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Suppression of cell cycle progression by a fungal lectin: activation of cyclin-dependent kinase inhibitors

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Abstract

The antiproliferative activity of a fungal lectin (VVL) isolated from the mushroom, *Volvariella volvacea*, was studied using a battery of cultured tumor cell lines. It was revealed that [^{3}H]thymidine incorporation into the cell lines was markedly reduced at 0.32 μM VVL. When S180 mouse sarcoma cells were incubated for 48 hr with doses of VVL ranging from 0.32 to 0.8 μM, prominent blebs on the cell surface and large vacuoles in the cytoplasm, but not apoptotic bodies, were observed under a fluorescence microscopy. VVL did not exert ribosome-inactivating activity or induce any changes in the expression of cyclins A, D1, and E. However, it did activate the expression of cyclin kinase inhibitors, namely *p21*, *p27*, *p53*, and *Rb*, in a dose-dependent manner. Flow cytometric analysis demonstrated an accumulation of cells in the G2/M phase in a time- and dose-dependent manner, indicating that VVL arrested cell proliferation by blocking cell cycle progression in the G2/M phase.

Keywords: *Volvariella volvacea* lectin; Tumor cell lines; Growth arrest; Cyclin kinase inhibitors

1. Introduction

The lectin VVL, a homodimeric protein with a molecular weight of 32 kDa, was purified from the edible mushroom, *Volvariella volvacea* [1]. VVL is a potent mitogen, as shown by its ability to stimulate uptake of [^{3}H]thymidine by cultured murine lymphocytes at nanomolar levels. It markedly enhanced transcriptional expression of interleukin-2 and interferon-γ, indicating that VVL possesses a potent immunomodulatory activity [1]. However, when tumor cell lines were exposed to VVL at submicromolar levels, a remarkable inhibition of cell proliferation was observed. Numerous plant toxins have been reported: some of them, such as mistletoe lectin from *Viscum album*, induced apoptosis [2], whereas others, including bolesatine from *Boletus satanas* Lenz, [3], Viscaceae lectin from *Phoradendron californicum* [4], and ricin from *Ricinus communis* [5], exerted their toxic activity by their ability to inactivate ribosomes [3–5]. The present study was therefore undertaken to investigate the molecular mechanism of antitumor activity of VVL in a number of tumor cell lines. The parameters investigated in VVL-treated tumor cells were: proliferative and morphological changes, gene expression of cyclins and cyclin-dependent kinase inhibitors, and changes in cell cycle distribution. The ribosome-inactivating activity of VVL was analyzed in an *in vitro* assay using rabbit reticulocyte lysate, and compared with that of ricin, a well-known ribosome-inactivating protein (RIP).

2. Materials and methods

2.1. Cell cultures

Cell lines were obtained from the American Type Culture Collection. The human prostate carcinoma LNCaP cell line (CRL 1740) was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 μg/mL of streptomycin, and 100 IU/mL of penicillin. The mouse sarcoma line, S180 (TIB 66), and the mouse melanoma line, B16 (NBL 6323), were cultured in supplemented Dulbecco's modified Eagle's medium and F10 medium, respec-

Abbreviations: VVL, *Volvariella volvacea* lectin; CDK, cyclin-dependent kinase; and CDI, cyclin-dependent kinase inhibitor.

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tively. The human breast carcinoma line, MCF-7 (HTB 22), was maintained in alpha minimum essential medium supplemented with 10 μg/mL of insulin and 10% FBS. All cell cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 [6].

2.2. Volvariella volvacea *lectin (VVL)*

The agglutinin (VVL, *Volvariella volvacea* lectin) was isolated from the edible mushroom *Volvariella volvacea*, as described previously [1]. It was initially dissolved in sterile distilled water to make a stock solution, from which appropriate concentrations were prepared with fresh cultured medium.

2.3. Cytotoxicity of VVL

Four tumor cell lines (1×10^5 cells/0.1 mL/well) were incubated with a serial dilution of VVL (4.0, 0.8, 0.16, 0.032, 0.0064 to 0.00128 μM) in 96-well culture plates for 42 hr, at which time 10 μL of [^{3}H]thymidine (0.25 mCi, Amersham) was added. The cells were incubated for a further 6 hr at 37° in a humidified atmosphere of 5% CO_2. The cells were then harvested with an automated cell harvester onto glass fiber filters, and radioactivity was measured with a Beckman model LS 6000SC scintillation counter [6]. All reported values were the means of triplicates.

