Author's response to reviews

Title: The immunomodulator PSK induces in vitro cytotoxic activity in tumour cell lines via arrest of cell cycle and induction of apoptosis

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Author's response to reviews: see over
Dear Editor,

Following the decision taken by the Editors and the comments made by the Reviewers, I am sending to you a new version of our paper “The immunomodulator PSK induces in vitro cytotoxic activity in tumour cell lines” (MS: 1185498167166850).

The revised version has a new title (suggested by reviewer) and three new tables. I am also including answers to the different critical comments of the Reviewers. We hope that now our paper can be considered for publication in BMC Cancer.

Looking forward to hearing from you,

Regards,

Angel Miguel Garcia
Response to Reviewer´s comments
Ref: MS 1185498167166850

Reviewer: Dr. RK Battacharya

1. “The findings are very preliminary. There is no originality in designing the research plan. PSK was shown to have cytotoxicity in all tumor cell lines tested. What is new? The compound is active against many tumors”

The originality of our research plan is that it was designed to investigate whether PSK has a direct cytotoxic effect on tumour cells in vitro. Although several groups have shown that PSK has an anti-tumour effect due to its immunomodulatory properties, there has been very little research into its direct cytotoxic activity. In fact, we have been able to find only one study, which reported the direct cytotoxic effect of PSK in vitro on one of various leukaemia cell lines (28). Other study reported that PSK augments docetaxel-induced apoptosis in human pancreatic cancer cells NOR-P1 (29). To our best knowledge, there has been no published study on the activity of PSK on the in vitro proliferation of tumour cell lines derived from different solid tumours.

What is new about our manuscript is that we describe for the first time the cytotoxic effect of PSK in vitro on proliferation of tumour cell lines derived from different solid tumours. Our results provide the first demonstration that the antitumor properties of PSK may be due to immunomodulatory properties and a direct cytotoxic effect.

We cannot agree that our findings are preliminary, since we analyzed the proliferation rate in seven tumour cell lines and two types of assay were performed: BrdU absorbance and viable cell count, with each repeated at least three times. Moreover, we analysed cell cycle, apoptosis and caspase-3. Hence, we do not believe that our findings can be considered preliminary.
2. “Besides, all cell lines were not subjected to all experiments, such as neuraminidase treatment, cell cycle analysis, and particularly annexin V experiment and caspase 3 activation. Moreover, it was not clear why neuraminidase treatment did not alter PSK cytotoxicity.”

After obtaining wide and clear evidence that PSK exerts a direct cytotoxic action on tumour cell lines in vitro, we then analysed the mechanisms underlying this action. In fact, we studied cell cycle, apoptosis and caspase-3 expression in all seven tumour cell lines, but we only included Figures and text on the two phenotypes found in these cell lines for the sake of simplicity. In the revised version (Tables 2, 3 and 4), we have now added the data for the other tumour cell lines.

In a previous publication (22), our group reported the action of neuraminidase on PSK. In the present paper, we studied whether the neuraminidase treatment of PSK modifies its cytotoxic and immunomodulatory properties. Results obtained showed that neuraminidase treatment did not modify the cytotoxic effect of PSK on the tumour cell lines, neither immunomodulatory effect on the NKL cells. In light of the reviewer’s comments, we have introduced a new paragraph into the Materials and Methods (page 5, line 4) and Discussion (page 13, line 7) on the action of neuraminidase on PSK.

3. “Apoptosis was measured in only two cell lines, that too using limited apoptotic parameters (expression of caspase 8, bax/bcl, cytochrome C and DNA fragmentation, morphologic characteristics has been ignored). It is stated that PSK induced apoptosis in certain cell lines (only two used) indicating cytotoxic effect by different molecular mechanism depending of histology of tumor. This conclusion is not validated by experimental results”

In fact, as commented above, we studied whether PSK induces apoptosis in all seven tumour cells. However, the Results and Figures only showed the two different phenotypes found in order to simplify the manuscript. We now include more tumour cell lines, showing the same two phenotypes (see Tables 3 and 4). We quantified apoptosis by using annexin-V flow cytometry. The DNA ladder
shows apoptosis, and we performed this analysis first. However, because this is not a quantitative assay, we only included the annexin-V findings. We also measured caspase-3 activation as indicator of apoptosis induction. A more in-depth analysis of the effects of PSK on the apoptosis process and signal transduction of apoptosis will be the subject of a future investigation. In this study, we show clearly for the first time that PSK induces apoptosis in tumour cell lines (four of seven cell lines analysed) and that caspase-3 is implicated in the induction of this apoptotic process.

4. “It was also not clear if PSK activity is tumor specific as no conclusion could be drawn from PBL experiments”.

Our results of a dose-response curve (section 2) showed that PSK does not exert cytotoxic effect on PBLs. These data were not shown because identical absorbance results and viable cell counts were obtained for PSK-treated and untreated lymphocytes. Moreover, Figure 2 shows that PSK had no effect on lymphocytes, only PSK had a synergistic effect with IL-2 on lymphocyte proliferation. We previously reported that PSK has the same effect as IL-2 on the induction of proliferation and activation of NKL cells (16). It is clear that PSK does not have a cytotoxic effect on lymphocytes or NKL cells.

5. “Figure numbers are absent under the figures, and it's difficult to comprehend from the legends as these do not describe what are being measured and how these are measured”

Figure numbers have been included, and the figure legends have been modified as requested.
Response to Reviewer’s comments
Ref: MS 1185498167166850

Reviewer: Dr. Kenichi Matsunaga

- Minor Essential Revisions

  1. The CM.101 has been corrected
  2. Figures 1, 2 and 3 have been corrected

- Discretionary Revisions

  1. The title has been modified as recommended.
  2. Differences in composition between PSK and neuraminidase-treated PSK were previously published (22). We have included a new paragraph in Materials and Methods describing these differences (page 5, line 4)
  3. A new paragraph has been included in the Discussion on this point (page 13, line 7)
  4. The statistical analysis has been included in Table 1 and Figures 1 to 3. A new section has been inserted in Materials and Methods (page 8, line 23)
  5. The anti-metastatic properties of PSK were previously attributed to immunomodulatory effects on NK cells. We hypothesise that the in vivo anti-metastatic effect of PSK may also derive from its cytotoxic properties. This paragraph in the Discussion has been revised accordingly (page 14, line 14).
  6. The reference number has been inserted and the reference has been added to the references list.
  7. The abbreviation (PSK) has been inserted on first use.
  8. All cell line names are now written in a consistent manner.
  9. The inclusion of IMIM PC-1 and U937 cell line names was an error, and they have been deleted.
  10. The sentence with RPMI has been changed.
  11. The Abbreviation List has been completed.