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Effect of honey bee venom on proliferation of K1735M2 mouse melanoma cells in-vitro and growth of murine B16 melanomas in-vivo

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Abstract

Bee venom has been reported to exhibit antitumour activity in-vitro and in-vivo. Apoptosis, necrosis and lysis of tumour cells were suggested as possible mechanisms by which bee venom inhibited tumour growth. The aim of this study was to investigate potential mechanisms by which bee venom inhibits K1735M2 mouse melanoma cells in-vitro and B16 melanoma, a transplantable solid melanoma in C57BL/6 mice, in-vivo. The proliferation of K1735M2 cells in-vitro was inhibited by bee venom in a concentration- and time-dependent manner. The inhibition was indicated by the arrest of the cell cycle at the G1 stage, as detected by flow cytometric measurements. The bee venom induced apoptosis-like cell death as identified by histological observations and by DNA fragmentation. In the in-vivo experiments, the bee venom (1.0, 3.0, 9.0 mg kg^{-1} of body weight, on days 1–12) was injected intraperitoneally into mice 24 h after the mice were inoculated with B16 cells. Inhibition of the solid tumour was observed. Apoptosis of the K1735M2 cells was suggested as the possible mechanism by which bee venom inhibited cell proliferation and induced K1735M2 cell differentiation in-vitro. The in-vivo experiment indicated that bee venom could be used as a chemotherapeutic agent against malignant tumours.

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Introduction

The venom of the honey bee, *Apis mellifera*, is a complex mixture of substances with biological activity (Habermann 1972). The major components of bee venom are melittin, apamine, polyamines, hyaluronidase and phospholipase. Previous studies have directly examined its effects on the cardiovascular system (Marsh & Whaler 1980; Cook & Hof 1988; Thomas & Hiley 1988), immune system (Reisma et al 1985; Jutel et al 1995; Bellinghausen et al 1997), nervous system (Luo et al 1998; Ren et al 1999; Stocker et al 1999; Chen et al 2001) and blood (Pogliani & Cofrancesco 1983). It was noted that apamine and catecholamines such as noradrenaline (norepinephrine) and dopamine caused neurotoxic effects and the degranulation of mast cells (Orlov 1979). Reports showed that melittin, a haemolytic peptide consisting of 26 amino acids, has effects on erythrocytes and mast cells (Katsu et al 1990), leukaemic cells (Hait et al 1985; Killion & Dunn 1986), mouse fibroblast cells (Shier 1979; Lo et al 1997), skeletal muscle cells (Fletcher et al 1996; Ownby et al 1997), rat thymocytes (Duke et al 1994; Sakamoto et al 1996; Shaposhnikova et al 1997) and the human lymphoblastoid cell line (Weston & Raison 1998). Experiments showed that melittin was capable of binding to calmodulin, which plays a key role in cellular proliferation (Cheung 1982). It has also been shown that melittin also inhibited the melanotropin receptor in M2R melanoma cell membranes (Gest & Salomon 1987). Recently, a more intriguing report showed that bee venom stimulated differentiation and inhibited growth of V79 and Hela cell lines (Orsolic et al 2001). Orsolic et al (2001) suggested that apoptosis, necrosis and lysis of the tumour cells were the possible mechanisms by which the bee venom inhibited tumour growth.

In this report, the effect of the bee venom on the proliferation of K1735M2 melanoma cells in-vitro is examined and the possible mechanisms of antitumour activity of the bee

venom are discussed. In addition, the effect of the bee venom on the growth of B16 melanoma cells in mice is studied.

