Molecular Alterations Which Induce Apoptosis To Cancer Cells By A Novel Antitumorigenic Protein From A Mushroom, Tricholoma Matsutake

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Abstract

A novel tumoricidal protein from *Tricholoma* matsutake, induced the morphological changes typical to apoptosis such as nuclear condensation, aneuploidity and DNA fragmentation at concentrations as low as 5-20ng/ml to cancer cells. Molecular alterations related to cell cycle, especially G1/S transition were investigated with a human keratinocyte transformed with oncoproteins, E6 and E7 of human pappiloma virus (HPV)-16. TTM didn't alter significantly an oncosuppressor p53 level, but induced hyperphosphorylation of pRB. Time-dependent change of G1 cyclins, cdk2 and cdk4 after addition of TTM showed that expression level of cdk inhibitors, INK4 family and $p27^{Kip1}$ did not altered, while that of $p21^{Waf1}$ was downregulated.

Introduction

Evidence has been accumulating that change or loss in function of the tumor-suppressing gene products, oncosuppressors such as p53 and retinoblastoma protein (pRB), are the most frequently observed phenomenon in many human cancers (tumor cells).

p53 plays a central roll in protecting cells from malignant transformation by retarding the progression of cell cycle from G0/G1 to S phase or inducing apoptosis depending on degree of the lesion[1)-4)]. The pRb functions as a tumour suppressor in a concerted fashion with p53 proteins through regulation of transcription. Since phosphorylation status of pRB is a main determinant of cell cycle progression through G0/G1, this hypophosphorylation facilitates liberation of a transcription factor E2F1 from pRB-E2F1 complex, which induces the expression of molecules necessary for the progression of cell cycle from G0/G1 to S phase[5)-9)].

For cancer prevention, it is an useful strategy to find substances that are preferentially cytotoxic against tumor cells, but not cytotoxic against non-tumorous normal counterparts. For this purpose,

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the cells transformed by DNA virus such as Simian Virus 40 (SV40), adenovirus, or human papilloma virus (HPV) are most appropriate since they express the oncoproteins, Large antigen, E1A and E1B, and E6 and E7, respectively, all of which have binding domains to p53 or pRB and inactivate them. Especially, HPV type 16 and 18 have assigned to be causable viruses for human cervical cancer.

Based on these considerations, we have been screened anti-tumorigenic substances in biological materials by using preferential cytotoxicity against SV40-transformed NIH/3T3 cell (SV-T2) comparing to its normal counterpart, NIH/3T3 cell The antitumorigenic activity was found in several mushrooms, and an antitumor protein was found and purified.

In the prersent study, some molecular characterization and the action profile of antitumor proteinto SV40-transformed NIH/3T3 cell (SV-T2) and a human HPV transformed keratinocyte[10] were discussed.

Materials and Methods

Cell Culture: A mouse fibroblast cell (A31) and its SV-40 transformant (SV-T2) were maintained in DMEM supplemented with bovine calf serum at 37 C in a 5% CO2-humidified incubator. A HPV16-immortalized human keratinocyte cell line, PHK16-0b was a generous gift of Dr. Shigeru Yasumoto (Kanagawa Prefectural Cancer Research Institute). PHK16-0b was maintained in the complete MCDB152 medium containing a hormone mixture and 0.5% chelated fetal calf serum. Cellular viability was determined by MTT assay in 96-well flat-bottomed tissue culture plates by measuring the absorbance of each well using a microplate reader[11].

Flowcytometric Analysis: Cellular DNA was stained with propidium iodide by the methods of Lars L. Vindeloveta[12] and was analyzed by fowcytometry with the FACSort/Cell FIT DNA system (BectonDickinson Co. USA).

Cytochemical Change: changes in morphology of nuclei were evaluated by staining with acridine orange. Cells were fixed in 3% fomaldehyde and washed three times in phosphate buffered saline (PBS). After treated with 0.5% tritonX 100 in PBS, those were stained with 1mg/ml of acridine orange in PBS and observed by fluorescence microscopy (Olympus).

Determination of DNA Fragmentation: Cells were harvested, washed with PBS and suspended in a lysis buffer. After incubation at 37 C for 12h, the intact genome DNA was removed by centrifugation. The DNA in the supernatant was extracted with phenol and chloroform-isoamylalcohol and precipitated with 70% ethanol. After RNaseA treatment at 37C for 12h, it was analyzed by electrophoresis in 5% agarose geland visualized by ethidium bromiide staining.

