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Full Length Research Paper

The effect of polysaccharide peptide and taxol on human solid tumor tissue *in vitro*

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Taxol has anti-tumor properties, polysaccharide peptide (PSP), an active substance of Yunzhi, is an effective immunopotentiator, which is used to supplement the chemotherapy and radiotherapy of cancers patients. The antitumor activity of polysaccharopeptides has been documented. In this study, the in *vitro* effect of PSP upon the metabolic rate of breast solid tumors was observed and this effect was compared with taxol, which is a well-known chemotherapeutic drug. 117 patients' tissues were treated with 4, 2 and 1 mg/ml of PSP and 4.27 µg/ml of taxol for 24 h. ATP bioluminescence assay was used to measure the *in vitro* metabolic rate of the breast cancer tissues and SPSS was used for statistical analysis. The estrogen receptor (ER), progesterone receptor (PR), HER-2, tumor grading and the age of patients were all analysed in the study. Taxol was significantly effective on tumors in younger aged, low tumor grade; ER and PR negative group's subjects. PSP demonstrated similar results and also suppresses the activity of breast solid tumor. ATP bioluminescence assay was successfully performed in determining tumor metabolic rate in a timely fashion such that chemotherapeutic treatment could be guided by the results.

Key words: Polysaccharide peptide, taxol, ATP bioluminescence, estrogen receptor, progesterone receptor.

INTRODUCTION

Taxol, a natural product extracted from pacific regions yew tree Taxus brevifolia, is a potent anti-neoplastic drug (Gelmon, 1994; Fu et al., 2009; Choi et al., 1995; Exposito et al., 2009; Watchueng et al., 2011). The growing demand for taxol greatly exceeds the supply that can be sustained by isolation from its natural source and alternative sources of the drug are being sought (Choi et al., 1995; Exposito et al., 2009; Watchueng et al., 2011). The most promising approach for the sustainable production of taxol and related taxoids is provided by plant cell cultures at an industrial level (Choi et al., 1995; Exposito et al., 2009; Watchueng et al., 2011). Taxol has been purified using modernized technique and machines. It is currently being clinically used against different tumour processes (Choi et al., 1995; Exposito et al., 2009; Watchueng et al., 2011). In the July 3, 1991 issue of the Journal, scientists were attempting to develop a synthetic formulation of taxol by studying its mechanism of action. In 1992, the FDA approved the use of taxol as a treatment for women with advanced ovarian cancer. Since then, it has also been approved for use in the United States to treat advanced breast cancer, lymph node-positive breast cancer, non-small-cell lung cancer and AIDS-related Kaposi's sarcoma. In contrast to other antimitotic drugs, such as cochicine, podophyllotoxin, vinblastine, maytansine and nocodazole, which inhibit microtubule assembly (Luduena, 1979), taxol promotes microtubules aggregation from tubulin dimmers and stabilizes them by preventing depolymerization with a concomitant induction of apoptosis (Jordan et al., 1993; Carré et al., 2002; Jordan et al., 1996; Derry et al., 1998; Schiff et al., 1979; Jordan and Kamath, 2007). In addition, taxol induces abnormal arrays or "bundles" of

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microtubules throughout the cell cycle and multiple asters of microtubules during mitosis (Rao et al., 1999; Yang et al., 2009), thus inhibiting cell replication predominantly at the G_0/G_1 and G_2/M phases (Donaldson et al., 1994; Portugal et al., 2010). Morphologically, taxol-treated cells form abnormal, disorganized microtubules, leading to an epithelioid cell shape (Pletjushkina et al., 1994). Previous experimental *in vitro* studies with taxol have been performed in tumor cell lines, epithelial cells and fibroblasts (Gloushankova et al., 1994; Matsuoka et al., 1994; Jin et al., 2009; Schiff and Horwitz, 1980; Jordan and Kamath, 2007). Neoplastic cells (MKN-28, MKN-45 and MCF-7), treated with taxol at a concentration of 0.01 mM, showed growth inhibition, low colony-forming efficiency and a prolonged G2+M phase accumulation (Matsuoka et al., 1994; Jin et al., 2009). Whereas, normal cells (Balb/c 3T3 cells and human fibroblasts originating from stomach cancer tissue), treated with taxol at a concentration of less than 0.5 µm, showed no growth inhibition and no decrease in colony-forming efficiency (Matsuoka et al., 1994; Jin et al., 2009).