Materials and Methods

Chemicals, cells and animals

Lyophilized honey bee venom was obtained from Sigma Chemical Co. (St Louis, MO). Thiazolyl blue tetrazolium bromide (MTT), phosphate-buffered saline (PBS), propidium iodide and other reagents were also obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise indicated. The K1735M2 mouse melanoma cells used in this study were obtained from Dr I. J. Fidler (Anderson Cancer Center, Houston, TX). B16 melanoma cells were obtained from the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). Tumour cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37°C. Chemicals for cell culture were obtained from GIBCO BRL Technologies Ltd (Paisley, UK). Only single-cell suspensions of more than 90% viability, as determined by trypan blue exclusion, were used in the in-vivo experiment. Forty female C57BL/6 mice (6 weeks old; weight: 18 ± 1 g), maintained in the Laboratory Animal Unit of Institute of Materia Medica, were used.

Cell proliferation assay

The cytostatic/cytotoxic effect of bee venom on K1735M2 cells was studied in a standard MTT assay as described by Lasek et al (1995). Tumour cells were dispensed in a 96-well flat bottom microtitre plate (Costar) at a concentration of 10^4 cells per well. After 1 day of culture, allowing cell attachment, they were treated with serial dilutions of 20 μL of bee venom of for 5 days. Four hours before the end of incubation, 20 μL of MTT solution (5.0 mg mL^{-1}) was added to each well. After 4 h, resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO, A.R.). The absorbance was measured on an ELISA reader (BIORAD Model 550). The cytostatic/cytotoxic effect was expressed as a relative percentage of inhibition calculated as follows:

$$\text{Relative \% inhibition} = \frac{\text{Control absorbance} - \text{Experimental absorbance}}{\text{Control absorbance}} \times 100 \quad (1)$$

Morphological observation in in-vitro experiments

K1735M2 cells (5×10^5 cells/cm^2) were incubated in MEM–10% FBS with and without 14.2 μg mL^{-1} of the experimental agent for 5 h. The cells were observed with an inverted phase-contrast microscope (Nikon TE300) using a digital camera (Cool Snap fx).

Flow cytometric assay of the cell cycle

K1735M2 cells (5×10^5 cells/cm^2) were incubated in MEM–10% FBS with or without 14.2 μg mL^{-1} of the experimental agent. Five hours later, incubations were terminated by rinsing the cells with PBS, and a trypan blue exclusion test was performed to ensure that 80% of the cells were viable. The cells were then fixed by placing on ice for 30 min in 80% ethanol, then washed again. After centrifugation at 800 *g* for 5 min at 4°C, propidium iodide (50 mg L^{-1}) in PBS solution with ethylenediamine tetraacetic acid and 50 mg mL^{-1} of RNAse (Sangon Genomic Company, China) was added and incubated in darkness for 30 min at 37°C. The cells were then filtered through a 35-mm strainer cap to ensure single-cell suspension, and analysed by a flow cytometer (EPICS ELITE, Coulter, USA) and CellQuest software. The Argon laser excitation wavelength was 488 nm, while emission data were acquired at wavelength 580 nm.

DNA fragmentation assay

K1735M2 cells (5×10^5 cells/cm^2) were incubated with or without 2.8 and 14.2 μg mL^{-1} of the experimental agent. After 5 h and 24 h of incubation, cells were trypsinized and collected by centrifugation at 800 *g* for 5 min at 4°C. A Genomic DNA Purification Kit (SK252, Sangon Genomic Company, China) was used for DNA extraction. Cells were suspended in 200 μL of Triton X-100 (0.05% w/v) and 0.1 mM ethylenediamine tetraacetic acid solution (pH 8.0), then lysed in cell lysis solution for 10 min at 65°C. Following chloroform extraction, 500 μL of supernatant was mixed with precipitation solution for 2 min. DNA was extracted by centrifugation for 2 min at 16000 *g* and 4°C. The sample was dissolved in 1.2 M NaCl by stirring gently. The DNA sample was treated with RNAse A (Sangon Genomic Company, China) for 1 h at 37°C, and treated with proteinase K (Sangon Genomic Company, China) for 1 h at 37°C. The DNA fraction was analysed by agarose gel electrophoresis (in a 1.0% agarose gel) and visualized by staining with ethidium bromide. DNA Mass Ladder (Life Technologies, Gaithersburg, MD) was used as a marker.

