Author's response to reviews

Title: IGF-I activates caspases 3/7, 8 and 9 but does not induce cell death in colorectal cancer cells

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Version: 3 Date: 4 December 2008

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

Thank you for sending us the reviewer’s comments to our paper. We found the reviewer’s constructive comments are helpful. We have carried out some additional work and obtain extra data. According to the new data we have revised the paper and included these new data in the new version of the paper. In the new version of the paper we also withdraw PI3 kinas/Akt and MAPK pathways work, we believe these two pathways are some how related to IGF-I activation of caspases, but we don’t have further evidence to prove it yet, therefore we are currently carrying more studies to clarify it. We think Akt/PI3 kinase and MAPK pathway studies could form another new paper after we obtain more new data.

In summary in the new version of the paper, a new figure (Figure 4) has been added, and three old figures (figure 4, 5 and 6) have been withdrawn. All these changes have been incorporated into the manuscript and the title of the paper has been changed to “IGF-I activates caspases 3/7, 8 and 9 but not induced cell death in colorectal cancer cells”. The following is our responses to the reviewer comments.

Reviewer’s report
Title: Insulin-like growth factor I activates apoptotic pathways in colorectal cancer cells
Version: 2 Date: 5 November 2008
Reviewer: Jack Youngren
Reviewer’s report:
Overview:
The authors present data suggesting that IGF-1 has pro-apoptotic effects in HT-29 colon cancer cells, with some corraboration in experiments with a couple other cell lines. They demonstrate a blockade of these effects by IGF-1R blockade employing specific receptor antibodies. These results are in direct contrast to published reports employing the exact same cell lines. The authors make mention of some of these contradictory studies in their introduction, referring to the "controversial" nature of the field. However, the authors do not take steps in their studies to ensure their validity in the face of contradictory reports.

Major Compulsory Revisions:
1. The authors must include other measures of apoptosis. While Caspase activation is a hallmark of apoptosis, only one type of assay is employed in these studies and the sole readout is luminescence with no way to assess the
untis. It is clear that the units employed to quantify caspase activity are relative and are highly different from graph to graph, which does not allow for a true understanding of the conditions of the cells. Preferable would be a measure of the percentage of apoptotic cells. While this might be overkill in another paper, the authors are operating at the disadvantage that previous authors have shown that small molecule inhibitors of the IGF-1R (Piao et al Molecular Cancer Therapeutics, 2008) and monoclonal antibodies against the IGF-1R (Zhang and Zhang, Cancer Investigation, 2008) both induce apoptosis in these same cell lines.

We agree with reviewer’s comments and have employed a previous reported cell death assessment method (Remacle-Bonnet MM 2005) to examine cell death following IGF-I treatment. And it was found that exogenous IGF-I actually not cause cell death even it activates caspases 3/7, 8 and 9. We also induced the cancer cell (HT-29 and SW620) to apoptotic death with a known apoptotic inducer (5-FU) and the dead cells were then examined with the cell death assessment method. In this way we validate our cell death assay method. According to the new finding we have re-written the paper. In the new version of paper all the new data and new validated methods have been included. A cell proliferation assay was also carried out and the new data was included in the new version of the paper.

2. To draw conclusions based on the use of downstream inhibitors, the authors should study the effects of IGF-1 incubation as well as the antibodies and kinase inhibitors on signaling through the respective pathways. If the reader is to believe that Akt is pro-apoptotic in these cells, a signaling scheme linking IGF-1 stimulation to pro-apoptotic pathways in the cell should be demonstrated.

We believe Akt signalling is somehow related to IGF-I activation of caspases, but we don’t have further evidence to prove it yet, therefore we are currently carry more studies to clear this up. But we decided in the new version of our paper we won’t include Akt data yet. We believe the Akt/PI3 kinase data could form another new paper after we obtain more new data.