Taxol has many clinical applications, especially in the treatments of cancer, for example, ovarian cancer (Vermorken, 2001; Fung-Kee-Fung et al., 2007), lymphoma (Zhou et al., 2000; Valavanis et al., 2001) and breast cancer (Seidman et al, 1994). It is not only being used as a single agent in the treatment of cancer but also in combination with other drugs, such us doxorubicin and etoposide in ovarian cancer (Hahn et al., 1993; Vadgama et al., 2000), rituximab, gemcitabine and vinorelbine in lymphoma (Emmanouilides et al., 2002) and trastuzumab, anthracyclines, docetaxel, vinorelbine and gemcitabine in breast cancer (Campone et al., 2003; Friedrichs et al., 2002; Nagourney, 2001).

Polysaccharide peptide (PSP) is an active substance of Yunzhi, which is isolated from the deep-layer cultivated mycelia of Coriolus versicolor COV-1 (Dong et al., 1996; Shang et al., 2003). Its polypeptide components are rich in glutamic and aspartic acids, whereas its poly-saccharide component is made up of monosaccharides, with alpha-1, 4 and beta-1, 3 glucosidic linkages (Dong et al., 1996; Ng, 1998; Fisher and Yang, 2002; Shang et al., 2003). PSP is effective at inhibiting cell proliferation through apoptosis (Chow et al., 2003). The mechanism for apoptosis may be through up-regulation of p21 and down-regulation of cyclin D1 (Chow et al., 2003). PSP stimulates both humoral and cellular immune responses and induces the production of gamma-interferon and interleukin-2 and increases T-cell proliferation (Chow et al., 2003). Detail mechanism is still not known but recent research has found that PSP actually contains relevant B- and T-cell epitopes and can act as a good carrier in improving an antipolysaccharide anamnestic immune response (Lett et al., 1994). This active substance is also a valuable adjunct in raising the white blood cell counts of mice and patients treated with chemotherapeutic drugs (Chow et al., 2003). It counteracts the immune-suppression induced by cytotoxic drugs such as cyclophosphamide (Chow et al., 2003). In double-blind trials, PSP significantly extended the five-year survival rate of patients with esophageal cancers (Kidd, 2000; Ferreira et al., 2010). The anti-tumor mechanisms of PSP upon breast cancer have not yet been fully investigated (Kidd, 2000; Ferreira et al., 2010). *In vitro* experiments have revealed that PSP can inhibit proliferation through apoptosis of leukemia, lymphoma, stomach and lung cell lines (Chow et al., 2003; Kidd, 2000; Ferreira et al., 2010). Other actions of PSP include observation of breast cancer cell growth after the treatment of PSP (Chow et al., 2003), leukemic cell-cycle progression and expression of interleukins (Hsieh et al., 2002; Nicandro et al., 2007), as multi-cytokine inducers that are able to induce gene expression of various immunomodulatory cytokines and cytokine receptors (Ooi and Liu, 2000; Ferreira et al., 2010; Kurbacher and Cree, 2005), boosts immune cell production, ameliorated chemotherapy symptoms and enhanced tumor infiltration by dendritic and cytotoxic T-cells (Kidd, 2000; Ferreira et al., 2010). Its mechanism of action is still under investigation and its clinical efficacy has not been properly evaluated.

The aim of this study is compare taxol as a standard reference in breast cancer treatment with the effect of PSP on breast solid tumor *in vitro*, by using ATP bioluminescence method to measure the metabolic rate of the tumors.

