Analysis of Cell Cycle Gene Expression Responding to Acetoxyscirpendiol Isolated from Paecilomyces tenuipes

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Paecilomyces tenuipes is believed to contain potential oncostatic and tumor-reducing components. Molecular mechanism, however, is poorly understood concerning the potential antitumor components and their biological function. We purified acetoxyscirpendiol (ASD) from methanolic extracts (MPT) of the fungus and tested the two compounds for the molecular profile of their antitumor potential. Using a differential display protocol, cyclin C and Mad-1 were identified as candidate genes responding to MPT. When a quantitative PCR was performed on the total RNA from MCF-7 treated by MPT or ASD, gene expressions of cyclin C and Mad-1 were greatly augmented. In terms of protein expression, cyclin C level increased up to 12 folds in response to ASD as well as MPT. Similar as MPT treatments, ASD-treated cells synthesize cyclin C as 2—4 fold compared to the control treatments. In terms of Mad-1 expression in cells treated with ASD, the level of Mad-1 expression increased up to 2.5 folds by MPT treatment. Cyclin C expression was compared with non-treated cells in various cell lines. MCF-7 cell was shown highly responsive to the MPT or ASD treatment. Taken together, these results strongly indicate that MPT contains potential antitumor components which might exert their action by modulating cell cycle-related genes such as cyclin C and Mad-1 in MCF-7. The major onciongenic component in MPT may be ASD which modulates cyclin C and Mad-1 expression.

Key words acetoxyscirpendiol; cell cycle gene; Paecilomyces tenuipes; cyclin C; Mad-1; gene expression

Paecilomyces tenuipes, a caterpillar fungus, derives nutrients from host bodies while growing in the host insects. This entomogenous mycelium serves a valuable source in medicinal remedies and in health promotion. In the Orient, this fungus has been used as herbal medicine to treat allergic diseases, asthma, cancer, and tuberculosis.\(^1,2\) P. tenuipes has been known to have novel ingredients inducing differentiation and to inhibit cell growth in various malignant cell types. Thus P. tenuipes is believed to contain potential oncostatic and tumor-reducing components. In addition, P. tenuipes has also been shown to induce apoptosis in human breast cancer and leukemic cell lines.\(^3\) However, a major shortcoming in the use of P. tenuipes in cancer treatment is the poor knowledge about antitumor components and their biological function. In order to identify a potential antitumor component, P. tenuipes was extracted in methanol and the extract was tested for its antitumor potential in terms of cell cycle related gene expression.

Numerous genes are modulated in their expression by antitumor agents. Only a minority of these genes has been identified to date. Only a few cell cycle regulated genes, such as the proto-oncogene c-fos and the cyclin-depenent kinase (CDK) inhibitor p21/WAF1/CIP1, are considered as candidates.\(^4—6\) Cell cycle progression from G1 to S phase is regulated by phosphorylation of proteins by cyclin/CDK complexes.\(^7\) CDKs are serine-threonine kinases that drive the cell cycle through phosphorylation of a number of key substrates. The most interesting of these distinct cyclins appears to be cyclin C, but no direct support in the promotion of cell cycle progression has been observed to date.

Nam and co-workers recently purified acetoxyscirpendiol (4β-acetoxyscirpine-3α,15-diol, ASD) from the methanolic fraction of fruit bodies of P. tenuipes (MPT).\(^3,4\) ASD is a trichothecone with a monoacetoxyl group at the C-4 position. All trichothecones in common have a 9,10 double bond and a 12,13 epoxide group. Trichothecones are biosynthesized in numerous fungi imperfecti and has been known as its cytotoxicity. Trichothecone family compounds cause apoptosis in vivo and in vitro.\(^9,10\) The cytotoxic activities of the trichothecones are well-documented in mammalian tissue culture resulting from inhibition of protein and DNA. Some trichothecones inhibit a variety of steps in translation including initiation process and elongation steps.