In-vivo experiments

Forty C57BL/6 female mice (Madison, WI) were used when they were 6 weeks old. The care and use of the mice were in accordance with institutional guidelines. Each group consisted of 10 mice. B16 murine melanoma cells (20 μL, 1×10^6 cells) were transplanted into the oxter of the right fore limb of C57BL/6 mice. After 24 h, the tumour-bearing mice (10 mice in each group) were treated with an intraperitoneal injection of the bee venom (1.0, 3.0 or 9.0 mg kg^{-1} daily) for 12 days. Control mice received mock injections with saline. The body weights of mice in each group were measured every 24 h during in-vivo experiments. The mice were killed on day 12 and the local tumours were removed carefully from the transplanted oxter and

the net mass weights of the tumours were weighed. Relative tumour inhibition was calculated as follows:

$$\text{Relative tumour inhibition (\%)} = \frac{(\text{Control mice tumour weight} - \text{Treated mice tumour weight})}{\text{Control mice tumour weight}} \times 100$$

Statistical analysis

Cell proliferation data were plotted and the 50% inhibiting concentration (IC50) of bee venom was obtained using the computer program KaleidaGraph (Synergy Software, Reading, PA) using a nonlinear Hill equation. Statistical Program for Scientific Studies (SPSS 10.0) package (for a Windows operating system) was used to perform statistical analysis of the data. A one-way analysis of variance was used to compare multiple groups. Cell-cycle data were analysed for significance with the Mann–Whitney *U*-test. Data are presented as mean values with the standard deviation (mean ± s.d.). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

The effect of honey bee venom on proliferation of K1735M2 cells in-vitro

Concentration-dependent cytostatic effects caused by bee venom were determined by the MTT method. After 24 h incubation, the honey bee venom caused concentration-dependent growth inhibition in K1735M2 cells with an IC50 value of 10.0 μg mL^{-1} (as shown in Figure 1). The concentration of bee venom in the range 1.4–28 μg mL^{-1} significantly inhibited growth compared with the control

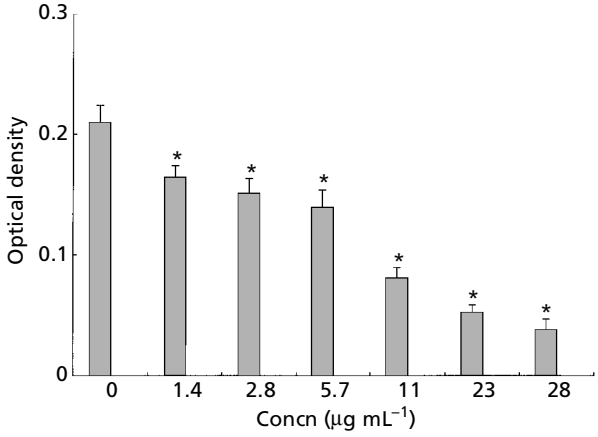

Figure 1 Concentration-dependent inhibition of the proliferation of K1735M2 cells by bee venom. K1735M2 cells were treated with various concentrations of the bee venom for 24 h. An MTT assay was performed according to the procedure described in the Materials and Methods section. Each bar represents the mean ± s.d. of six experiments performed in triplicate. *$P < 0.01$, vs untreated controls.