MATERIALS AND METHODS

Subject selection

117 patients with age ranging from 27 to 81 were recruited for this study. Written consents, approved by University of Hong Kong, Clinical Research Ethics Committee, were obtained from all participants. The tissues were obtained from the operation thereafter in the Queen Mary Hospital and collected in sterilized bottle containing Hanks' Balanced Salt Solution (HBSS).

Pathological results of patients

Immunohistochemical staining for estrogen receptor, progesterone receptor and c-erbB-2 expression was performed. Lymphovascular permeation and histological grading of tumor differentiation were also identified.

Tissues and culture

After washing the breast cancer specimens with HBSS and penicillin 100 units/ml, streptomycin 10 µg/ml and amphotericin 2.5 µg/ml (Sigma, Missouri, USA), the specimens were minced into 1 mm^2 pieces. The samples were grown for 24 h in 24 well plates and soaked in DMEM, Dulbecco's Modified Eagle's Medium (Invitrogen, California, USA), with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate and supplemented with: 10% feta bovine serum. This medium is formulated for the usage in a humidified incubator with an

Table 1. Fluctuations of cell metabolic rate caused by different concentrations of taxol and PSP in 117 patient's tissue.

Group	Mean of ATP [rlu]
Control	6.0124
Taxol 4.27 µg/ml	1.4747*
PSP 4 mg/ml	1.9661*
PSP 2 mg/ml	2.2503*
PSP 1 mg/ml	2.5279*

* *P* value = 0.05.

Table 2. Proportions of different grades of breast solid tumor specimens with percentage inhibition over 50 upon the treatment of PSP and taxol.

Drug	Grade 0	Grade I	Grade II	Grade III
Taxol 4.27 µg/ml	45/63 [71%]*	13/18 [67%]*	15/24 [63%]*	9/12 [75%]*
PSP 4 mg/ml	39/63 [63%]*	9/18 [50%]	5/24 [20%]	5/12 [38%]
PSP 2 mg/ml	37/63 [58%]*	9/18 [50%]	7/18 [40%]	6/12 [50%]
PSP 1 mg/ml	33/63 [53%]	17/18 [94%]*	7/18 [38%]	4/12 [33%]

* *P* value = 0.05

atmosphere of 5% CO_2 at 37°C. Capsules of PSP were prepared in serial dilutions and tested in mono-layer cells. The concentration which could cause 50% cell death was 2 mg/ml.
The percentage inhibition was calculated as:

$$\text{The percentage inhibition} = \frac{\text{Mean ATP of control - Mean ATP of test group}}{\text{Mean ATP of control}} \times 100$$

Determination of ATP levels in tumor tissue

The specimens were segregated (each group consisting of 3 wells) were as follows: control, taxol 4.27 µg/ml (Peak Plasma Concentration), PSP 4 mg/ml, PSP 2 mg/ml and PSP 1 mg/ml. The specimens were incubated for another twenty-four hours then distributed in microtubes and homogenized by a sonicator (Sonics and Materials Inc., Connecticut, USA). ATP bioluminescence assay kit (Roche Mannheim, Germany) was used for the determination of ATP activity in the tissue specimens, as well as cancer cells in the supernatant of the culture medium.

Statistical analysis

Data are reported as mean±SD and all statistical analyses were performed using SPSS 15.0. Data were analyzed using the one way ANOVA. Differences between the experimental and control groups were regarded as statistically significant when $p < 0.05$.

RESULTS

Specimens from 117 patients were being tested on their activities using PSP and taxol. After applying PSP and taxol to solid breast tumors, the ATP absorbance (rlu) results are shown in Table 1. The calculated suppression effect of PSP and taxol shows that the concentration of PSP and the metabolic rate were inversely proportional. The mean values of ATP were statistically significant. The optimum concentration of PSP in the treatment of breast cancer is currently unknown, thus three concentrations of PSP (4, 2 and 1 mg/ml) were used in order to determine the optimal concentration reference. A comparison between the tumor activity reduction effect of PSP and taxol, and the solid tumor grading are summarized in Table 2. The presence or absence of ER and PR on the effect of PSP and taxol has been compared in Table 3. The median age of patients in our study was 54 years (range 27 to 81). The median age of patients in our study was 54 years (range 27 to 81) and the results show the relationship between age and tumor grading (Table 4). The increase in tumor grading leads to a general decrease in both the effect of PSP and taxol and the presence of the hormonal receptors also reduces their effects. From our data, the specimens from younger patients tend to have greater chance to be of higher grading in this study (Table 4). All the comparisons among the groups shows statistical significant with p value less than 0.05.