Northern Blotting: Total RNA of cells ($2 \times 10^4/cm^2$) was extracted by adding guanidinum thiocyanate-phenol-chloroform method. RNA was electrophoresed in a 1.2% agarose gel followed by capillary transfer to HybondN membranes. RNA was crosslinked by UV irradiation. The blottedmem-

brane was prehybridized at 42 C for 2h and hybridized in a buffer containing probes at 42 C for 12 to 16h. [32]p DNA probes were prepared by randompriming. The *c-myc* expression was analyzed with human cDNA fragment. Glyceraldehyde 3-Phosphate dehydrogenase (G3PDH) expression was analyzed with 1.1kb human cDNA probe. Quantification of autoradiograms was carried out with Bas 2000.

Immunoprecipitation and Immunoblotting: Cells were harvested and lysed in an extraction buffer with 1% NP-40 and a protease inhibitor cocktail. The extracts were applied to SDS-PAGE[13] and electrotransferred to a PVDF membrane. The blotted membrane was incubated with anti-pRb, anti-p53, anti-cyclinE or Dl, or anti-cdk2 or -cdk4 antibodies, and then with anti-mouse or anti-rabbit horseradish peroxidase-conjugated whole antibodies. The antibody protein complexes were detected by ECL detection system. Antigen-antibody complexes were immunoprecipitated with proteinA-Sepharose beads and the precipitated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Cdk2 or Cdk4 was identified by those antibodies described above and detected by ECL system. Protein concentration was detemined by a dye-binding assay kit (BIO-RAD500-001).

Results and Discussion

Apoptosis induction in PHK16-0B

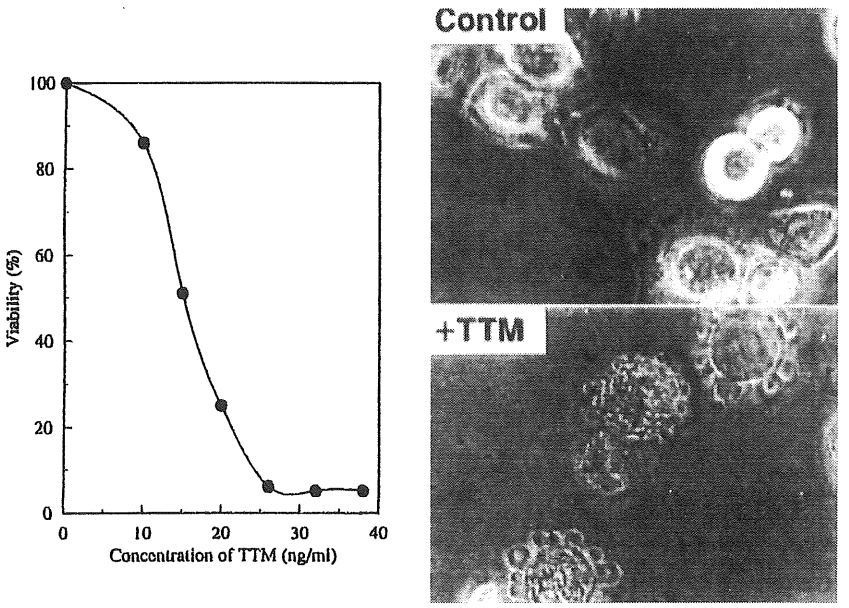

Fig. 1 Cytotoxicity against PHK16 cells with a LD50 value of 15ng/ml (left). In the cells treated with TTM, the disruption of the cellular membrane, blebbing from cytoplasm, cell shrinkage and chromatin condensation (acridine orange staining) were observed microscopically (right).

TTM had intense cytotoxicity against PHK16 cells with a LD50 value of 15ng/ml (Fig 1, left). In the cells treated with TTM, the disruption of the cellular membrane, blebbing from cytoplasm, cell shrinkage and chromatin condensation (acridine orange staining) were observed microscopically (Fig. 1-right). These changes were typical features of apoptosis[14].

The appearance of the sub-G1 population is often considered to be alternative marker of apoptosis. DNA histograms of PHK16-0b with flowcytometric analysis showed appearance of hyperploidy at l0hr after addition of TTM, suggesting that TTM induced the change in structure of nuclear DNA.

A' DNA ladder' of oligonucleosomal unit on gel electrophoresis is another hallmark of apoptosis[15]. Degradation of nuclearDNA was observed in PHK16-0b cells treated with TTM and with mitomycin C for 24h. However, typical DNA ladders were not observed in both cases. Oberhammer et al reported that in some epithelial cell lines. Cleavage of DNA to 300 and /or 50bp fragments occurred in the apoptotic process because of release of chromatin loop domain prior to internucleosomal fragmentation. This might be the case in the action of TTM and also of mitomycin C[16], which resulted in unclear laddering.

Change in *c-myc* expression by T TM

Cellular oncogenes appear to play key roles in the biochemical pathways controlling cell proliferation and death[17]. Especially, elevated *c-myc* expression is implicated in proliferation of epithelial cells[18)-20)]. To examine the involvement of c-Myc in cell death induced by TTM, *c-myc* expression was analyzed in PHK16-0b cells before and after addition of TTM.