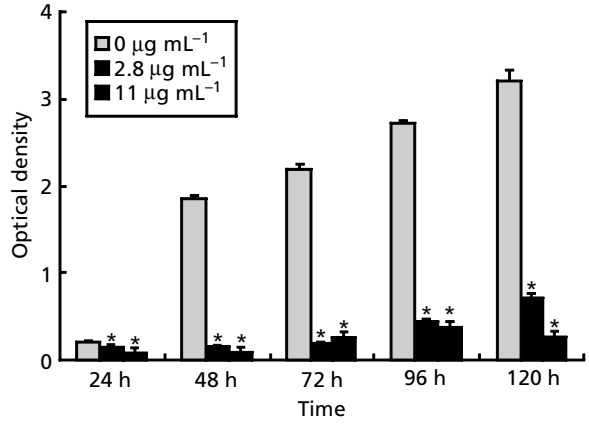

Figure 2 Time-dependent inhibition of the proliferation of K1735M2 cells by bee venom. K1735M2 cells were incubated with bee venom (2.8 or 11.0 μg mL^{-1}) for serial time intervals. Cell viability was determined by the MTT assay. Each bar represents mean ± s.d. of six experiments performed in triplicate. *$P < 0.01$, vs untreated controls.

($P < 0.01$, one-way analysis of variance). However, the effectiveness of the bee venom was maintained for a longer duration in-vitro. Thus, when MTT assays were performed daily for 5 days, the time-course of cell death after the bee venom treatment was monitored (Figure 2). K1735M2 cells were incubated with the bee venom (2.8 or 11 μg mL^{-1}) for 120 h. Significant inhibition ($P < 0.001$, one-way analysis of variance test) occurred at different times for K1735M2 cells treated with both concentrations of the bee venom.

Morphological observation in in-vitro experiments

When observed with a phase-contrast microscope, the K1735M2 melanoma cells showed a subconfluence and formed a sheet of polygonal cells (Figure 3A). Treatment with 14.2 μg mL^{-1} of the bee venom for 5 h induced morphological differentiation of murine melanoma cell line K1735M2 so that all contacted cells were separated from each other (Figure 3B). Fragmentation of high-density materials from the cells was recognized under the phase-contrast microscope using the same magnification (400×) as in Figure 3A. Cells treated with the bee venom showed loss of the regular confluence and destruction of a sheet of polygonal cells. There was a high density of chromosomes, individual cells lost their original shape and long dendrite-like structures were formed. A 24-h treatment of K1735M2 cells showed an even greater loss of their original morphologies (data are not shown).

Analysis of DNA content of K1735M2 cells by flow cytometry

K1735M2 cells were incubated with a constant amount of the bee venom (14.2 μg mL^{-1}) for 5 h. The cells were stained with propidium iodide (50 mg L^{-1}) for 30 min. Table 1

