4. Discussion

4.1. Bioassay-guided fractionation of traditional Chinese medicine

Although traditional Chinese medicine (TCM) has demonstrated its effectiveness in the treatment of ailments and diseases throughout its long history of empirical use, only recently have there been studies designed to determine the mechanisms by which some compounds may act its results and to identify its active ingredients. The search for drugs active against tumours is a promising strategy, judged by the number of publications describing bioactive plant-derived compounds in the last few years.

Our work deals with the anti-tumour activity of compounds from two sources specific traditional Chinese medicines with two main focuses: immunomodulation and the inhibition of the growth of tumour cells. According to traditional use in China, eight crude extracts of traditional Chinese medicines, including *Polygonum cuspidatum,* and *Ganoderma lucidum,* were chosen to screen for inhibition activity of tumour cells and the activation of immune cells. Among them, the crude extract *Ganoderma lucidum* showed higher activity in stimulating B lymphocytes and macrophages and the crude extract of *Polygonum cuspidatum* strongly inhibited the growth of tumour cells. The crude extracts were sequentially fractionated, each fraction and/or pure compound being subjected to bioassay and toxicity studies in cells. The fraction which was most active was used for further fractionation. Through bioactive-guided fractionation, the active fraction GLIS, isolated from *Ganoderma lucidum* through several chromatographic steps, had the highest capacity to activate macrophages. The active compound HZ-3-1-b, purified from *Polygonum cuspidatum,* could significantly inhibit the growth of tumour cells.

4.2. Anti-tumour activity of GLIS

Ganoderma lucidum has demonstrated anti-tumour activity, but the mechanism still remains unclear. Some types of immunomodulating polysaccharide from mushrooms, including lentinan, schizophyllan and PSK, possess anti-tumour properties through the stimulation of host immune reactions. In our experiment, GLIS had no direct anti-tumour effect, but could activate macrophages to secrete cytokines and NO, to enhance phagocytosis, and to stimulate B cells to secrete IgM via plasma cells. These functions are related to the anti-tumour activities of host immune reactions.

It is well known that macrophages play an important role in many primary defense mechanisms. In our experiment we found that GLIS could activate macrophages to kill the tumour cells *in vitro*. The mechanism may be related to the enhancement of phagocytosis by GLIS and the active compounds secreted by macrophages. Phagocytosis represents the final and most indispensable step of the immunological defence system (Van der Goes, 1999), especially in the defence against tumour cells (Popov, 1999). The ability to stimulate NO

formation is important because NO has a wide range of biological activities, not only in the vascular systems but also in the non-specific host defence against invading micro-organisms and tumours (Bredt et al., 1994). Macrophage-derived cytokines have been shown to be cytotoxic for a range of tumour cells. IL-1β has been demonstrated to be cytotoxic and cytostatic for tumour cells. It has been demonstrated that IL-1β can act synergistically or additively with TNF-α in killing tumour cells as well. A crucial role for TNF-α in macrophage-mediated anti-tumour cell cytotoxicity has been demonstrated by Al-Sarireh et al. (2000).

GLIS could significantly enhance IgM production not only *in vitro* but also *in vivo*. Natural or spontaneous antibodies against toxins, bacteria and erythrocytes are present in the sera of normal non-immunized humans and mice. Within the last 15 years, cytotoxic antibodies have been shown to enable tumour cell lysis *in vitro* and *in vivo* by activating antibody-dependent cytotoxity. Yoshimura et al. (2001) found that transfected M2A PaCa-2 cells and huH-7 cells were effectively lysed by human natural antibody. Ollert et al. (1997) discovered the presence of natural IgM antibodies in the sera of healthy adults with high specificity for neuroblastoma (NB). These antibodies elicit effective killing of neuroblastoma cell by both complement activation and apoptosis. In nude rats with human NB xenografts, anti-NB IgM inhibits tumour growth. Of particular interest is the observation that antibody titers in neuroblastoma patients are significantly lower or absent.