The decrease in the level of expression of *c-myc* mRNA started at 12h after addition of TTM. After 19h, *c-myc* expression decreased to about 75% that of the control. This down-regulation of *c-myc* was dose-dependent. Kimura et.al. reported that down-regulation of *c-myc* expression could decrease the proliferation of HL60 cells and that some of the cells underwent apoptosis. We also observed that TTM induced the Gl arrest of SV-T2 cells temporarily prior to cell death. The down-regulation of *c-myc* by TTM in PHK16-0b might include the G1 arrest before cell death. ,However, the Gl arrest in PHK16-0b could not be confirmed by flowcytometric analysis probably because hyperploid subpopulation appeared in DNA histogram at the early stage in apoptotic process.

Phosphorylation status of pRb.

A number of studies have indicated that the transcription of *c-myc* can be modulated by release of E2F from pRb-E2F complex, which is dependent on phosphorylation of pRb[21)-22)]. The level of phosphorylation of pRb was increased by TTM in a dose-dependent manner. The pRb gradually sifted to hyperphosphorylated form until 12h, but the amount of pRb and the ratio of hyperphosphorylated pRb decreased after 18h, though the reason for that was not clear. This temporally enhanced phosphorylation of pRb was inconsistent with the previous results in that though the phosphorylation of pRb could be an event leading to increase of *c-myc* expression in normal cell cycle advance of keratinocytes the

level of *c-myc* was depressed with time by TTM. There might be a possibility that the depression of *c-myc* expression caused by TTM is independent of transcriptional regulation mediated by pRb. These results indicated that the action of TTM to PHK16-0b cells was in coflict with the mechanism of cell cycle progression.

Status of cyclin E dependent kinase, cdk2

To clarify whether inhibition of dephosphorylation of pRb and/or activation of cdks causes acceleration of pRb phosphorylation by TTM, we monitored the levels of G1 cyclins, cdk4 and cdk2 , and the phosphorylation state of pRb in the extracts from PHK16-0b cells by probing with specific antibodies against cydinDl and E, Cdk2, Cdk4, and pRb. At 3h after the addition of 60ng/ml of TTM, hyperphosphorylated pRb appeared. However, the elevation of the level of hyperphosphorylated pRb by TTM was transient. At 6h, the level of hyperphosphorylated pRb in PHK16-0b was less than that in control. The level of cyclinD was slightly decreased after addition of TTM. The level of cyclinE was decreased at 6h after addition of TTM, which seemed correlated with the decreased level of hyperphosphorylated pRb in the cells. No significant change was observed in the amount of cdk4 and cdk2. These results indicated that the level of cyclinD, cyclinE, cdk4, and cdk2 was independent on the level in phosphorylation of pRb at 3h after addition of TTM.

In cell cycle regulation, cdks require association with cyclin partners and concomitant phosphorylation/dephosphorylation at specific residues. As shown in Table 1, the levels of cyclinD-associated cdk4 and cdk2 and cydinE-associated cdk2 were not changed, which did not coordinate with the change of phosphorylation state of pRb. However, the change of the ratio of the hyperphosphorylated form in cyclinE-associated cdk2s, which migrated more rapidly on SDS-PACE than the hypophbspho-

Table 1. The changes in cell cycle related molecules by the addition on AP

Cell	A31		A31		SV-T2	
time	3h	6h	3h	6h	3h	6h
Ip						
	w/cyclin D1		w/cdk4		w/cyclin D1	
cyclin D1	↓	↓	ND		↓	↓
cdk4	→	→	→	→	→	→
p21	→	→	→	→	ND	
p27	→	↓	↓	↓	↓	↓
p15&p16	ND		ND		ND	
cyclin E	→	→	→	→	→	→
Simple western						
pRb	→	→	→	→	→	→
p53	ND		→	↓	↓	↓
c-myc	→	→			↓	↓
NFkB	→	→			→	→
IkB	→	→			→	→

rylated form, was consistent with the phosphorylation state of pRb.

The hyperphosphorylated cdk2 corresponds to catalytically active state of this protein. These suggested that TTM activated the cyclinE-associated cdk2, which cause elevation of the level of the hyperphosphorylated pRb.

We have presented evidence that PHK16-0b cells treated with TTM undergo apoptosis. TTM induced phosphorylation of pRb temporarily because of activation of cyclin E-associated cdk2 and depressed the *c-myc* expression. TTM appeared to direct the paradoxical behaviors to PHK16-0b by promoting and arresting cell cycle in the same time. This unbalanced control of cell cycle induced by TTM might lead to apoptosis in tumorigenic cells, though the signal transduction pathways to apoptosis by TTM has to be studied in more details.

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