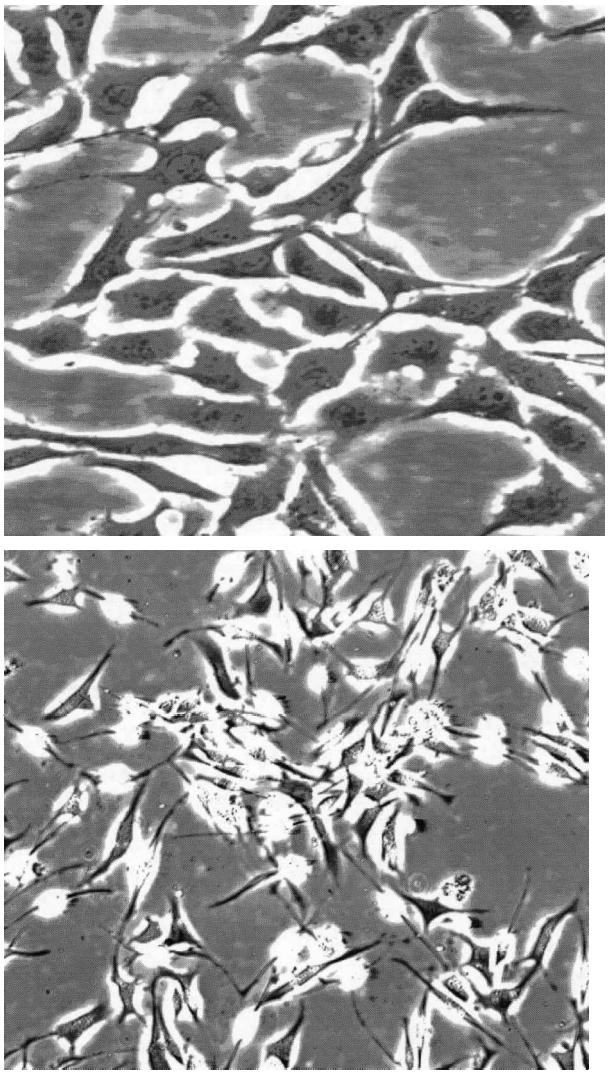

Figure 3 Morphological changes in K1735M2 melanoma cells observed by phase-contrast microscopy (magnification, ×400). A. Control K1735M2 cells are a sheet of polygonal cells with no dendrite-like projections or lipid granules. B. Following a 5-h treatment with 14.2 μg mL^{-1} of bee venom, long dendrite-like projections were observed and lipid and dark granules accumulated. These experiments were performed in triplicate.

Table 1 Effects of bee venom on the cell cycle of K1735M2 mouse melanoma (percentage of cells in the G1, G2-M and S phases of the cell cycle).

	G1 (%)	G2 (%)	S (%)
Control	54.90±0.05	10.90±0.08	35.30±0.15
Bee venom	64.70±0.07*	5.60±0.09	29.40±0.04

K1735M2 cells (10^7) untreated or treated with 14.2 μg mL^{-1} bee venom for 5 h were fixed in ethanol and then proteins and nucleic acids were stained with propidium iodide (50 mg L^{-1}). The percentage of cells in the G1, G2 and S phases was calculated using the software program CellQuest. Data are mean±s.d. from three different experiments. *$P < 0.05$, vs control (Mann–Whitney U-test).

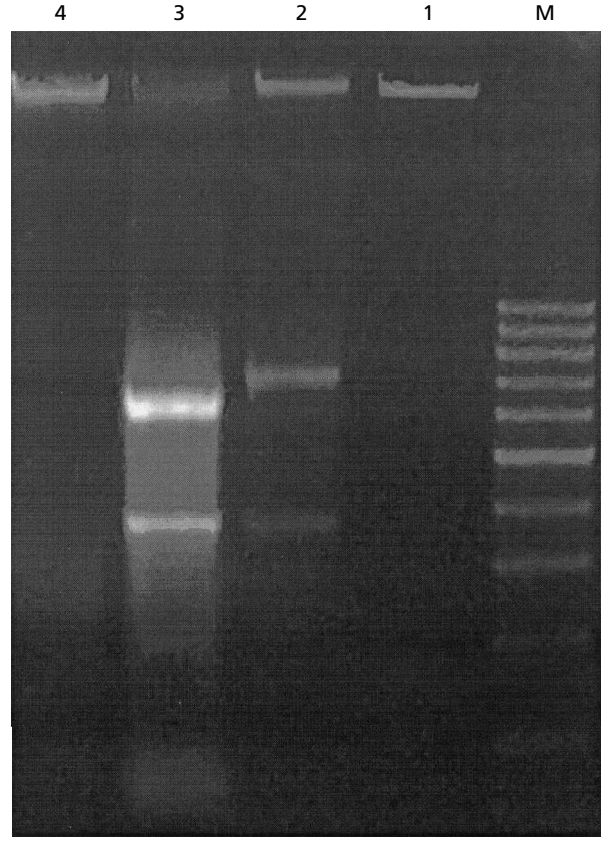

Figure 4 Agarose gel electrophoresis of DNA extracted from K1735M2 cells treated with bee venom. Lane M, molecular weight DNA marker; lanes 1 and 4, control; lane 2, DNA from cells treated with 2.8 μg mL^{-1} of bee venom; lane 3, DNA from cells treated with 14.2 μg mL^{-1} of bee venom. These experiments were performed in triplicate.

provides evidence of the impact of bee venom on the distribution of the K1735M2 cells between the G1 and S phases of the cell cycle. Compared with untreated cells, the bee venom induced a significant increase in the percentage of cells in the G1 phase and a decrease in the percentage of cells in the S phase and G2 phase. Cell death by apoptosis was seen as a sub-G1 peak when analysing a DNA histogram by flow cytometry (data are not shown). The arrest of the cell cycle was consistent with the observed suppression of cell proliferation.