Immune suppression in the tumour-bearing host is postulated to be the basis for the growth of antigenic tumours despite anti-tumour immune response. This hypothesis is based upon a variety of data showing that the anti-tumour immune response is demonstrable in the early phase of tumour growth, but is down-regulated upon continued growth. In recent years a number of scientists have attempted to describe the phenomenon of tumour-induced immune suppression in biochemical terms. We found here that GLIS was more effective on macrophages or B cells from tumour-bearing mice than that of normal mice. It is promising that the suppressed immunity could be recovered or enhanced by this compound. It has been reported that PSP enhanced immune functions in old mice, but not in young mice, and restored TNF-α production that suppressed by cytotoxic anti-tumour agents such as 5-fluorouracil, cyclophosphamide or bleomycin. Some soluble fungal glucans have been applied clinically for tumour immunotherapy, such as lentinan, derived from an edible mushroom (Chihara et al., 1969), and schizophyllan (i.e., SSG or Sizofiran), isolated from the culture filtrate of Schizophyllum commune (Miyazaki et al., 1995).

Although the data presented in this report yielded an incomplete picture concerning the effect of GLIS on the immune system, we have demonstrated that GLIS resulted in the activation of macrophages, the stimulation of the release of cytotoxic mediators by macrophages, and enhanced IgM secretion. Further studies with animal models are necessary to clarify how this activation occurs and to what extent it occurs *in vivo*.

4.3. GLIS selectively activates B cells and macrophages but not T cells

One of the objectives of this study was to characterise the immunomodulatory activity of GLIS. In previous work, it was found that GLIS stimulated the proliferation of mouse spleen lymphocytes, resulting in a three- to four-fold increase in the percentage of B cells (Zhang, 2002). The present work showed that GLIS could directly stimulate purified B cells to proliferation and activation, but could not activate CD3 positive cells (T cells) like LPS. After stimulation by GLIS, B cells showed an increased expression of CD25 and CD71, respectively, and became plasma cells to secrete IgM and IgG. Antigens are classified according to their B-cell activation characteristics. There are T cell dependent and T cell independent antigens. The GLIS responder cells were characterised to be B cells. After depletion of macrophages and T cells, the B cells could still be activated by GLIS. These results suggest the GLIS can be classified in the category of T cell independent antigens stimulating B cells.

Here it could be shown that GLIS activates macrophages in a dose-dependent manner. After stimulation, the macrophages spread and elongated, secreted TNF-α and IL-1β, and produced reactive nitrogen intermediates (NO). The capacity of phagocytosis was markedly increased. These results demonstrate that GLIS selectively activates B cells and macrophages, but not T cells.

The polysaccharides obtained from many natural sources represent a structurally diverse class of macromolecules, and this structural variability can profoundly affect the biological activities of B cells, T cells and macrophages. Lentinan was extracted from the mushroom *Lentinus edodes*, which stimulates T cells, macrophages and NK cells. Hamuro et al. (1978) reported that lentinan mediated an increase of alloreactive murine cytotoxic T-lymphocytes. Moreover, lentinan stimulates NK cell activity (Nanba et al., 1987) and activates macrophages into a release of TNF-α (Kerekgyarto et al., 1996), IL-1β (Chihara et al., 1987) as well as superoxide anion production, phagocytosis (Abel et al., 1989) and cytotoxity. PSP, a polysaccharide component, is made up of monosaccharides with α-1,4 and β-1,3 glucosidic linkages. It increased the secretion of IFN-γ and IL-2 production, T cell proliferation and NK cell function (Ng, 1998). Activation of peritoneal macrophages by PSP was observed by Liu et al. (1993).

Some polysaccharides stimulate B cells and macrophages but not T cells. Han et al. (2001) isolated a polysaccharide from a radix of *Platycodon grandiflorum* (PG) which increased the proliferation of B cells and activated inducible nitric oxide synthase (iNOS) transcription and NO production of macrophages. Angelan (10 kDa) is a polysaccharide recently purified from the root and cell culture of *Angelica gigas Nakai* (Ahn et al., 1996; Han et al., 1998) reported to directly activate macrophages and B cells. Some active pectic polysaccharides from *Beuleurum falcatum* are B cell activators (Sakurai et al., 1999). *Phellinus linteus* (PL), which is composed of glucose, galactose, mannose, arabinose, xylose, and uronic acid, showed broad action profiles on B cells, T cells, and macrophages (Song et al., 1995). In the present

study, we demonstrated that GLIS is an immunostimulator that selectively activates B cells and macrophages similar to angelan or PG, and is different from lentinan, PSP, and PL.

