Medicinal mushroom extracts inhibit ras-induced cell transformation and the inhibitory effect requires the presence of normal cells

W.L. Wendy Hsiao1,2,4, You Quan Li2, Tin Lap Lee2, Ning Li2, Marilyn M. You3 and Shu-ting Chang3

1Biomedical Science, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, 2Department of Biology, Hong Kong University of Science and Technology, Hong Kong and 3Department of Biology, Chinese University of Hong Kong, Hong Kong, China

Previously, we developed a simple Rat 6 (R6) cell system by which the inhibitory effects of non-cytotoxic chemicals can be assessed by focus formation assay upon transfection of ras oncogene to the host cells. Using this system, two well studied medicinal mushrooms Ganoderma lucidum and Tricholoma lobanense with anticancer potential were examined for their possible advent effects on cell transformation induced by ras oncogene. Results indicated that both species of mushrooms yielded strong inhibitory effects on ras-induced cell transformation. Further study on T. lobanense indicated that the DEAE-column-bound, polysaccharides (PS)-peptide enriched, but not the unbound fraction, showed strong inhibition in a dosage-dependent manner. Subsequent time course study revealed that the continued presence of the extract in the transfected cultures was required for a maximum inhibitory effect. At the same time, we also observed that significant levels of inhibition occurred even when the application of the extract was delayed until day 12 after transfection. Using a stable transformed cell line, R6/GFP-Ras expressing green fluorescent protein-ras fusion protein in a co-culture assay with normal R6 cells, we demonstrated that R6/GFP-Ras cells grew into green fluorescent foci with striking transforming morphology in the absence of extracts. However, in the presence of extracts, R6/GFP-Ras cells, in most cases, remained as small colonies compiled with only a few green fluorescent cells. Moreover, the inhibitory effect requires the presence of R6 cells. In our study, mushroom extracts have no effect on the growth of individually cultured normal or transformed R6 cells. It is noteworthy that the extracts do not affect the level, or the subcellular localization of the Ras protein. Collectively, the data strongly suggest that the inhibitory effect of the mushroom extracts is not due to a direct killing of the transformed cells, rather, it may be mediated through the surrounding normal R6. While the general understanding of the antitumor effect of PS and PSPC is mediated through the cytokines released by activated macrophages and T-lymphocytes, our data may provide a novel alternative mechanism that the mushroom PS peptides may exert anticancer effect by targeting the ras-mediated signaling pathway.

Introduction

The antitumor effect of mushrooms has long been observed in Asia, especially in China and Japan. The analysis of various species of mushrooms has resulted in the identification of a family of high molecular weight, hot-water-soluble polysaccharides (PS) and polysaccharide-peptide complexes (PSPC), which have tested positive for antitumor activities in animal studies (1). NMR analysis reveals that the antitumor PS are composed of a variety of linear and branched glucans. They appear in various conformations, and some are in the gel state. As a result of the complexity and heterogeneity of PS and PSPC and limited suitable bioassay systems, the mechanism of action of these PS remain obscure. Until now, research on the antitumor activities is based mainly on the results derived from experiments with implanted Sarcoma 180 or chemically induced tumors in mice (2–6). Antimitotic tests in tumor cell lines have been, so far, rather inconsistent or negative (1, 2, 4, 7–9). Aside from their antitumor effects, mushroom-derived PS and PSPC seem to function as immunomodulators; this was observed in animals as well as in cultured macrophages and T-lymphocytes. It has been postulated that the antitumor effect of PS and PSPC may be mediated through the cytokines released by activated macrophages and T-lymphocytes, instead of through direct cytoidal effects on tumor cells (4, 8–11). In order to gain a more comprehensive understanding, it is necessary to investigate the antitumor activity of mushrooms in a wider range of cell systems.

Previously, we have demonstrated that the established rodent cell line R6 is resistant to transformation induced by a potent c-H-ras (T24) oncogene in a focus formation assay. The transforming efficiency of T24, however, can be modulated by treatment with various tumor promoters and factors (12, 13). Using this R6/ras assay system, we have assessed the inhibitory or enhancing effects of various chemicals on T24 induced-transformation (14–16). In the current study, we explored the antitumor activity of mushrooms using the focus formation assay built around the R6/ras model system.