2.4. Fluorescent staining

S180 sarcoma cells were treated with a serial dilution of VVL for 48 hr, washed briefly, and spread onto clean glass slides, using a cytospin centrifuge (Cytospin 3, Shandon). The cells were fixed with buffered formalin, stained with 0.01% acridine orange in 0.06 M phosphate buffer, pH 6.0, and differentiated with 0.1 M calcium chloride. Nuclear morphology was assessed using a fluorescence microscope (Axioskop, Zeiss) with a 450- to 490-nm excitation block filter and a 520-nm barrier filter.

2.5. Ribosome-inactivating activity

The inhibitory activity of VVL on *in vitro* protein synthesis was analyzed as described [7]. Briefly, rabbit reticulocyte lysate was co-incubated at 30° for 30 min with [^{3}H]leucine and a serial dilution of VVL. The ^{3}H-labeled translation product was collected on a glass fiber filter, and the radioactivity was measured using a Beckman liquid scintillation counter. The IC_{50} value was expressed as the concentration of VVL required to inhibit 50% of the translation activity of the rabbit reticulocyte lysate, and compared with that of ricin (L8505 Sigma), a well-known ribosome-inactivating protein.

2.6. Flow cytometry

Only VVL-treated S180 cells were subjected to flow cytometric analysis. Cells were cultured with VVL for 24, 42, and 48 hr, lysed with Nonidet P-40 for preparation of nuclei [8], RNA removed with 200 μg/mL of RNase A (R4875 Sigma), and stained with 20 μg/mL of propidium iodide (PI, Sigma P4170) at 4° for 15 min. The red fluorescence of DNA-bound PI in individual cells was measured at 575 nm with a Coulter ALTRA™ flow cytometer, and the results obtained from 10,000 cells were analyzed using Expo II software (Beckman Coulter, Inc.).

2.7. Reverse transcription–polymerase chain reaction analysis (RT–PCR)

S180 sarcoma cells (5×10^6 cells) were treated with different doses of VVL for 48 hr before their total cellular RNA was isolated using the acid guanidine thiocyanate–phenol method [9]. One microgram of total RNA was directly reverse-transcribed at 42° for 50 min using a Superscript™ preamplification system (18089-011, GIBCO/BRL). Each RT product was subjected to PCR using Thermoprime DNA polymerase and primers (GIBCO/BRL and Table 1) in a 9700 Perkin Elmer thermal cycler. The PCR products were separated on a 1.2% agarose gel, and band intensity was measured by a Chemiluminescence Imaging Analysis System with FluorChem software (Alpha Innotech Corp.). The intensity of each band was normalized to that of the internal control, β-actin.

3. Results

3.1. Cytotoxicity of VVL

All cell lines maintained normal proliferative activity when incubated for 48 hr with VVL concentrations from 1.28 nM to 0.16 μM. However, a drastic decrease in proliferation was observed between 0.32 and 4 μM (Fig. 1). S180 cells were the most sensitive to VVL, followed by MCF-7 and B16, but the prostate cell line LNCaP was relatively insensitive, maintaining 50% of its proliferative activity at the highest dose of VVL (4 μM). Since S180 cells were most susceptible to the antiproliferative activity of VVL, its mechanism of growth inhibition was investigated further by a number of different bioassays in S180 cells only.

3.2. Morphological changes

Microscopic examination of the cultures showed that S180 cells had a smooth cell surface and a round nucleus in homogeneous cytoplasm, when they were cultured for 48 hr either in the absence of VVL or in the presence of 0.16 μM VVL (Fig. 2a). Blebs on the plasma membrane and large

Table 1
The primer sequences and fragment sizes of the investigated genes

Gene	Primer sequences	Fragment size (bp)	Ref.
Cyclin A	5′-TACTTCCTGCACCTGCAGCCT-3′ 5′-AGCATGGACTCCGAGCGACT-3′	399	20
Cyclin D1	5′-AGGCGGATGAGAACAAGCAGA-3′ 5′-CAGGCTTGACTCCAGAAGGG-3′	269	20
Cyclin E	5′-CCCAGCAGTAAGAAGGCAGAG-3′ 5′-CAGCTTCTGGAGCACTCAGTG-3′	287	20
p21	5′-CTTTGACTTCGTCACGGAGAC-3′ 5′-AGGCAGCGTATATCAGGAGAC-3′	253	20
p27	5′-AAGCACTGCCGGGATATGGA-3′ 5′-AACCCAGCCTGATTGTCTGAC-3′	297	20
p53	5′-GTGGCCTCTGTCATCTTCCG-3′ 5′-CCGTCACCATCAGAGCAACG-3′	429	21
Rb	5′-AGTCCAGCTGTGCAGAAACTTC-3′ 5′-ACCAATAGTGCAGTGTCTGCAG-3′	491	20
β-actin	5′-CCTTCTACAATGAGC-3′ 5′-ACGTCACACTTCATG-3′	594	22

