of cells at \(G_{0}/G_{1}\) phase was increased and that
at G2/M and S was decreased ($P<0.01$). The rate of apoptotic cells treated with 5 µmol/L of MG-132 for 48 and 96 h was 31.7% and 66.4%, respectively (Table 3, Figure 3).

**Table 3** Effect of MG-132 on cell cycle of EC9706 cells (mean±SD, %)

| Groups | 48 h | 96 h |
|--------|------|------|
|        | $G_0$/$G_1$ | S | $G_2$/$M$ | $G_0$/$G_1$ | S | $G_2$/$M$ |
| Control | 40.4±3.9 | 46.5±4.3 | 13.1±1.0 | 44.6±4.1 | 40.9±2.3 | 14.5±1.4 |
| MG-132 | 67.5±5.1 | 29.3±2.8 | 3.2±0.2 | 73.1±5.2 | 24.6±2.1 | 2.3±0.1 |

$^bP<0.01$ vs control group.

**DNA ladder**

Agarose electrophoresis showed marked ladders in MG-132 group, while the ladder was not detected in control group. Furthermore, DNA fragmentation was more apparent at 96 h (Figure 4).

**Discussion**

Proteolytic degradation by ubiquitin-proteasome system involves ATP-dependent covalent attachment of a macromolecular chain of ubiquitin (Ub) molecules to the target protein, followed by degradation through the multicatalytic 26S proteasome. The conjugation of Ub, a highly conserved 8.6 kDa protein, to its target protein is mediated by the serial actions of three enzymes. E1, the Ub-activating enzyme, activates Ub in an ATP-dependent manner. E2, the Ub-conjugating enzyme, catalyzes the attachment of Ub to the substrate protein. E3, the Ub-ligase, serves as a scaffold between E2 and the substrate and provides recognition specificity of the substrate. A protein tagged with a polyubiquitin chain is recognized and degraded by the 26S proteasome complex. This complex is composed of a 19S regulatory subcomplex and two 20S catalytic subcomplexes. UPP is extensively involved in physiological and biochemical processes. Some experiments showed that the low expression of some anti-oncogene including p53, p27\textsuperscript{kip1} in tumor cells was associated with the increasing activity of ubiquitin proteasome which leads to degradation of expression products of anti-oncogene, and have proved that deubiquitination of p53 is an important pathway for p53 stabilization\textsuperscript{11,12}. Moreover, the degradation accommodation of some transcription factors was regulated by UPP, such as NF-kB, c-fos, c-jun, c-mos, c-myc and MATa\textsuperscript{13-17}. So UPP is closely associated with the occurrence and development of malignant tumor.

Ubiquitin proteasome inhibitors include peptide aldehyde, borofax peptide and 3, 4-dichloro isocoumarin. MG-132, also known as carboxbenzoxyl-L-leucyl-L-leucyl-L-leucinal, a reversible, effective and specific peptide aldehyde inhibitor of ubiquitin-proteasome, could block UPP through inhibiting ubiquitin-mediated proteolysis by binding to and inactivating 20S and 26S proteasomes\textsuperscript{18-22}.

In our study, esophageal cancer cell line EC9706 was exposed to MG-132 to observe whether UPP could be inhibited. We found that the proliferation of cells was obviously inhibited in a dose- and time-dependent manner. The results also revealed some anti-tumor mechanisms of MG-132. First, MG-132 could up-regulate the expression of p27\textsuperscript{kip1}. p27\textsuperscript{kip1} was recently found\textsuperscript{23} as a anti-oncogene with function of negative regulation in tumor cells, inducing cell differentiation and apoptosis, enhancing cell’s adherence and regulating the resistance to medicines for noumenal tumors. p27\textsuperscript{kip1} protein is a cyclin dependent kinase inhibitor (CDKI) that could block G1/S transition of cell cycle by inhibiting the action of cyclin E-CDK2 complex and cyclin D-CDK4 complex\textsuperscript{24-26}. p27\textsuperscript{kip1} expression decreases in esophageal cancer and it may correlate with the histologic differentiation. Reduction of p27\textsuperscript{kip1} has been considered to be an independent prognostic indicator of esophageal cancer\textsuperscript{27-30}. The nuclear localization signal of p27\textsuperscript{kip1} contains a protein kinase B (PKB/Akt) consensus site at threonine 157, and phosphorylation of p27\textsuperscript{kip1} by PKB/Akt has been found to impair its nuclear import\textsuperscript{31,32}, which is a key procedure to play its functional role\textsuperscript{33}. We found that p27\textsuperscript{kip1} protein localized in cytoplasm of EC9706 cells showed low expression, but that localized both in cytoplasm and nuclei ($>200$).

**Expression of p27\textsuperscript{kip1}**

In the control group, the cytoplasm was stained in brownish yellow and the nuclei were stained in blue. In the experiment group, both the cytoplasm and nuclei were stained in brownish yellow, indicating that the expression of p27\textsuperscript{kip1} in EC9706 cells was increased after treated with MG-132 (Figure 5).
and nuclei of EC9706 cells showed high expression after treated with MG-132. Our previous studies demonstrated that the growth of EC9706 cells and tumors implanted in nude mice was obviously inhibited, apoptosis was induced and cell cycle was arrested in G1 phase by up-regulating p27kip1. Second, MG-132 could depress telomerase activity. The activation of telomerase was closely associated with cyclin. It has been reported that inhibition of UPP could not only increase the expression of p27kip1, but also increase the expression of p53[135]. Moreover p27kip1 and p21 regulated by p53 could inhibit cyclin and result in decreased telomerase activity[16,37]. Third, MG-132 could cause G1 arrest, which may be involved in changes of cell cycle regulatory factors such as p27kip1. Fan et al.[30] obtained the same results as ours. But Ling et al.[39] tended to consider ubiquitin-proteasome inhibitors to cause G1 arrest. The difference may be involved in the different types of cells. The last, MG-132 could induce apoptosis, which may be closely associated with the functions mentioned above.

In conclusion, MG-132 can obviously inhibit proliferation of EC9706 cells and induce apoptosis. The mechanisms include upregulation of p27kip1 expression, G1 arrest and depression of telomerase activity. The results indicate that inhibiting UPP is a novel strategy for esophageal carcinoma therapy.

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