In the study, we focused on two medicinal mushrooms: Ganoderma lucidum and Tricholoma lobanense. Both mushrooms exhibit antitumor activities, based mainly on animal studies (1–4, 17). Tricholoma lobanense is a native Hong Kong species. Ganoderma lucidum is an important traditional medicine in China and Japan, used for promoting health and treatment of various diseases, including cancer. Ganoderma lucidum is also a better documented natural product in terms of its pharmacological and chemical properties. Our results showed that ras-induced transformed foci were effectively inhibited by the addition of extracts of G. lucidum and T. lobanense in dosage-dependent and time-dependent

Abbreviations: DMEM, Dulbecco’s modified Eagle medium; D10CS, Dulbecco’s modified Eagle medium supplemented with 10% calf serum; GFP, green fluorescent protein; PS, polysaccharides; PSPC, polysaccharide-peptide complexes; T24, activated human c-H-ras oncogene.
manner. Data also revealed that the PS fraction of \textit{T. lobayense} would only exert an inhibitory effect on Ras-transformed cells when cells were co-cultivated with normal R6 cells, suggesting a novel mechanism in which the inhibitory effect of PS is mediated through the surrounding normal R6 cells.

Materials and methods

Preparations of mushroom samples

Fruiting bodies of \textit{G. lucidum} were homogenized and extracted with boiling distilled water for 6 h to obtain the PS-enriched preparations. After centrifugation to remove the insoluble portion, the water-soluble extracts were lyophilized, then kept at room temperature for later usage. Liquid mycelium cultures of \textit{G. lucidum} were also used to obtain PS-extract. Prior to extraction, the cultures were filtered and precipitated with ethanol according to Liu et al. (17). The precipitates were dissolved in distilled water, centrifuged to remove the insoluble, then lyophilized and designated as mycelium filtrate. \textit{Tricholoma lobayense} was originally isolated and established in cultures by S.T.Chang’s Laboratory at the Chinese University of Hong Kong, Hong Kong. \textit{Tricholoma lobayense} was cultured in nutrient broth as described above. Liquid cultures containing the secreted fungal PS were prepared as above. The water-soluble, PS-enriched components were lyophilized and designated as mycelium filtrate. Part of the filtrate was further fractionated into the unbound Fraction A1 and the salt-eluted bound fraction A2 using a DEAE-cellulose ion exchange column chromatography (17). Both fractions were dialyzed against ddH$_2$O and lyophilized for later use.

Cell cultures and plasmids

The Rat 6 (R6) cell line was a subclone of the Fisher rat embryo fibroblasts originating from Freeman’s Laboratory (18). R6/T24 cell line is a clonal R6 cell line transformed by the activated human c-Ha-ras oncogene (12). The R6 green fluorescent protein GFP-Ras cell line is a transformed clonal cell line established from a transformed focus derived from R6 cultures transfected by a GFP-ras fusion vector in our lab. Cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% calf serum (D10CS) and 1% ASP, designated as mycelium filtrate. Both the total, and the DEAE-column-bound fraction of \textit{G. lucidum} were used to obtain PS-extract. Prior to extraction, the mushroom preparations were lyophilized and designated as mycelium filtrate. Both the total filtrate and the DEAE-column-bound fraction of \textit{G. lucidum} were used to obtain PS-extract. Both the total, and the DEAE-column-bound fraction of \textit{G. lucidum} were used to obtain PS-extract. Both the total, and the DEAE-column-bound fraction of \textit{G. lucidum} were used to obtain PS-extract. Both the total, and the DEAE-column-bound fraction of \textit{G. lucidum} were used to obtain PS-extract. Both the total, and the DEAE-column-bound fraction of \textit{G. lucidum} were used to obtain PS-extract.
DEAE-column-bound fraction A2 of *T. lobayense* markedly inhibited *ras*-foci, while the unbound fraction produced no effect (Table III and Figure 1). The preparations had no or slightly toxic effect on either normal or transformed R6 cells (Table IV). The nutrient broth used for the *T. lobayense* cultures alone presented no inhibitory effect.

**Dosage and time course studies of Tricholoma filtrate on ras-induced transformation**

The inhibitory effect of the *Tricholoma* filtrate was further explored with regard to dosage and duration of treatment. Results revealed that the inhibitory effect of *Tricholoma* filtrate was dosage-dependent. Extracts in concentration as low as 1 μg/ml exerted a 19% inhibitory effect on the formation of *ras*-foci (Table V). Time course study indicated that the maximal effect was obtained when transfected cultures were treated with *Tricholoma* filtrate from days 4 to 20 after the transfection. Interestingly, a 32% reduction in foci number was still obtained when the treatment was delayed until day 12 after the transfection of the *ras* oncogene (Figure 2).