4.4. Characterisation of active fraction GLIS

Although GLIS and LPS demonstrate similar modes of action on B cells and macrophages, our study reveals that they have different properties, as indicated by the inability of polymyxin B, an LPS inhibitor, to attenuate the activity of LPS but not that of GLIS. We also found the different functional mechanisms of GLIS and LPS using C3H/HeJ mice which are LPS hyperesponsers. GLIS, but not LPS, could stimulate NO production by macrophage from C3H/HeJ.

Intensive studies are dealing with the immunomodulating effects of *Ganoderma lucidum*, but are rarely focusing on the composition of the active fractions. He et al. (1992) reported that two polysaccharide components, a glucan and an arabinogalactan, were obtained from the fruit bodies of *Ganoderma lucidum*. Both consist of β-1,3 and β-1,6 linked glycosides. In addition, two fractions of Ganoderma B, a glucan-protein, and Ganoderan C, a galactoglucan-protein, were separated from *Ganoderma lucidum* with Mr 400 k by (Hikino, 1985). The polysaccharide part of both these fractions is chiefly composed β-1,6 and β-1,3 glycosides.

Our study presents the immunomodulating GLIS isolated from the fruit bodies of mushrooms as a protein-bound polysaccharide fraction, which contains more than 90% carbohydrates. The carbohydrate portion is composed of eight different monosaccharides, predominantly D-galactose, D-glucose and D-mannose in the molar ratio of 2:4:1.5. The active component GLIS was purified by HPLC, and has a Mr of about 2000 k. It seems evident that GLIS has different chemical properties compared to that of the known active components in *Ganoderma lucidum*.

The activity of GLIS for the stimulation of lymphocytes was reduced significantly by $NaIO_4$ but not by pronase E treatment, suggesting that this activity is associated with the polysaccharide but not the protein moiety. Further investigations show that digestion of GLIS with β-1,3 glucanase reduced its stimulatory activity. It might be postulated that the β-1,3 glycosyl group is important for its biological activity. Subsequent detailed analytic studies should elucidate the structure of this highly active polysaccharide.

4.5. Interaction of B cells and macrophages

Our results furthermore showed that B cells could be stimulated by GLIS directly; the survival of B cell and production of IgM were significantly lower than those of mouse spleen cells. In the presence of macrophages, the survival of B cells and IgM secretion was significantly increased after stimulation with GLIS. It seems that the effect of GLIS on spleen B cells includes interaction with macrophages.

Marginal zone macrophages are important for the induction of anti-virus TI-1 and TI-2 and anti-bacterial TI-2 antibody response (Spencer et al., 1998). Bondada et al. (2000) thought

that T independent antigens elicit antibody response in the absence of carrier specific T helper cells, but require signals from accessory cells (macrophages and dendritic cells) or specific cytokines. The relationship between adherent cells and B cells was also reported by Takemoto et al. (1994). They found that the mitogenic activity of F-5-2, a pectic polysaccharide fraction isolated from medical plants, was abolished by removing the resident adherent cells from spleen cells. Re-addition of those cells to the culture system restored the activity of F-5-2. The adherent cells seem to be essential for the proliferation of spleen cells by F-5-2. For B cell stimulated by GLIS, macrophages are not essential but benefical for IgM production. This indicates that the macrophages play an important role in maintaining survival or escape apoptosis of B cells and force them to secrete IgM.