DISCUSSION

ATP bioluminescence uses the ATP dependency of the light emitting luciferase catalyzed oxidation of lucerferin for the measurement of the metabolic rate in normal and cancer cells. A production of ATP reflects an abnormally In addition to ER, low tumor grade, younger patient age, and a lack of PR expression all showed a large reduction in tumor activity measured by ATP bioluminescence upon

Table 3. Proportions of ER and PR negativity with percentage inhibition over 50 upon the treatment of PSP and taxol.

Drug	ER [-ve]	ER [+ve]	PR [-ve]	PR [+ve]
Taxol 4.27 µg/ml	35/51 [69%]*	46/66 [69%]*	51/72 [71%]*	30/45[67%]*
PSP 4 mg/ml	33/51 [65%]*	24/66 [36%]	42/72 [58%]*	15/45 [33%]
PSP 2 mg/ml	30/51 [59%]*	30/66 [45%]	45/72 [63%]*	15/45 [33%]
PSP 1 mg/ml	27/51 [53%]	27/66 [41%]	33/72 [46%]	21/45 [47%]

**P* value = 0.05.

Table 4. Grading of breast tumor patients in three different age groups.

Age group	Grade 0	Grade I	Grade II	Grade III
20 to 49	9/51 [18%]	0/51 [0%]	45/51 [29%]	27/51 [53%]
50 to 69	12/42 [29%]	3/42 [7%]	12/42 [21%]	18/42 [43%]
70 to 99	6/24 [25%]	3/24 [13%]	6/24 [25%]	9/24 [38%]

high metabolic rate of cancer cells (Liu et al., 2000; Kurbacher and Cree, 2005). This assay is also highly sensitive to the presence of small amounts of ATP (Khan, 2003). It is a reliable and convenient method for the analysis of metabolic rate of cells.

PSP acts by directly suppressing tumor cell growth both *in vitro* and *in vivo* (Dong et al., 1996; Shang et al., 2003). Its prevention of *in vivo* growth of tumor mass is probably mediated through its immunomodulating effect (Dong et al., 1996; Shang et al., 2003). Taxol also acts by the direct suppression of tumor cell growth both *in vitro* and *in vivo* (Matsuoka et al., 1994; Jin et al., 2009; Schiff et al., 1979; Jordan and Kamath, 2007; Zhou et al., 2000; Valavanis et al., 2001), but it lacks the ability to boost immune response *in vivo*. Both PSP and taxol have a direct suppressive effect on tumor cells through apoptosis (Chow et al., 2003; Yang et al., 2003; Blankenberg, 2009). High dose taxol lead to side effects, such as myalgia/arthralgia and neutropenia (Ng, 1998; Fisher and Yang, 2002). In view of the immunomodulating effect of PSP, we can propose that the combined action of both taxol and PSP may likely be compensated on the decrease in the amount of white blood cells brought upon by high dosage taxol treatment alone, because the presence of B- and T-cell epitopes on PSP can induce an anamnestic antipeptide response (Lett et al., 1994). Follow up studies are required to support the above adjuvant hypothesis.