vacuoles in the cytoplasm were prominent in S180 cells treated with VVL at concentrations of 0.32 to 0.80 μM (Fig. 2b). Cell disintegration and cell death occurred when the cells were exposed to 0.80 μM VVL, but no apoptotic bodies were observed, indicating that VVL-induced cell death is unlikely to involve apoptosis.

3.3. Ribosome-inactivating activity

A ribosome-inactivating activity of IC_{50} = 29 μM was found for VVL. This is at least 10,000-fold lower than the IC_{50} = 1 nM found for the well-known ribosome-inactivating protein, ricin.

3.4. Gene expression

The expression of genes encoding cyclins A, D1, and E is crucial for cells to maintain a normal growth cycle. Not

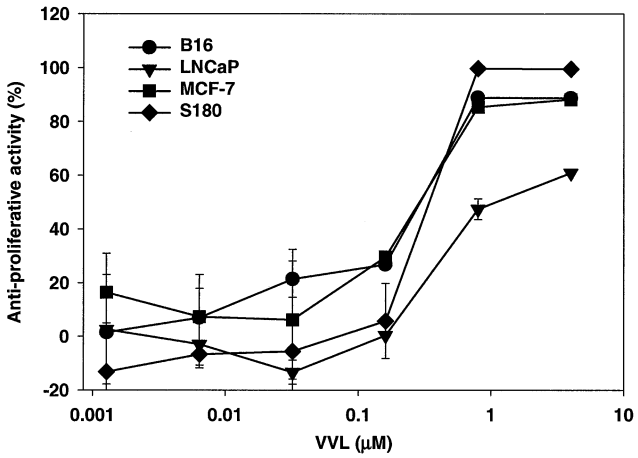

Fig. 1. Antiproliferative activity of VVL on various cell lines. Effect of *in vitro* VVL treatment, ranging from 1.28 nM to 4 μM, for 48 hr on proliferation of cultured tumor cell lines. Each data point represents the mean (± SD) of triplicate determinations using separate wells.

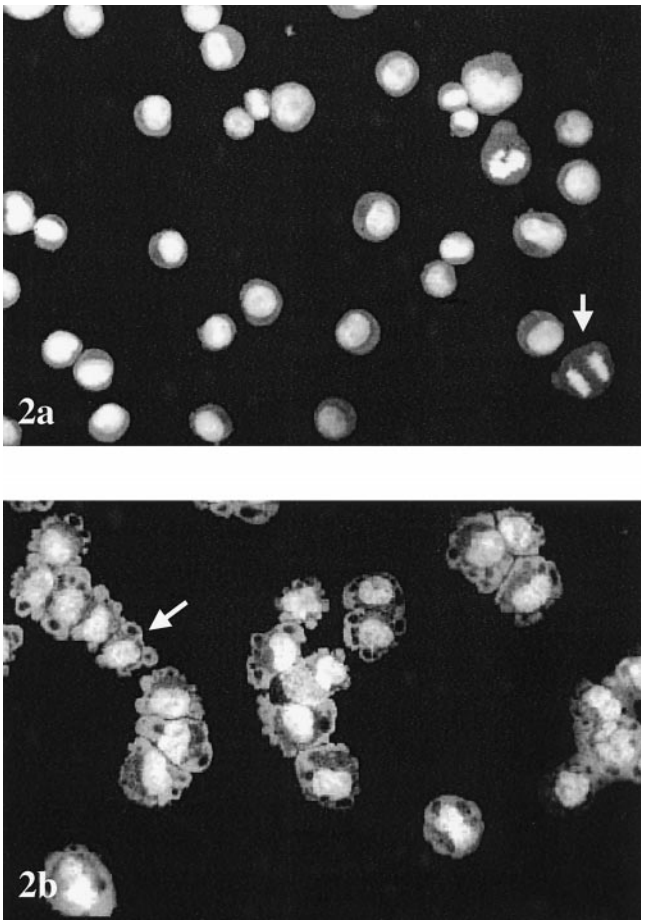

Fig. 2. Morphological changes in S180 cells after exposure to VVL for 48 hr. (a) S180 cells were round-shaped with a spherical nucleus and a smooth cell surface. Mitotic figures (arrow) were occasionally observed in normal culture or cells treated with VVL up to 0.16 μM. (b) Prominent cytoplasmic blebs and vacuoles (arrow) and irregular nuclei were observed in S180 cells treated with VVL from 0.32 to 0.8 μM for 48 hr. No apoptotic cells were observed (800×)