**Effect of Tricholoma filtrate on R6/GFP-Ras cells co-cultivated with normal R6 cells**

To further explore the nature of the inhibitory effect of *Tricholoma* filtrate, we reconstituted the focus formation assay by seeding 500 R6/GFP-Ras cells on a 90 mm culture plate pre-seeded with 2.5 × 10^5 normal R6 cells 24 h earlier. The co-cultures were then maintained in D5CS medium in the presence and absence of *Tricholoma* filtrate. The effect of *Tricholoma* on colony formations of individual R6 and R6/GFP-Ras cell lines were conducted in parallel with the coculture assay. Results indicated that addition of *Tricholoma* did not affect the colony formation (Figure 3A and B), nor the morphology of R6 or R6/GFP-Ras cells (data not shown). In the co-culture of R6 and R6/GFP-Ras (Figure 3C), the R6/GFP-Ras cells formed many dense transformed colonies on the top of the monolayer of R6 cells in the absence of treatment, resembling the formation of transformed foci shown in Figure 1. Addition of *Tricholoma* filtrate (200 μg/ml) effectively blocked the formation of the GFP-Ras transformed colonies. Indeed, under the fluorescent microscope, the green fluorescent colonies, representing the R6/GFP-Ras cells, were severely retarded in the presence of the *Tricholoma* filtrate, while grown to sizable colonies in the absence of the drug treatment (Figure 3). It is worthy to note that treatment with *Tricholoma* does not affect the initial plating efficiency of R6/GFP-Ras cells as nearly the same number of GFP-positive cells were observed on both the treated and untreated plates under a fluorescent microscope, 24 h upon the addition of the filtrate. Additional evidence came from the fact that early withdrawal of the treatment

![Image](image_url)
substantially reduces the inhibitory effect of the filtrate, resembling what we observed in the focus formation assay (data not shown).

**Tricholoma filtrate posted no effect on the expression of GFP-Ras protein**

To determine whether the retardation of R6/GFP-Ras colonies is due to the suppression of Ras protein, protein extracts derived from the treated and untreated R6 and R6/GFP-Ras cells were examined for Ras protein expression using western blot analysis. Result showed that addition of *Tricholoma* filtrate to the cultures did not reduce the level of GFP-Ras fusion protein expression identified with either anti-Ras or anti-GFP antibody (Figure 4). The same blot was hybridized with anti-actin antibody as a protein loading control. Aside from the protein level, *Tricholoma* treatment did not alter the normal subcellular localization of the GFP-tagged Ras protein, nor the transforming morphology of GFP-Ras cells as it shows in Figure 5. In that, the GFP-Ras proteins are correctly localized in the inner surface of the plasma membrane of the untreated (Figure 5A–C) as well as of the treated (Figure 5D–E) R6/GFP-Ras cells co-cultivated with R6 cells as described in the experiments shown in Figure 3C. No alteration of subcellular localization of GFP-Ras proteins was seen when R6/GFP-Ras cells were grown alone and treated with *Tricholoma* filtrate (data not shown).

**Discussion**

The antitumor effects of PS and PSPC of higher fungi have long been investigated in tissue cultures, animal models and clinical patients, yet the mechanism underlying the action of PS is unclear. Considerable evidence suggests that the antitumor effect of PS is mediated through the cytokines released by activated macrophages and T-lymphocytes, instead of through direct cytocidal effects on tumor cells (4,8–11). In order to explore other possible cell-mediated responses to PS, we employed a non-lymphocytic *in vitro* cell system and tested the antitumor activity of PS against cell transformation induced by a defined ras oncogene. The results showed that both the fruiting bodies and the cultured mycelia of *G. lucidum*, a medicinal mushroom well known for its antitumor activity, markedly inhibited the formation of ras-induced transformed foci assessed by the focus formation assay in the R6 embryo fibroblast cell line. Interestingly, none of the PS preparations were toxic to either normal or ras-transformed R6 cells. Thus, the inhibitory effect of the mushroom extracts is not due to a direct cell killing of the transformed cells used in the study. This finding is consistent with the observations made in lymphatic cell system in which no cytotoxicity was detected under the treatment of *G. lucidum* (4,9,10).

The inhibitory effect was not restricted to *G. lucidum*. When we examined culture filtrate from *Tricholoma*, similar results...

---

**Fig. 2.** Time course studies of the effect of PSPC-enriched fraction A2 of *Tricholoma* filtrate on the number of transformed foci in R6 cultures transfected (at day 0) with pT24 plasmid DNA. Where indicated, transfected cultures were treated with 100 μg/ml of A2 fraction of *Tricholoma* filtrate described in Table III. Experiments were terminated at day 20, and stained with Giemsa stain for scoring. Relative number of foci is the ratio of foci obtained in the presence of drug to that in the absence of drug (i.e. no treatment control).