DNA fragmentation assay

To confirm whether bee venom induces apoptosis in K1735M2 cultures, DNA ladder analysis was performed (Figure 4). Oligonucleosome-sized fragments identical to those observed during apoptotic cell death were found in both the K1735M2 cells treated with 2.8 μg mL^{-1} of the bee venom (lane 2) and 14.2 μg mL^{-1} of the bee venom (lane 3). A nucleosome ladder of DNA fragments was not found in

Table 2 The effect of bee venom on the growth of B16 mouse melanoma cells in C57BL/6 mice.

Bee venom (mg kg^{-1})	Number of mice		Body weight (g)		Tumour weight (g)	Inhibition (%)
	Beginning	End	Beginning	End		
–	10	10	17.70±0.82	21.00±1.63	3.05±0.92	
1.0	10	10	17.70±0.95	20.60±1.58	2.44±1.38	20.00±1.66
3.0	10	10	17.80±0.63	19.90±1.73	1.44±1.19*	52.80±1.50
9.0	10	10	17.80±0.79	18.70±1.77	1.52±1.60*	50.20±1.85

Data are mean±s.d. *$P < 0.05$, vs untreated control group (one-way analysis of variance test).

the untreated cells (lanes 1 and 4), unlike the characteristic ladder pattern in lane 2 and lane 3.

Effect of treatment with the bee venom on growth of B16 cells in mice

The impact of bee venom on the growth of implanted B16 melanoma was evaluated. The treatment with bee venom inhibited primary tumour growth of B16 melanoma cells on the oxters of C57BL/6 mice after intraperitoneal injection. Table 2 shows that the relative tumour inhibition obtained with 1.0, 3.0 and 9.0 mg kg^{-1} of bee venom was 20.0, 52.8 and 50.2%, respectively. The tumour weight of mice treated with 3.0 and 9.0 mg kg^{-1} of the bee venom was significantly reduced ($P < 0.05$, one-way analysis of variance test) compared with that of the control group. During the in-vivo experiment, physiological behaviour, appetite and body weight were not disturbed.

Discussion

In this study, we demonstrated that bee venom inhibited proliferation or had a cytostatic effect on K1735M2 mouse melanoma cells in-vitro. The bee venom inhibited the proliferation of K1735M2 cells in a concentration- and time-dependent manner (as shown in Figures 1 and 2). As the bee venom concentration increased, the relative percentage of inhibition of K1735M2 cells also increased. There was a significant inhibition over the concentration range 1.4–28 μg mL^{-1} of bee venom ($P < 0.01$, one-way analysis of variance test) compared with the control group. The IC50 value for bee venom in K1735M2 cells was 10.0 μg mL^{-1}, while the same dose of the bee venom killed more than 70% of Hela cells and 90% of V79 cells (Orsolic et al 2001). In addition, at the same bee venom concentration (2.8 or 11.0 μg mL^{-1}), the relative percentage of inhibition of K1735M2 cells increased over time. Significant inhibition ($P < 0.001$, one-way analysis of variance test) was observed at different times for K1735M2 cells treated with both 2.8 and 11.0 μg mL^{-1} of the bee venom (Figure 2).