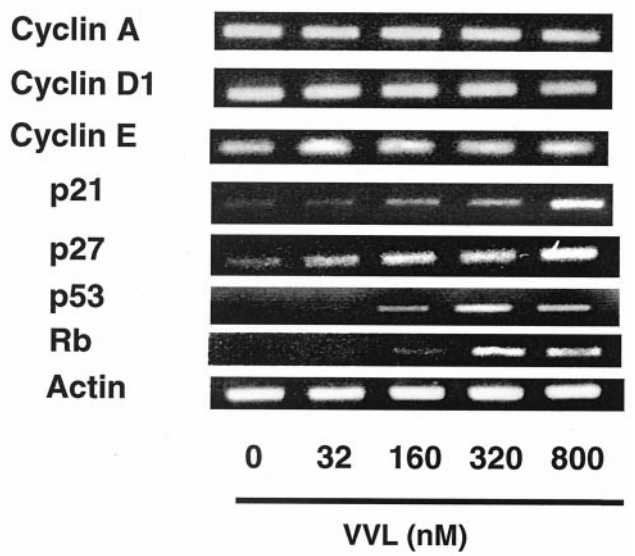

Fig. 3. Expression of cyclin and inhibitor genes in VVL-treated S180 cells. Changes in gene expression in S180 cells treated with VVL for 48 hr. The expression of cyclins A, D1, and E remained at a relatively constant level at doses of VVL up to 320 nM, with only a slight decrease detected at 800 nM. On the other hand, dose-dependent induction of cyclin kinase inhibitors *p21*, *p27*, *p53*, and *Rb* was observed.

surprisingly, similar levels of expression of these three genes were observed in S180 cells exposed to VVL concentrations up to 0.32 μM. The expression was slightly decreased ($<$ 10%) when the dose of VVL was 0.8 μM (Fig. 3). On the other hand, expression of genes for the cyclin kinase inhibitors *p21*, *p27*, *p53*, and *Rb* was extremely low until the cells were exposed to 0.16 μM VVL. At higher doses, expression of these genes showed a dose-dependent increase, compared with that of the internal control, β-actin.

3.5. Cell cycle distribution

Since S180 cells lost their proliferative capacity so dramatically after 48 hr of VVL treatment (approximately 100% at 0.16 μM VVL vs 0% at 0.8 μM VVL), cells were exposed to varying doses of VVL for either 24 or 48 hr and then subjected to flow cytometric analysis. In agreement with the proliferative assay and morphological observations, S180 cells cultured in the presence of VVL up to 0.16 μM exhibited a DNA pattern similar to that of cells without VVL treatment (Fig. 4). However, more than 50% of cells were found in the G2/M phase when the cells were exposed to 0.8 μM VVL for 24 hr, but is decreased to approximately 20% at 48 hr because more than half of the cells had died with $>$50% of the cells were in subG1 phase.

4. Discussion

Many of the heterodimeric cytotoxic lectins consist of a cytotoxic subunit A and a sugar-binding subunit B [10]. The

