Author's response to reviews

Title: Chondromodulin-1 directly suppresses growth of human cancer cells

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Author's response to reviews: see over
We are grateful to Reviewer 1, Ian Cree, for the critical comments and useful suggestions that have helped us to improve our paper considerably. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the version of our paper.

Comments by Reviewer 1, Ian Cree.

Comment #1
The authors should undertake further experimental work to support their contention that the Jak-STAT pathway is involved.

Response
As you pointed out, we should undertake further experiment to dissect the mechanism in the action of ChM1, particularly in the Jak/STAT pathway. A luciferase reporter assay of STAT, GAS, and ISRE was performed and added in the revised manuscript. The reporter assay demonstrated that ChM1 suppressed the transcriptional activities of STAT and GAS but not of ISRE (Figure 5), and we have indicated that the Jak/STAT signaling pathway is one of the targets of ChM1 action. However, we do not know how ChM1 activates intracellular signaling pathways. As described in the discussion, ChM1 may act through one or more of the following mechanisms: 1) by recruiting protein tyrosine phosphatase family members such as SHP which inactivate Jak; 2) by recruiting SOCS and/or PIAS to degrade STAT dimers; or 3) by directly or indirectly inhibiting cofactors that form complexes with STAT dimers [24, 35].

Comment #2
The considerations about the possibility that ChM1 could induce expression of other growth factors.

Response
We have considered the possibility that ChM1 may alter the expression level of any cytokines and growth factors. We performed RT-PCR of VEGF (vascular endothelial growth factor) and cell cycle-related genes, and subtraction differential gene expression (gene fishing), but no gene was found to be differentially expressed by ChM1. Therefore, we examined signaling molecules such as AKT and cell cycle-related protein at the protein level.
Comment #3
Further discussion of the controls used in the experiments performed, particularly with the adenoviral vector, would be helpful.

Response
I agree with you that we should always consider the controls, particularly when using adenoviral vector. As stated in the manuscript, the preliminary experiment for adenoviral infection and cytotoxicity have already been performed. As described below, cells that express the coxackie-adenovirus receptor (CAR), that can bind with adenovirus and transfect the adenovirus gene to the cell, were selected. We confirmed the infection and expression of the transfected gene with the Xgal assay. The multiplicity of infection (MOI) was gradually increased, and the MOI at the point that the level of expression was the highest was used. Cytotoxicity or any effects of Ad-LacZ in cells was not observed in each cell.

Comment #4
The authors should be more circumspect in their interpretation of the data for following a therapeutic approach.

Response
We agree with you that our results do not indicate a therapeutic approach. Therefore, as shown discussion, our results suggest that this molecule warrants further in vivo study in the future. Of course, an in vitro study should be performed and elucidate the mechanism in the action of ChM1.

Comment #5
Why were these particular cell lines chosen over the many others available?

Response
As described above, because we used the adenovirus vector to observe the effect of ChM1, and we had to choose the cells to be infected by adenovirus. As described in the manuscript, recombinant protein was scarce for some experiments. The effect of ChM1 on endothelial cells but not the effect on fibroblast. We used HUVECs(Human umbilical vascular endothelial cells) and NHDFs(neonatal human dermal fibroblasts). The HepG2 cell line, from hepatocellular carcinoma, was chosen as hyper vascular tumor clinically. Experiments using HepG2 were performed both in vivo and in vitro on culture plates. Obvious effects of ChM1 were observed in both conditions, so we decided to use the HepG2 cell line as the representative tumor cell. Next, the effect of ChM1 was observed in some tumor cells, chosen as described above, cultured on plate. Only in HeLa cells,
human uterine cervical adenocarcinoma, was the effect of ChM1 in plate culture not observed. Therefore, HeLa was chosen as the representative of the cell from which the effect was not seen in plate culture. It was an unexpected result that the effect of ChM1 was seen with HeLa cultured in agarose gel, and it was difficult first to explain these seemingly contradictory results.

