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

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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 lobayense* with anticancer potential were examined for their possible advert 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.lobayense* 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 and 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 marcrophages 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.

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.

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 Sarcoma180 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 cytocidal 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 lobayense*. Both mushrooms exhibit antitumor activities, based mainly on animal studies (1–4,17). *Tricholoma lobayense* 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.lobayense* in dosage-dependent and time-dependent

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manners. Data also revealed that the PS fraction of *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 *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 *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. *Tricholoma lobayense* was originally isolated and established in cultures by S.T.Chang's Laboratory at the Chinese University of Hong Kong, Hong Kong. *Tricholoma lobayense* was cultured in nutrient broth as described (17). 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_2O and lyophilized for later usage.

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) (Invitrogen, Carlsbad, CA). Cultures were maintained in a humidified incubator at 37°C with 5% CO_2 in air and fed twice a week with fresh medium. Plasmid pT24 contains a 6.4 kb *Bam*HI fragment corresponding to the coding sequence of the human bladder c-Ha-*ras* oncogene. The plasmid pT24 was obtained from M.Wigler's Laboratory.

Focus formation assay

The standard focus formation assay and treatment of the cultures were performed as described earlier (12). In brief, 5×10^5 cells seeded in 90 mm plate were transfected with 1 mg T24 plasmid DNA and 20 μg R6 genomic DNA as carrier DNA by DNA-mediated transfection procedure based on Bacchetti and Graham (19) and Wigler *et al*. (20), with slight modifications (12,13). To determine the effects of mushroom extracts on ras-induced focus formation, transfected cultures were fed with DMEM plus 5% fetal calf serum (D5FCS) in the presence and absence of test sample on day 2 upon transfection, then continued feeding with each respective growth medium twice a week throughout the experiment. Lyophilized samples of mushroom preparations were weighted, dissolved in boiling ddH_2O, then centrifuged at 10 000 r.p.m. for 20 min to remove the residues. The supernatant was sterile filtered, then added to the growth medium at designated concentrations. All experiments were performed in six replicate plates.

Cytotoxicity assay

The cytotoxic effect of each sample was performed on both R6 and R6/T24 cell lines. Cells were seeded in triplicate at 10^4/60 mm plate in D10CS. The next day, the test sample was added to the cultures and kept for 5 days. At the end of treatments, cells were trypsinized and counted using a Coulter Counter Fullerton, CA. Cytotoxicity was expressed as percent survival, i.e. cell counts of the treated cultures divided by cell counts obtained from the untreated cultures.

Colony formation and co-culture assays

For a better quantitative assessment of the inhibitory effect for drug testing, we designed a co-culture assay to simulate the focus formation assay. The assay was set up by seeding 500 *ras*-transformed R6 cells on a 90 mm plate in triplicate pre-seeded with 2.5×10^5 normal R6 cells 24 h earlier. A day after the seeding of the transformed R6 cells, the test sample was added to the co-cultures of normal and transformed R6 cells grown in DMEM plus 5% calf serum (D5CS). At the end of 2 weeks, culture plates were fixed with 10% formaldehyde, stained with Giemsa stain and photographed. In order to distinguish the transformed from the neighboring normal R6 cells, a transformed cell line, R6/GFP-Ras expressing green fluorescent GFP-Ras fusion protein was used in the co-culture assay. In a parallel experiment, the possible toxic effect of mushroom extracts on the growth of R6 and R6/GFP-Ras cells were tested by seeding 500 of each cell line separately in D10CS in the presence and absence of test chemical for the duration of 12–14 days. Cultures were stained and scored for total number of colonies per plate. All the experimental cultures were fed twice a week with or without the mushroom extract.

Western blot analysis

R6 or R6/GFP-Ras-transformed cells were seeded 2.5×10^5 per 90 mm plate in DMEM plus D10CS. The next day, cultures were fed with fresh medium in the presence and absence of Tricholoma filtrate A2 fraction (500 μg/ml) and fed twice a week. Cells were washed with cold PBS three times and lysed in 400 μl NET buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, pH 8.0, 1 mM APMSF, 1 μM E-64, 1 μM pepstatin, 100 mM $NaVO_5$ and 10 μg/ml aprotinin) plus 1% NP-40 on ice according to Lu *et al*. (21). For western blotting analysis, 40 μg of protein extracts were loaded on a 12% SDS–PAGE. After separation, the proteins were transferred to a Hybond-C nylon membrane (Amersham, Piscataway, NJ), reacted to either anti-Ras (Santa Cruz) or anti-GFP antibodies (Clontech, Palo Alto, CA) and visualized by the ECL detection kit (Amersham) according to the manufacturer's manual. Blots were hybridized with anti-actin antibodies (Santa Cruz, Santa Cruz, CA) to normalize the gel loading.