Although it has been studied extensively, questions still remain about the mechanism that regulates Ig secretion. Goodrich et al. (1998) suggested the intestinal epithelial cells might secrete IL-6 and TGF-β to regulate local B cells antibody secretion, and their effects may be highly dependent on the activation state of the epithelial cells. Guo et al. (2000) found that when normal B cells from mice spleens were cultured with a pectic polysaccharide bupleuran 2IIc in the presence of anti-IL-6 neutralizing antibodies, the enhanced IgM secretion by bupleuran 2IIc was reduced. When B cells were stimulated with this sample, their IL-6 secretion and transcription of IL-6 mRNA were enhanced. Among the cytokines capable of stimulating B lymphocytes, IL-6 was reported to induce B cell proliferation and the secretion of large amounts of IgM (Markine-Goriaynoff et al., 2001). The present study showed that IL-6 can enhance the production of IgM, but has no synergetic effect with GLIS. The reason may be that IL-6 can be produced by GLIS-stimulated B cells themselves.

The supernatant of macrophages increased IgM production by plasma cells, but the effect of the supernatant of macrophages on B cells is not as strong as that of macrophages. The results suggest that in the presence of GLIS, the influence of macrophages on B cells is not only through the substances secreted by the macrophage but also through direct interaction. Blys secreted by macrophages was recently reported to be involved in B cells activation or survival. Blys is a member of the tumour necrosis factor (TNF) ligand superfamily that is functionally involved in B cell proliferation. Soluble Blys binds specifically to B cells and promotes proliferation of B cells costimulated by antibodies to immunoglubulin M (Moore et al., 1999; Schneider et al., 1999). Blys also promotes the survival of both resting and activated B cells (Khare et al., 2000; Do et al., 2000). Transgenic expression of blys in mice results in the enlargement of spleen and lymph nodes, and an increase in mature B cells that show an activated phenotype and production of autoantibodies (Gross et al., 2000).

4.6. The receptor of GLIS

Previous studies reported that FITC-labled glucans can be used to assess receptor binding by flow cytometry (Ainsworth, 1994). In our experiment, fluorescein-labeled GLIS (FITC-GLIS) was found to bind to macrophages and macrophage cell lines J774 and U937. This indicates that GLIS stimulates macrophages first through binding to the receptor.

Many biological functions of carbohydrates and glycoconjugates are often realised by the interaction with their receptors. β-(1,3)-glucan polysaccharides isolated from fungi can bind to the lectin site of CR3 with high affinity and prime the receptor for subsequent cytotoxic activation by iC3b-tumour cells that are otherwise inert in stimulating CR3-dependent cytotoxicity (Vetvicka et al., 1996; Vetvicka et al., 1997). Several different receptors for β-glucan have, in fact, been identified on leukocytes, including 160- and 180- kDa proteins on the surface of human monocytes and U937 cells (Szabo et al., 1995).

GLIS is a heteropolysaccharide. We found that when β-glucan and mannose receptor antagonist laminrin and α-mannan were added to the culture, the NO production by the macrophage stimulated with GLIS was decreased. This study indicates that GLIS triggers macrophages activation through interaction with the β-glucan receptors and mannose receptors. In addition, we found that GLIS could stimulate B cells and macrophages like LPS, and the anti-CD14 antibody could also block the NO production by macrophages stimulated with GLIS.

4.7. Chemoprevention of HZ-3-1-b

Li (1999) found that the extract of *Polygonum cuspidatum* could significantly inhibit the proliferation of different tumour cells. In this work, HZ-3-1-b was found to be one of the active compounds through bioassay-directed fractionation. HZ-3-1-b could induce SW620 cell apoptosis *in vitro*, which appears to account for its growth inhibitory and anti-proliferate activities. After treatment with HZ-3-1-b, SW620 cells could not transit from S phase to G_2/M, were arrested in S phase, and DNA fragmentation occurred immediately.

HZ-3-1-b is identified in this work as the compound resveratrol, which is a well-known component in grape or red wine (Tinhofer et al., 2001). Our results demonstrated that resveratrol is an active compound in *Polygonum cuspidatum*, which inhibits the growth of tumour cells and induces apoptosis in SW620 cells. The finding of clear and effective compounds of TCM will help to elucidate the mechanism of this ancient medicine. *Polygonum cuspidatum* was traditionally used as a folk medicine for the treatment of skin burns, hepatitis and gallstones, etc., and recent studies have found that *Polygonum cuspidatum* exhibits anticancer activity by acting as an antimutagen or a inhibitor of protein-tyrosine kinase (Su et al., 1995; Jayatilake et al., 1996). This identifies a new active compound of *Polygonum cuspidatum* for tumour therapy. Moreover, our isolation procedure provides a simple and plentiful source for resveratrol. The quick purification process, using two steps of extraction and HPLC, provides a new technology for resveratrol preparation.