Comment #6

How was the concentration of ChM1 chosen?

Response

The concentration of the rhChM1 that could reduce the DNA synthesis of HUVECs by half was at most 25µg/ml, so the effective doses were 10µg/ml. However, that concentration of rhChM1 did not affect on the DNA synthesis of NHDFs (Fig. 1B) as previously described[8]. The reasons why effective doses of rhChM1 became higher are that the rhChM1 protein deteriorated or the cell conditions were different. Although there is not sufficient rhChM1 protein and IC50 was not determined, we used a similar concentration. rhChM-1 inhibited the DNA synthesis in HepG2 in a dose dependent manner (Fig. 1C), but not in HeLa cells (Fig. 1D) cultured on plate. Our result, for the first time, showed that ChM1 has a direct effect on specific cancer cells cultured on either plates or in agarose, and, possibly, on many other cancer cell types.

Comments #7

The figures are of poor quality and should be improved.

Response

We agree with your comment, so we have improved the quality of all figures.

Comment #8

The conclusion that further in vivo work is required is perhaps true, but surely more in vitro work should be performed to elucidate the mechanisms of action of this molecule first.

Response

More in vitro work concerning this molecule should be performed. That STAT signaling pathway is suggested as the candidate for the target of ChM1 and our results clearly indicate that this molecule warrants further study in vivo.
We are grateful to Reviewer 2, Vasilis Vasiliou, for the critical comments and useful suggestions that have helped us to improve our paper considerably. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the version of our paper.

Comments by Reviewer 2, Vasilis Vasiliou.

Comment #1
The authors should give a more mechanistic approach on the actions of the ChM1.

Response
As you pointed out, we should undertake further experiments to dissect the mechanism of action of ChM1, particularly in the Jak/STAT pathway. A luciferase reporter assay of STAT, GAS, and ISRE was performed and added in the revised manuscript. The reporter assay demonstrated that ChM1 suppressed the transcriptional activities of STAT and GAS but not of ISRE (Figure 5), and we have indicated that the Jak/STAT signaling pathway is one of the targets of ChM1 action. However, we still do not know how ChM1 activates intracellular signaling pathways. As described in the discussion, ChM1 may act through one or more of the following mechanisms: 1) by recruiting protein tyrosine phosphatase family members such as SHP which inactivate Jak; 2) by recruiting SOCS and/or PIAS to degrade STAT dimers; or 3) by directly or indirectly inhibiting cofactors that form complexes with STAT dimers [24, 35].

Comment #2
The authors present the alteration of the protein levels of cell cycle-related proteins by ChM1, but from figure 2D this is not obvious. This paper will become stronger the effects on cell cycle proteins will be more clear.

Response
We agree with your opinion that the alteration of the protein levels of cell cycle-related proteins by ChM1 should be quantified. We measured the densitometry and, as shown in figure 2D(b), levels of proteins for cyclin D1, cyclin D3, and cdk6 were significantly decreased by Ad-ChM1. In contrast, Ad-ChM1 caused up-regulation of p21 cip1, a cdk inhibitor, at 12hours and 36 hours. The reason why the levels of proteins are much higher at 12hrs is not known, but the proliferative activity of the cells in very high because of the few cells on the plate at 12hrs. Repeated experiments showed similar results. The signal contrasts of those proteins were different due to exposure conditions of each membrane. This is why statistical analysis was not performed.
Comment #3
The discussion should include at least speculation of how ChM1 exhibits its antitumor effects.

**Response**
As described above, we added the result of the luciferase reporter assay that the Jak/STAT signaling pathway is a target of ChM1 action, and speculated in the discussion of revised manuscript that ChM1 may act through one or more of the following mechanisms: 1) by recruiting protein tyrosine phosphatase family members such as SHP which inactivate Jak; 2) by recruiting SOCS and/or PIAS to degrade STAT dimers; or 3) by directly or indirectly inhibiting cofactors that form complexes with STAT dimers [24, 35].

Yours sincerely,