Results

Effect of extracts of G.lucidum on ras-induced transformation

In the primary experiment, extracts of fruiting bodies and filtrate of *G.lucidum* were tested. While both the fruiting bodies and mycelium filtrate were effective in inhibiting foci formation induced by c-Ha-*ras* oncogene, the latter seemed to be more effective (Table I). At 200 μg (dry weight)/ml and above (Table I,B), mycelium filtrate showed nearly 100% reduction of foci. On the other hand, the fruiting body extract-treated cultures exhibited 56% inhibition at 200 μg/ml and 80% at a dose of 500 μg/ml (Table I,A). Neither samples posted cytotoxicity on either the host R6 or R6/T24 cells (Table II). Thus, the inhibitory effect of the mushroom extracts on *ras*-transformation is not due to a direct killing of cells transformed by *ras* oncogene in the focus formation assay.

Effect of Tricholoma filtrate on ras-induced transformation

Both the total, and the DEAE-column-bound fraction of *T.lobayense* filtrate were tested in R6 cells upon transfection of the *ras* oncogene. Data showed that the total filtrate and the

Table I. Effect of hot water extracts of *G.lucidum* on *ras*-induced transformation

Material tested	Conc. (μg/ml)	Number of foci	Relative number of foci[a]
A. Extract of fruiting bodies			
Mock control		0	
No treatment	0	14.7 ± 4.0	1
Extract	10	13.7 ± 4.5	0.93
	100	8.3 ± 2.9	0.56
	500	3.0 ± 1.0	0.2
B. Extract of mycelium filtrate			
Mock control		0	
No treatment	0	18.3 ± 2.3	1
Extract	100	2.7 ± 1.2	0.15
	200	1.0 ± 1.0	0.05
	500	1.3 ± 1.5	0.07

[a]Relative number of foci; the ratio of foci obtained in the presence of drug to that in the absence of drug (i.e. no treatment control).

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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 co-culture 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/GPF-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

Table II. Cytotoxicity of hot water extract of *G.lucidum*

Treatment[a]	Conc. (μg/ml)	R6 cells % survival[b]	R6/T24 cells % survival[b]
No treatment	0	100	100
Fruiting body	500	93	98
Mycelium filtrate	500	89	98

[a]The duration of the treatment was 5 days for all treatment groups.
[b]% Survival = cell counts of treated cultures/cell counts of untreated cultures ×100%.

Table III. Effect of extracts of *Tricholoma* filtrate on *ras*-induced transformation

Material tested	Conc. (μg/ml)	Relative number of foci
Mock control	–	0
No treatment	0	1
Total mycelium filtrate	100	0.04
	200	0.10
DEAE-column purified fraction A2[a]	100	0.04
DEAE-unbound fraction A1[a]	50	0.96
	100	0.91

[a]The liquid cultures of *T.lobayense* was filtered, concentrated and precipitated with ethanol. The precipitate was dissolved in distilled water. The water-soluble fraction was then applied to a DEAE-cellulose column. The unbound fraction A1 was eluted with distilled water, and the retained components (A2) were eluted with a salt gradient of NaCl (0–2 M) (5).

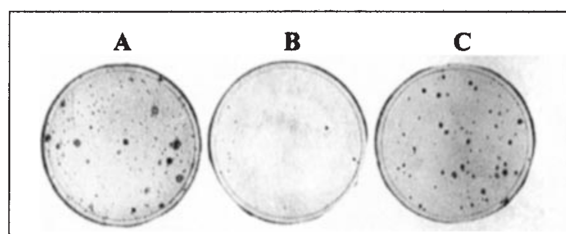

Fig. 1. Giemsa stain of transformed foci in R6 cell cultures transfected with pT24 plasmid DNA. Transfected cultures were grown in normal medium alone (**A**); normal medium with 100 μg/ml DEAE-column-bound fraction A2 (i.e. PSPC-enriched fraction) (**B**); or normal medium with 100 μg/ml DEAE-column-unbound fraction A1 (**C**) of *Tricholoma* filtrates. Experiments were carried out as described in Table III.

Table IV. Cytotoxicity of extracts of *Tricholoma* filtrate

Treatment[a]	Conc. (μg/ml)	R6 cells % survival	R6/T24 cells % survival
No treatment	0	100	100
Total filtrate	400	98	91
DEAE-column-bound fraction A2	100	80	93
DEAE-column-unbound fraction A1	100	101	105

[a]The duration of the treatment was 5 days for all groups.