Estrogen receptors (ER) are expressed in approximately 65% of human breast cancers, implying that this sex steroid plays an important role in the development and propagation of the disease (Dickson and Lippman, 1987; Lippman and Dickson, 1989; Hayashi, 2007). Approximately one third of women with breast cancer respond to ablative endocrine therapy (Davidson and Lippman, 1988, Pritchard, 1998; Goel et al., 2009), and anti-estrogens positively influence the course of established breast cancer (Stuart et al, 1996; Gibson et al., 2009) or prevent the development of primary disease (Early Breast Cancer Trialists' Collaborative Group, 1998; Clarke, 2008). Based upon *in vitro* and *in vivo* data, estrogen probably acts as both a growth factor and a survival factor for breast cancer (Dickson and Lippman, 1987; Lippman and Dickson, 1989; Hayashi, 2007).

In the early course of breast cancer establishment, a cytokine response could induce apoptosis of the cancer cells via the activation of cell surface receptors for tumor necrosis factor (Jäättelä et al., 1995; Stegh et al., 2002). Estrogen acts through a putative plasma membrane ER (Pietras and Szego, 1977; Pietras and Szego, 1980; Fleming and Gurpide, 1980; Toran-Allerand, 2005) that has been shown to have strong functional evidences (Chen et al., 1999; Farhat et al., 1996; Razandi et al, 1999; 2000; Marino and Ascenzi, 2008). In our *in vitro* study, breast cancer solid tumor treatment with taxol and PSP is markedly less effective in the present of estrogen receptor (ER). The novel and rapid mechanisms, mediated through the plasma membrane estrogen receptor, by which estrogen prevents taxol-induced apoptosis of breast cancer has been reported (Pritchard, 1998; Goel et al., 2009). It tells us that taxol cannot overcome the anti-apoptotic effect of estrogen and ER. From the clinical data that we obtained, for taxol (4.27 µg/ml), 69% of the ER (-ve) samples have an ATP-count percentage decrease greater than 50 and 75% for ER (+ve) samples. Comparing to that of PSP, concentrations 4 and 2 mg/ml showed 65 and 59%, respectively of percentage decrease greater than 50% for ER (-ve) samples, but only 36 and 45%, respectively for ER (+ve)

ones. The data suggests that PSP may not be able to overcome the effect of estrogen and ER.
the addition of PSP and taxol. Higher grade tumors may exhibit an *in vitro* proliferation advantage over those with a lower grade and younger women tend to have higher grade breast cancers than older women (Albain et al., 1994; Kidd, 2000; Ferreira et al., 2010) (Table 4). A relationship between tumor growth and negative PR status has previously been described in the human tumor clonogenic assay (Hug et al., 1986; Gonzalez-Angulo et al., 2007). It is likely that this association is simply a reflection of the lower proliferation rates in hormone receptor positive tumors (Silvestrini et al, 1993; Fisher et al., 2004; Wenger et al., 1993; Gazic et al., 2008) and that may mirror the tumor activity measured in our study.

Moreover, receptor HER-2 (c-erbB-2) is believed to be involved in the development of breast cancer (Duffy et al., 2003; Keshava et al., 2004). HER-2 is a self antigen, but antibodies and T-cells specific for HER-2 have been isolated from cancer patients, suggesting HER-2 may be a good target for active immunotherapy. Over-expression of the growth factor receptor HER-2 (c-erbB-2, neu) has transforming potential and occurs in approximately 20 to 30% of breast and ovarian cancers (Renard et al., 2003; Baxevanis et al., 2010). From our data, neither c-erbB-2 positive nor negative gave much effect on both the actions of Taxol and PSP. For taxol, c-erbB-2 (-ve) and c-erbB-2 (+ve) gave out the percentage of 92.86 and 100% respectively; for PSP, they are 53.33 and 55.56%, respectively.

In summary, we found that PSP has a prominent suppressive effect towards early onset breast solid tumor activity *in vitro* by using the ATP bioluminescence measuring method. Such method would provide an alternative way in checking tumor activity and give us the effect of the target chemotherapeutic drugs. Such assay success occurred in all specimens obtained, these results maybe helpful when consideration is given to designing future studies. PSP may be applied as an alternative anti-tumor agent to patients reducing the side effects of the conventional chemotherapeutic drugs.

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