According to a recent report, ASD from the fruiting bodies of Isaria japonica induces apoptosis by activating caspase-3 in human leukemia HL-60 cells.\(^11\) Some trichothecones induced apoptosis by activating c-Jun N-terminal kinase and p38 mitogen-activated protein kinase.\(^10,12\) Other trichothecones also induced apoptosis by activating mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated protein kinase (ERK), p38 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in myeloid cells.\(^9\)

This study was designed to identify cell cycle related genes which are modulated by compounds in P. tenuipes. When cells were treated with MPT or ASD, the differential display procedure revealed that the cyclin C and Mad-1, cell cycle genes, were shown upregulated. These genes may serve as candidates responding to the components in MPT or ASD. Analyses on mRNA and protein expression confirmed that they acted as primary genes responding to components in P. tenuipes.

MATERIALS AND METHODS

Compounds and Cell Culture Conditions Methanolic extracts (MPT) were prepared from fruit bodies of P. tenuipes, according to Nam et al.\(^3\) Acetoxyscirpendiol (4β-acetoxyscirpine-3,15-diol, C₂₃H₃₄O₆, FW = 324) was isolated from the methanolic extracts. HeLa, MCF-7, HepG2 and Hep293 cells were maintained routinely at 37.5 °C in DMEM supplemented with 2 mM l-glutamine, 100 U/ml penicillin-
streiitol, and 200 U of M-MLV reverse transcriptase, in a final
thus providing a value for relative cyclin C and Mad-1
ratio of PCR products and unincorporated primers was nor-
electrophoresis on 2% agarose gels. The gels were dried and
confirmed by restriction analysis. Samples were subjected to
0.5% TBE buffer. Specificity of PCR products was con-
unincorporated primers on 5% non-denaturing primers on
within its linear range.12) First-strand cDNA synthesis was
100 U of M-MLV reverse transcriptase, in the presence of
10 μg of RNA, 0.25 μg of oligo(dT11) primer (AAGCTTGG),
0.5 mM each of dNTPs, and first strand buffer (25 mM
Tris–HCl pH 8.3, 37.5 mM KCl, 1.5 mM MgCl2, 5 mM dithio-
threitol, and 200 U of M-MLV reverse transcriptase, in a final
preparation was as follows: After initial denaturation for 5 min
(H11032)_3; cyclin-C, 5′-ACACCAA-GATGAGCCAACGT-3′;
actin, 5′-TGAAAGATGATGATGCCTG-3′ and 5′-CCA-
ACCTGCTCAGATACATC-3′.

Amplified PCR products were labeled at the 5′-end using
[33P]-ATP and T4 polynucleotide kinase and separated from
unincorporated primers using a 6% non-denaturing polyacrylamide gel in a TBE buffer. At the termina-
tion of electrophoresis, gel was subjected to the silver
staining. Bands with varying intensity of expression patterns
in comparison to control lanes were excised and eluted in
5% non-denaturing primers on
10% low fat milk. After copious washing with
PBST, the immunoblots were further incubated for 1 h at
room temperature with either a secondary horseradish perox-
idasyme conjugated anti-rabbit IgG antibody in PBS containing
10% low fat milk, or a secondary anti-goat IgG. Subse-
quent ECL detection was performed according to the manu-
facturer’s recommendations (Amersham). Levels of protein
expression were quantified by the use of a Packard Cyclone
reader. Equivalent loading of protein samples was ensured by
immunodetection with an anti-actin polyclonal antibody and
additional Coomassie blue staining of respective blots.

RESULTS

Isolation and Sequence Analysis of Primary MPT Re-
spounding Genes Gene expression pattern was analyzed ac-
cording to the differential display method on the total RNA
isolates from MCF-7 cells treated with MPT at 0.1—0.5%
(w/v) in the incubation media for 2h. Differential display
analysis revealed that various genes varied their expression
under the MPT treatment. Several of the cDNA bands repre-
senting gene transcripts disappeared or decreased in MCF-7,
whereas others increased or were exclusive to the latter. Fig-
ure 1 shows such bands with varied intensity of expression patterns in comparison to the control lane representing cells
treated only with DMSO. Seventeen candidate bands were
recognized using 9 different combinations of upstream and
downstream primers. Among the positive bands, 6 promising
bands were further selected based on the difference in the
band intensity. Each DNA was isolated, subcloned and se-
querenced. Sequence data indicated cyclin C and Mad-1 (mi-
obrotic arrest deficient like-1) as the cell cycle-related genes re-
sponding to components in MPT. Other four candidate genes
are as yet unidentified or insignificant and omitted from fur-
ther study.