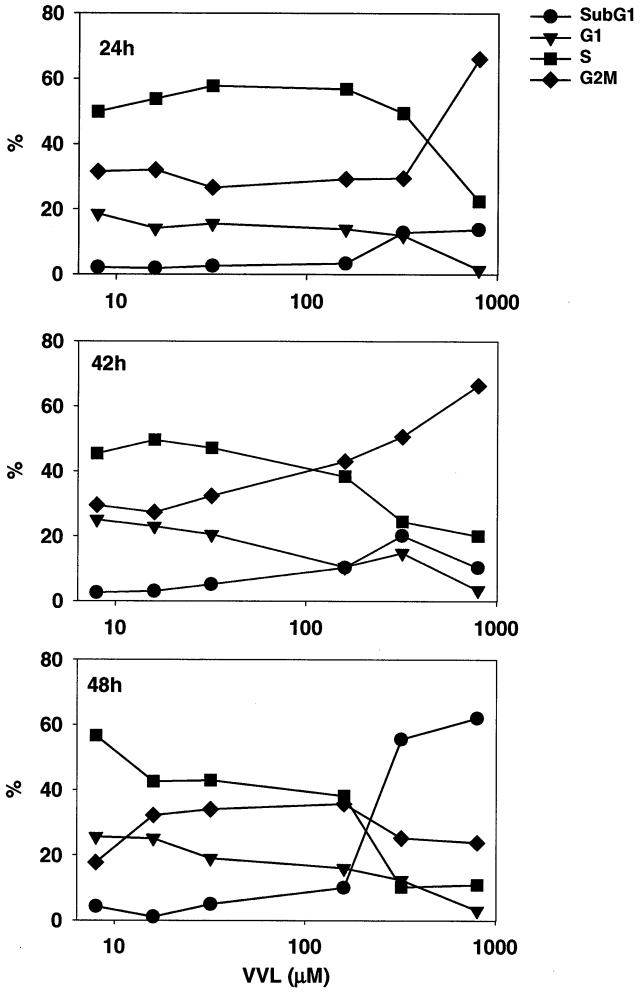

Fig. 4. Flow cytometric analysis of S180 cells treated with various doses of VVL for 24, 42, and 48 hr showing that the DNA patterns of the cells were similar to the control until the doses of VVL increased from 0.32 to 0.8 μM.

B chain binds cell surface glycoproteins, facilitating entry of the toxic A chain into the cytoplasm, where it then exerts its toxic effect on the target cell. A number of toxic lectins that kill cells at nanomolar levels have been identified. These include ricin, mistletoe lectin (ML-I), and abrin [11–13]. The IC_{50} of ricin on rat hepatoma cells is 2.2 nM [14], that of mistletoe lectin (ML-I) on K562 cells is 0.65 μM [15], and that of abrin on human colorectal cancer cells is 1.5 nM [16]. VVL, a fungal lectin of molecular mass 30 kDa isolated from the straw mushroom *Volvariella volvacea*, is a homodimer [1] that possesses immunomodulatory activity on mouse splenocytes, but that also exerts strong antiproliferative activity on cultured tumor cell lines at submicromolar levels ($IC_{50} \approx 0.35$ μM, Fig. 1).

One factor underlying the mechanism of VVL cytotoxicity could be interference with protein translation. For example, the A chain of abrin is an *N*-glycosidase that inactivates the 60S subunit of eukaryotic ribosomes by cleaving the adenine–ribose bond of residue 4324 in 28S rRNA [5]. This results in inhibition of protein synthesis and

confers on abrin the designation of 'ribosome-inactivating protein' (RIP). Our data with VVL demonstrate only low levels of ribosome-inactivating activity compared to the potent RIP, ricin (IC_{50} = 1 nM for ricin vs 29 μM for VVL), thus excluding the possibility of ribosome inhibition as a major factor in the mechanism of VVL's antiproliferative activity.

Evidence was found for the point in the cell cycle at which VVL-treated cells were blocked. A normal cell cycle was measured when S180 cells were exposed to low doses of VVL (Fig. 4), but a 2-fold increase in cells arrested at the G2/M phase of the cell cycle was noted when the concentration of VVL was increased from 0.16 to 0.8 μM.

Three sets of factors involved in governing progress through the cell cycle are cyclins, CDKs, and their inhibitors, CKIs. VVL had very little effect on expression of cyclin genes A, D1, and E. Normal levels of expression were maintained until a dose of 0.8 μM VVL, where total lethality was observed. On the other hand, expression of the CKI genes *p21*, *p27*, *p53*, and *Rb* exhibited a dramatic, dose-dependent increase from nearly undetectable in untreated cells to much higher levels at VVL concentrations of 0.16 μM or above (Fig. 3).

There are two families of CKIs, namely the INK4 family (p15, p16, p18, and p19) and the Cip/Kip family (p21, p27, and p53) [17]. The INK4 family members inactivate the cyclin–CDK complex, while Cip/Kip members recognize a broad range of cyclin–CDK targets. In normal cells, p21 forms a quaternary complex with a cyclin, a CDK, and proliferating cell nuclear antigen (PCNA) [18]. p21 not only controls CDK activity, but also directly inhibits PCNA-dependent DNA polymerase activity [19], even in the absence of a cyclin/CDK [20]. The tumor suppressor gene, *p53*, controls the expression of p21, thus further interfering with DNA replication [20]. Based upon the present findings, perturbations in some of these molecular interactions could be involved in the mechanism underlying the cytotoxic action of the straw mushroom lectin, VVL.

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