Table V. Dosage effect of DEAE-column purified fraction of *Tricholoma* filtrate on *ras*-induced transformation

Test sample	Conc. (μg/ml)	Relative number of foci
Mock control (no T24 DNA)	0	0
No treatment	0	1
DEAE-fraction A2[a]	1	0.81
	5	0.78
	10	0.87
	20	0.56
	50	0.47
	100	0.32

[a]DEAE A2 fraction was prepared as described in Table III legend.

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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

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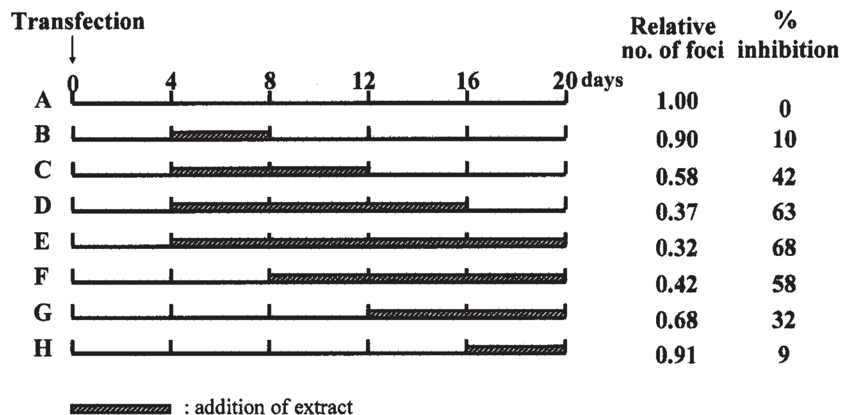

Fig. 2. Time course studies of the effect of PSPC-enriched fraction A2 of *Tricholoma* filtrate on the number of transformed foci in R6 cultures transfected (at day 0) with pT24 plasmid DNA. Where indicated, transfected cultures were treated with 100 μg/ml of A2 fraction of *Tricholoma* filtrate described 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 *Ticholoma* filtrate A2 fraction (200 μg/ml). (**C**) 500 R6/GFP-Ras cells were seeded on a lawn of 2.5×10^5 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. 2.5×10^5 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 manufacture 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.

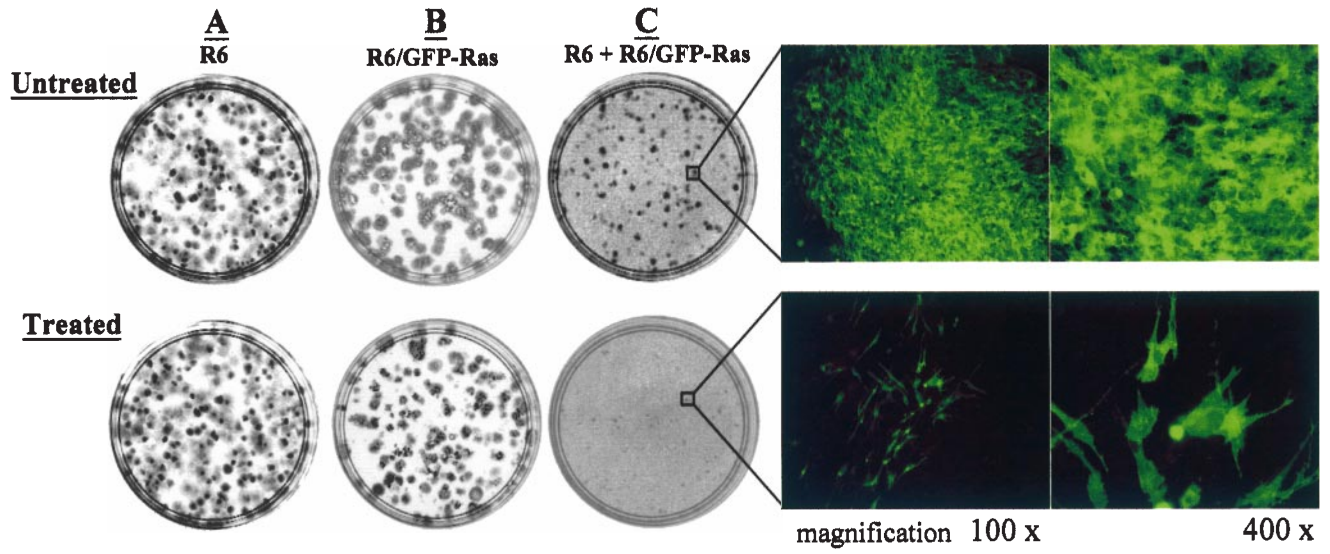

Fig. 3.