Quantitative Assessment of mRNA Expression by RT-
PCR Quantitative PCR analysis of Mad-1 showed that
Mad-1 transcription was significantly upregulated by MPT or
ASD in MCF-7 cells. MCF-7 cells were treated with 0.5%
MPT or 5 mM ASD and total RNA was isolated following
various treatment periods. The total RNA extracts were re-
verse-transcribed into cDNA and subjected to the quantita-
tive PCR. The relative mRNA expression is calculated as the
ratio of each expression against control and normalized for
actin mRNA expression. Figures 2 show the time course of
cyclin C and Mad-1 mRNA expression in response to the
MPT or ASD treatment. MCF-7 cells were treated for 1, 2, 4,
and 24 h with MPT and were compared with control cells incubated in the media containing 0.1% DMSO for 2 h. Cyclin C mRNA expression appeared to be upregulated by MPT (Fig. 2A). In the case of ASD, very similar expression pattern was observed during the treatment despite less dramatic difference in comparison to the MPT treatment. Fig. 2B shows that Mad-1 mRNA was upregulated with MPT. Similar as MPT treatment, an increased level of Mad-1 transcription was also apparent when MCF-7 cells were treated with ASD. This consistent expression pattern, between the two classes of compound, strongly implies that ASD may be the major functional components modulating genes of cyclin C and Mad-1 in the MPT fraction.

**Western Blotting** Western blot analyses were performed in order to quantify the affected expression of these genes at the protein level during time course experiments (Fig. 3). Actin was used as standard for the total amount of proteins loaded on the gel. The relative protein expression was calculated with the level of protein expression for the non-treated as 1 and corrected according to the actin signal. Results showed that cyclin C expression was greatly increased in MCF-7 cell during MPT treatment. This increase coincides with that of Mad-1 while the two proteins remained less affected in other cell lines used in this study. Under ASD treatment, similar increase in the expression of the two proteins was also observed. These results indicate that components in MPT not only increased the mRNA expression of these genes but also increased the expression of the protein. Most likely, ASD is the major functional component in modulating cyclin C and Mad-1 expression.

The degree of cyclin C expression varied significantly among the cell lines tested, being elevated in MCF-7. As for Mad-1, similar expression pattern was observed. Fig. 4 shows that ASD and MPT induce cyclin C and Mad-1 expression especially in MCF-7 among other cell lines. In HeLa (cervical cancer), HepG2, and human embryonic kidney (HEK) 293, less significant change was observed in terms of expressed cyclin C.

Among cell lines, relative protein expression was mea-
Cells were treated for 24 h with MPT or ASD as delivered in methanol. Cyclin C expression was compared among four different cell lines. Protein expression was quantified by fluorimaging analysis and normalized to actin protein expression. Values of relative expression were obtained as fold induction against control treatments including 0.1% DMSO only. Columns refer to mean values of Western blots (n=5); bars, standard deviations (S.D.).

As in Fig. 5, Mad-1 expression in cells treated with MPT or ASD was compared among the cell lines. Cellular extracts were subjected to immunoblotting following electrophoresis on a 12% SDS PAGE. Relative protein expression was quantified and normalized to actin protein expression. Values obtained for 24 h treatments were expressed in comparison to the fold induction of control treatments (0.1% DMSO). Columns, mean values of Western blots; bars (n=5); bars, S.D.

FIG. 5. Cyclin C Protein Expression in Various Cell Lines

Fig. 6. Mad-1 Protein Expression Comparison

As in Fig. 5, Mad-1 expression in cells treated with MPT or ASD was compared among the cell lines. Cellular extracts were subjected to immunoblotting following electrophoresis on a 12% SDS PAGE. Relative protein expression was quantified and normalized to actin protein expression. Values obtained for 24 h treatments were expressed in comparison to the fold induction of control treatments (0.1% DMSO). Columns, mean values of Western blots; bars (n=5); bars, S.D.