Apoptosis is a form of programmed cell death, characterized by several morphological and biochemical aspects, which differs from necrosis. Apoptosis can be triggered by a variety of extrinsic and intrinsic factors, such as physiologic activators, damage-related inducers and therapy-associated agents (Wyllie et al 1980; Raff 1992; Williams & Smith 1993; Thompson 1995). The results presented in this study show that the bee venom induced marked morphological differentiation of the murine melanoma cell line K1735M2, which included production of dendrite-like structures and induction of granules (Figure 1B). Morphological changes associated with apoptosis are usually accompanied by activation of endonucleases that cleave internucleosomal DNA and yield the characteristic ladder pattern on agarose gel electrophoresis. The result obtained in the DNA fragmentation was confirmed by agarose gel electrophoresis of DNA isolated from K1735M2 cells after 5 h of incubation (data are not shown). A clear ladder pattern can be seen on the agarose gel after 24 h incubation with 2.8 and 14.2 μg mL^{-1} of the bee venom (Figure 4). In addition, the results also demonstrated that bee venom induced alterations in the cell cycle kinetics (Table 1). Honey bee venom induced cell arrest of K1735M2 cells in the G1 phase of the cell cycle, caused apoptosis-like cell death and caused an increase in the sub-G1 fraction upon cytometric analysis of the DNA content by propidium iodide staining (data not shown). Accumulation of bee-venom-treated cells in the G1 phase of the cell cycle seems to be responsible for these cytostatic effects. All these results suggested that bee-venom-induced cytostatic effects caused the induction of apoptotic events. However, there are alternative explanations. Simple contact of tumour cells with the bee venom could directly change the conformation of the tumour cell membrane. Conformational changes of a protein directly affect its function (e.g. enzyme activity, membrane receptor sensitivity and ion-channel transductivity). The cell membrane not only controls the exchange of substances and information into or out of the cell body, but it also reflects the comprehensive changes taking place inside the cell. Further study by the authors showed that the coil proportion of the tumour cell membrane protein decreased significantly (circular dichroism; data not shown). The changes in membrane protein conformation would suggest that it has a much stronger reaction with membrane lipids and induces protein modifications in the signal transduction pathway (Gest & Salomon 1987; Duke et al 1994). So it has been proposed

that apoptosis of the tumour cells might be the possible mechanism by which bee venom inhibits tumour growth.

In comparative analysis, the K1735M2 cell line does not seem to be an appropriate model for melanoma. The B16 mouse melanoma cell line is an appropriate model for tumours with a low level of MHC-1, even though B16 expresses melanoma-associated antigens (Peter et al 2001). The same was also evident from our experiments. Therefore, a B16 cell line was used for the in-vivo study. The in-vivo results showed that systemic administration of 1.0 and 3.0 mg kg^{-1} of bee venom resulted in significant retardation of B16 melanoma growth ($P < 0.05$, one-way analysis of variance test) with the relative tumour inhibition being 20.0 and 52.8%, respectively. Administration of 9.0 mg kg^{-1} of bee venom resulted in almost the same antitumour effect as that of 3.0 mg kg^{-1}. The in-vivo experiment indicated that bee venom played a role in the successful completion of primary tumour proliferation or growth and also had a beneficial therapeutic potential for treatment of a solid tumour. The effect might be due to its cytostatic activity against tumour cells. Further research on the B16 melanoma in-vitro is currently being undertaken. The possible mechanisms of bee-venom inhibition of B16 melanoma are being investigated.

Conclusion

The venom in the bee sting contains a number of active ingredients. The major one is melittin, a molecule that destroys cells by slicing through the cell membrane. In this report, the antitumour effect of the bee venom was studied in-vitro and in-vivo. Apoptosis of the tumour cells was found to be the possible mechanism by which the bee venom inhibited tumour growth in-vitro. This is a fantastic opportunity to make some fundamental research observations and use them to develop a potential drug to treat a major disease, such as cancer. Further research is required to define the molecular mechanism of cell death in-vivo, and also to determine the death factors for melanoma cell, the receptors for these factors, the intracellular signalling pathway activated by the receptors, and so on. Further study is required before clinical application can be considered, but the current results suggest optimism for the future.

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