**Fig. 3.** Effect of PSPC-enriched fraction A2 of *Tricholoma* filtrate on colony formation of normal and transformed R6 cells. (A and B) 500 of R6 or R6/GFP-Ras-transformed cells per 90 mm plate were seeded in DMEM plus D10CS in the presence and absence of *Tricholoma* filtrate A2 fraction (200 μg/ml). (C) 500 R6/GFP-Ras cells were seeded on a lawn of 2.5 × 10⁵ normal R6 cells plated in 90 mm plate 24 h earlier. The *Tricholoma* filtrate A2 fraction (200 μg/ml) was then added to the co-cultures of normal and transformed R6 cells grown in D5CS 24 h after the seeding of the transformed cells. At the end of 2 weeks, culture plates were fixed with 10% formaldehyde, stained with Giemsa stain and photographed. GFP-Ras-colonies derived from the co-cultures were viewed and photographed under a fluorescent microscope at 100 and 400× magnifications.

**Fig. 4.** Effect of PSPC-enriched fraction A2 of *Tricholoma* filtrate on Ras protein expression in normal and transformed Rat 6 cells. 2.5 × 10⁵ R6 or R6/GFP-Ras-transformed cells per 90 mm plate were seeded in DMEM plus D10CS. Next day, cultures were fed with fresh medium in the presence (+) and absence (−) of *Tricholoma* filtrate (A2) (200 μg/ml) and fed twice a week. At the end of 14 days, cells were washed with cold PBS, lysed in NET buffer plus protease inhibitors and collected for western blot analysis as described in the Materials and methods. Protein extracts obtained from the treated and untreated R6 and R6/GFP-Ras cultures were loaded 40 μg/lane and separated on 12% PAGE gel by electrophoresis. The resulting blot was hybridized sequentially with anti-Ras (Santa Cutz), anti-GFP (Clontech) and anti-actin (Santa Cutz) antibodies and visualized using Amersham ECL Western Blotting Detection Kit according to the manufacturer manual.

**Fig. 5.** Effect of PSPC-enriched fraction A2 of *Tricholoma* filtrate on the subcellular localization of GFP-Ras protein in GFP-ras-transformed R6 cells co-cultivated with normal R6 cells. The co-cultures were prepared as described in Figure 3 legend, and treated (+) or untreated (−) with *Tricholoma* filtrate (200 μg/ml) for 7 days. (A–C) Fluorescent microscopic views of three individual R6/GFP-Ras colonies derived from the untreated co-cultures; (D–F) three individual colonies derived from the treated co-cultures.
Mushroom extracts inhibit cell transformation by ras

Fig. 3.

Fig. 4.

Fig. 5.
were obtained. In the previous study, an antitumor component was identified in the culture filtrate of *T. lobayense* (17). The active component, based on tumorigenesis studies in animals, was found to reside in the DEAE-cellulose ion exchange column bound fraction, but not in the unbound fraction. Further characterization of the bound fraction showed that the fraction is a PSPC with a molecular weight of 154 kDa. It is intriguing that the levels of inhibitory activity of the DEAE-bound and -unbound fractions of *Tricholoma* assessed by focus formation assay were remarkably similar to those obtained by animal tumorigenesis test studied by Liu *et al.* (17) (Table VI). The inhibitory activity of the bound fraction appeared to be dosage-dependent. Based on the time course study, early withdrawal of the component impaired the full activity of the PSPC as shown in Figure 2B–D. On the other hand, a 32% inhibition was still observed when the addition of the chemical was delayed until day 12 (Figure 2G). In fact, our study shows that the duration, rather than the time of application, dictates the efficacy of the compound, as demonstrated by the relative number of foci of groups B and H, C and G and D and F. Each group of the pair received the same duration, but different time frame of treatment, yet each yielded a similar number of foci. We also observed that the relative foci decreased from 0.32 to 0.04 when the experiment was carried out for 24 (Table III), instead of 20 days (Figure 2). This result reiterates the tentative conclusion that it is the length, not the time frame, of treatment that is more critical in determining the extent of inhibition of transformed foci.