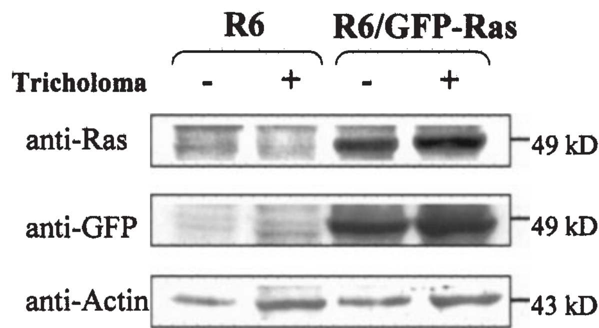

Fig. 4.

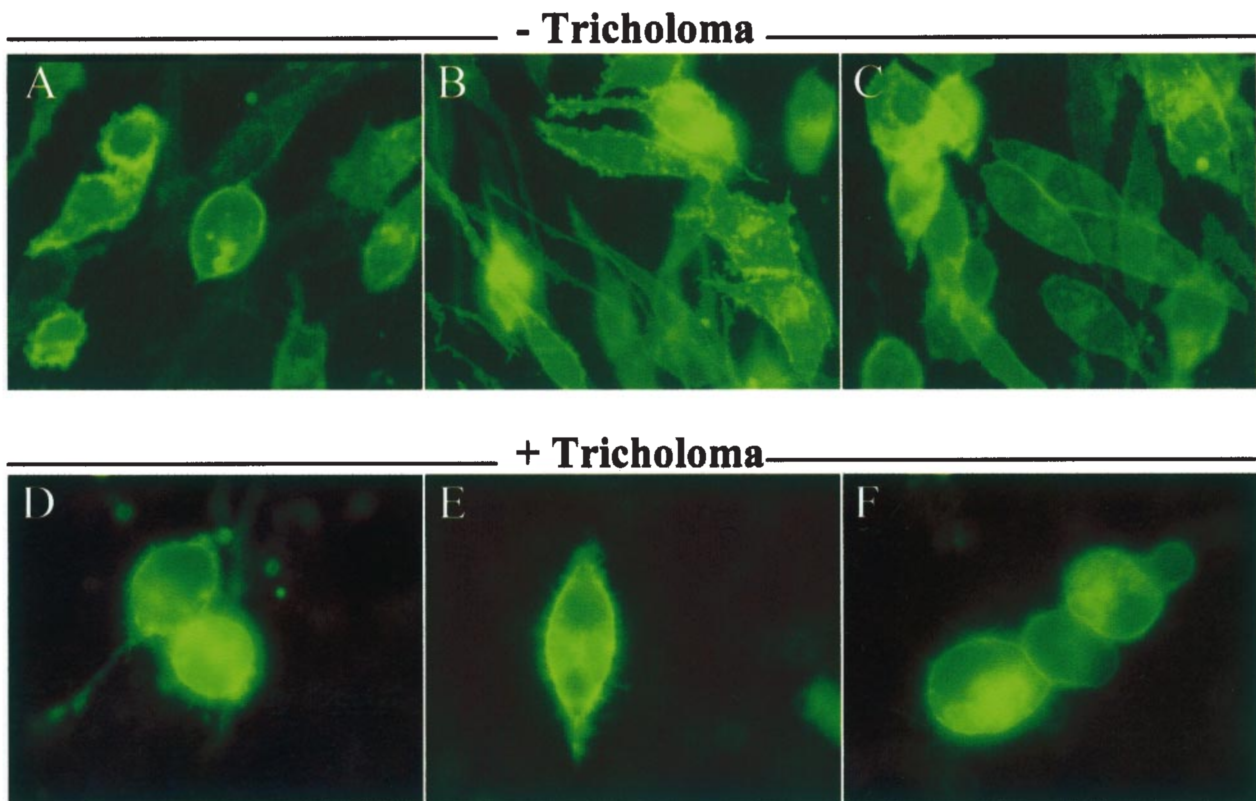

Fig. 5.

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Table VI. Comparisons of anticancer effects of filtrates of *T.lobayense* cultures assessed by animal test versus *in vitro* focus formation assay

Material tested	ICR male mice[a] (% of inhibition)	Balb/c male mice[a] (% of inhibition)	Focus formation assay (% of inhibition)
Crude extract	84%	66%	90% (100 μg/ml)
DEAE-column purified fraction (A2)	96%	50%	96% (100 μg/ml)
DEAE-unbound fraction (A1)	40%	6%	9% (100 μg/ml)
No treatment	0%	0%	0%

[a]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 [[a]animal data was obtained from Liu *et al*. (17)].

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 cytocidal 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* oncoprotein 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 PSCP 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 PSCP-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

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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.

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