The mitotic arrest deficient like-1 (Mad-1) is a key component of the mitotic checkpoint system. According to recent reports, mutation and decreased expression of mitotic checkpoint genes including Mad-1 are evident in cancer cell lines with defective mitotic checkpoint. The biological implications of the overexpressed Mad-1 in cancer cells are unknown. Overexpression of Mad-1, however, inhibited cell proliferation, S-phase entry, and colony formation, changes that were accompanied by a reduction in cdc20 activity.

Interaction of Mad proteins in the checkpoint is currently unclear. When hyperphosphorylated, however, Mad1 helps forming Mad2 multimers at unattached kinetochores. Mad1 affects the ability of Mad2 and Mad3 to interact stably with cdc20, a substrate adaptor and activator of the anaphase-promoting complex (APC). The Mad1-Mad2 complex could provide a structural framework for the assembly of Mad protein complexes. The structural framework could regulate their ability to interact with the APC and its associated regulators cdc20. The overexpression of Mad-1 observed in this study would play an important role in inhibiting APC activity by promoting the Mad1-Mad2 complex, eventually retarding to elucidate simultaneous upregulation of cyclin C and Mad-1 under MPT or ASD treatment. No report is available to date that cyclin C expression affects Mad-1 expression or vice versa. As a potential explanation, the two genes may have a common upstream responsive element to ASD. Future study is necessary to dissect the upstream regions or to locate transacting elements whose expression is modulated by ASD.

Cyclin C belongs to the cyclin family of proteins that control cell cycle transitions through activation of specific catalytic subunits, the cyclin-dependent kinases (CDKs). The periodic degradation of typical cyclins is crucial for cell-cycle progression and depends on the catalytic activity of the associated CDK. No direct evidence, however, is apparent that cyclin C and cdk8 play an important role in promoting cell cycle progression. Cyclin C-cdk8 rather controls the RNA polymerase II transcription machinery considering the cyclin C-cdk8 complex is found associated with the RNA polymerase II. Upregulation of cyclin C upon MPT treatment may promote RNA polymerase II activity, thus eventually inducing expression of genes for cell growth arrest, differentiation and even apoptosis. This may provide a novel insight into the mechanisms of cell cycle gene products leading to general transcriptional regulation by MPT and ASD.

Upregulation of cyclin C under MPT treatment provides unprecedented outlook for the mechanisms of cell cycle and general transcriptional regulation by MPT and ASD. This study presents MPT and ASD are effective especially in ER-positive MCF-7 breast cancer cell lines. Both agents are able to retard the progression of breast tumors pointing to the possible therapeutic application of these analogues in breast cancer. Compared to MCF-7, however, neither of Mad-1 and cyclin C expression was shown significantly modulated in other cell lines tested in this study. The difference is somewhat puzzling. Among many potential explanations, ASD may have been taken up by MCF-7 cells with better efficiency than other cell lines. Receptors specific to MCF-7 may act differently to ASD and other related functional components in MPT. Further study may be required to determine the difference of ASD uptake among various cell lines.

The study would play an important role in inhibiting APC activity by promoting the Mad1-Mad2 complex, eventually retarding...
Taken together with the expression pattern of cyclin c expression under MPT or ASD treatment, these results suggest that Mad-1 plays an important role in inhibiting cell proliferation of MCF-7, especially. According to the data presented, the cell cycle gene serve as a prime factor for the inhibitory effect of MPT and ASD against cancer cells. In conclusion, these results suggest that tumor suppression may be achieved through the concerted action of cyclin C and Mad-1. These genes may represent molecular targets whose modulation may trigger a tumor suppressive potential that is otherwise dormant in cancer cells. Further, these components have the eligible profile to be tested as therapeutic agents for the treatment of hyperproliferative diseases such as breast cancer.

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