The mechanism underlying the inhibitory effect of PS or PSPC against ras-foci remains unclear. In this study, the ras-transformed cells in focus formation were effectively inhibited during the early stage of transformation, and were equally inhibited when the stably transformed cells were mixed with normal cells, then treated with PS extract in the co-culture assay (Figure 3C). Three key findings in this study may shed light on the possible mode of inhibition against ras-foci. First, treatment with the extracts posts no cytotoxic effect on either normal or established ras-transformed cell line assessed by the cell proliferation test and colony formation assay (Table II and Figure 3A and B). Secondly, mushroom extracts do not block the expression (Figure 4), nor alter the membranous localization and the transforming activity of the ras oncogene tagged with GFP displayed in R6/GFP-ras cells (Figure 5). Thirdly, the inhibitory effect of mushroom extracts against ras-transformed cells requires the presence of normal cells. The last point was well illustrated in the co-culture experiment, in which the colony formation of R6/GFP-ras cells was only inhibited in the present, but not in the absence of the co-cultured normal R6 cells (Figure 3C). As mentioned earlier, treatment with *Tricholoma* does not affect cell adhesion as the number of seeded R6/GFP-Ras cells found in the treated cultures was similar to that found in the untreated cultures. Thus, the mushroom extract seems to exert its opposing effect on cell expansion, rather than on cell adhesion of the transformed cells. An early report indicated that certain triterpenoids from *G. lucidum* inhibited farnesyltransferase activity of Ras protein and retarded the growth of k-ras transformed cells (22). In our case, based on the clear display of the membranous GFP-tagged Ras protein under the treatment with *Tricholoma* filtrate observed in vivo, the PS extract does not seem to act as farnesyltransferase inhibitor. Taking all these observations together, our data suggest that the antitumor effect of PS or PSPC from *G. lucidum* and *T. lobayense* is very likely mediated through the normal Rat 6 host cells, by direct or indirect cell contact. Based on our preliminary investigation, however, inhibition of the *Tricholoma* filtrate on the growth of transformed cells was not apparent when normal and transformed cells were each grown on an individual chamber (upper and lower) separated by a microporous membrane using a Transwell culture chamber system, suggesting that the inhibitory effect of mushroom filtrate may require a direct cell-to-cell contact (data not shown). However, determining the precise target of the PS and PSPC requires further investigation. Our previous works indicate that the transforming ability of the activated ras oncogene can be modulated by various factors and compounds (14–16). Early works by others suggested that the antitumor effect of fungal PS and PSPC is mediated through the cytokine released from the host cells. Later, Wang *et al.* presented evidence that treatment with *G. lucidum* stimulated macrophages and T lymphocytes to release TNF-α and IFN-γ, both of which were cytotoxic to HL-60 and U937 (9,23). Other related studies that may shed light on the mechanism of mushroom extract are the recent works on glucan, a natural PS product widely distributed in fungi. Glucan has been reported to act as immunomodulator and cell response modifier. Binding of glucan to its specific glucan receptors can elicit a serial cellular response through the modulating of activities of various factors including IgE, cytokines, chemokines, transcriptional factors and growth factors (24–26). Interestingly, the bioactive glucan receptors are present in human fibroblasts (26). Whether a similar mechanism applies to the inhibitory effect of mushroom extracts in our cell system warrants further investigation.

This study is the first to demonstrate that the PS- and PSPC-enriched mushroom extracts can inhibit cell transformation induced by a defined oncogene through a novel non-cytocidal route. Ras proteins play a pivotal role in regulating cell growth and the development of human cancer. The demonstration of

<table>
<thead>
<tr>
<th>Material tested</th>
<th>ICR male mice (%) of inhibition</th>
<th>Balb/c male mice (%) of inhibition</th>
<th>Focus formation assay (% of inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>84%</td>
<td>66%</td>
<td>90% (100 μg/ml)</td>
</tr>
<tr>
<td>DEAE-column purified fraction (A2)</td>
<td>96%</td>
<td>50%</td>
<td>96% (100 μg/ml)</td>
</tr>
<tr>
<td>DEAE-unbound fraction (A1)</td>
<td>40%</td>
<td>6%</td>
<td>9% (100 μg/ml)</td>
</tr>
<tr>
<td>No treatment</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Mice were injected intraperitoneally for 10 consecutive days with 20 mg of each tested sample/kg/day or distilled water as a negative control.

Antitumor activities of each sample were tested against the growth of solid S-180 tumor transplanted in both ICR and Balb/c male mice [*animal data was obtained from Liu *et al.* (17)].
the inhibitory effect of mushroom extracts on ras-induced transformation in this current study may have broad implications for cancer prevention and treatment and may provide a better understanding of the underlying mechanism of the cancer inhibitory effect of mushroom PS.

References

Received November 25, 2002; revised December 17, 2003; accepted February 4, 2004