Resveratrol is one dietary chemopreventive phytochemical that has recently attracted considerable interest because of its remarkable multi-functional inhibitory effects on multistage carcinogenesis. One of the plausible mechanisms that could account for the chemopreventive activity of resveratrol is its suppression of prostaglandin biosynthesis. Prostaglandins are known to play pivotal roles in pathogenesis of carcinogenesis, and certain NSAIDs have protective effects on experimental carcinogenesis. Furthermore,

epidemiological data indicate a reduced risk of colorectal cancer among individuals who regularly ingest aspirin or other NSAIDs which inhibit cyclooxygenase. Suppression of prostaglandin biosynthesis through selective inhibition of COX is thus regarded as an important cancer chemopreventive strategy (Cuendet et al., 2000; Moreno, 2000; Brzozowski et al., 2000).

Common epithelial cancers such as colon, breast, prostate, and lung remain the dominant cause of cancer deaths in developed countries. Treatment of these tumours in their advanced stages, with the current emphasis upon cytotoxic chemotherapies, has made little impact upon survival or quality of life (Landis et al., 1999). One promising strategy is chemoprevention. Chemoprevention is the use of natural or synthetic compounds to block, reverse or prevent the development of invasive cancers. Effective chemopreventive agents cause growth arrest and, in some cases, induce apoptosis of tumours (Manson et al., 2000). Our present investigation clearly demonstrates that resveratrol can induce apoptosis of SW620 cells in culture. These findings suggest that resveratrol is a potential chemoprevention agent in human colon cancer.

4.8. Inhibition of the enzyme activity glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by resveratrol

In the present study, a 38 kDa protein was found to have affinity to resveratrol using resveratrol affinity chromatography, which identified the protein as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by MALDI-TOF-analysis. GAPDH is a key regulatory enzyme of glycolysis, catalysing the formation of 1,3-bisphosphoglycerate, and has been commonly considered as a constitutive housekeeping gene. However, several lines of evidence indicate that GAPDH is a multifunctional enzyme, involved in various biological processes such as endocytosis, the control of gene expression, DNA replication and repair, and neuronal apoptosis (Sirover, 1999).

Our results showed that resveratrol could significantly inhibit GAPDH activity. In various origins of human cancers such as lung, pancreas and cervical carcinomas, GAPDH expression is substantially increased compared with normal tissues (Tokunaga et al., 1987; Schek et al., 1988; Kim et al., 1998). Antisense oligodeoxynucleotide of GAPDH on cultured carcinoma cells is associated with the apoptotic process, including increased DNA fragmentation (Kim et al., 1998). Revillion et al. (2001) found that the overall survival and relapse-free survival were significantly reduced in patients whose tumours showed an enhanced level of GAPDH expression.

It is interesting that antisense oligonucleotide of GAPDH can induce apoptosis of tumour cells, whereas it inhibits apoptosis of neuronal cells, indicating a neuroprotective effect. GAPDH gene expression was specifically increased during programmed neuronal cell death. GAPDH antisense oligodeoxyribonnucleotide blocks age-induced expression of GAPDH and effectively inhibits neuronal apoptosis (Ishitani et al., 1998). There are not only many reports about apoptosis induced by resveratrol (Joe et al., 2002; Lee et al., 2002; Dorrie et al., 2001; She et al., 2001), but also several reports about the neuroprotective activity of resveratrol

(Nicolini et al., 2001; Jang et al., 2001; Sinha et al., 2002). This means that resveratrol is capable of blocking the apoptotic process of neuronal cells, but, in contrast, can induce apoptosis of tumour cells. Further studies should show whether the two functions of resveratrol are related with GAPDH activity.