3. The discussion should be expanded to discuss the directly contradictory studies and to offer possible explanations for the discrepant results. A discussion of the limitations of their study must be included. Any suggestion that IGF-1 derivatives might make ideal anti-cancer agents should be tempered by a thorough discussion of the body of work demonstrating the negative aspects of IGF-1 on colorectal cancers.

The discussion section has been re-written according to the new finding and reviewer’s comments regarding to this have been considered during the re-write paper.
Reviewer's report
Title: Insulin-like growth factor I activates apoptotic pathways in colorectal cancer cells
Version: 2 Date: 23 October 2008
Reviewer: Leon A Bach
Reviewer's report:
MAJOR COMPULSORY REVISIONS
1. The major flaw of this work is that rates of apoptosis are not shown. Did IGF-I increase apoptosis in all cell lines? If so, were all cell lines equally sensitive? For example, we recently published that IGFs promote apoptosis in LIM 1215 colon cancer but not RD rhabdomyosarcoma cells (Fu, J Cell Biochem, 100, 58-68, 2007. What about proliferation and, in the myoblasts, differentiation?

We agree with reviewer’s comments and have employed a previous reported cell death assessment method (Remacle-Bonnet MM 2005) to examine cell death following IGF-I treatment. And it was found that exogenous IGF-I actually not cause cell death even it activates caspases 3/7, 8 and 9. We also induced the cancer cell (HT-29 and SW620) to apoptotic death with a known apoptotic inducer (5-FU) and the dead cells were then examined with the cell death assessment method. In this way we validate our cell death assay method. According to the new finding we have re-written the paper. In the new version of paper all the new data and new validated methods have been included. A cell proliferation assay was also carried out and the new data was included in the new version of the paper.

2. The authors use SB203580 as a MAPK inhibitor. However, there are several distinct MAPK pathways and this compound is an inhibitor of p38 MAPK not MEK1 as stated in the discussion. IGF-I receptor signalling is predominantly via the MEK/ERK pathway.

We believe Akt signalling is some how related to IGF-I activation of caspases, but we don’t have further evidence to prove it yet, therefore we are currently carrying more studies to clear this up. But we decided in the new version of the paper we won’t include Akt data yet. We believe the Akt/PI3 kinase data could form another new paper after we obtain more new data.

3. Statistical methods need clarification. It is stated that experiments were performed in triplicate (I presume within each experiment) and repeated on 2 cultures. I interpret this to mean that there were only 2 independent experiments and statistics were performed on n=2, not n=6. Confirmation is needed that this is the case. Of course, statistical analysis on n=2 is marginal. Also, which multiple comparison test was used after ANOVA?

We confirm that the statistical analysis was perform on n=6 samples and Bonferroni’s Multiple Comparison Test was used after ANOVA.
4. The text and the figures are often inconsistent regarding the statistical significance of between-group comparisons, e.g. Fig 4 IGF-I ± LY. Also, the statistical methods indicate that ** signifies p<0.01, whereas the figure legends indicate p<0.001; which is correct?

In the new version of paper Figure 4 has been left out. But we still like state that in the method we indicated ** means P< 0.01. But in the figure legends 0.001 is an actually P value. Of course when the figure is used in a new paper we will make sure our write won’t cause any confusion.

5. The IGFIR antibody only partially inhibited IGF-I effects. Either the antibody did not completely suppress IGFIR activation or another receptor is involved. The effect of the antibody on IGF-I-induced receptor activation (e.g. IGF-I receptor phosphorylation) should be directly measured to distinguish between these possibilities.

Thanks to reviewer’s paying very more detail to the IGF-I R ab actions. We believed that IGF-I antibody partially inhibited IGF-I effects may be due to another receptor involvement. The inhibition effect of IGF-I R ab on IGF-I is more effective in SFM media conditions than in SCM conditions seems confirmed this. We have included this in our discussion.

6. Although caspase-8 and -9 are components of the extrinsic and intrinsic apoptotic pathways, there is cross-talk between them, so it is not possible to conclude that both pathways are involved without more detailed studies.

This has been changed in the new version of paper.

7. The authors state that SB203580 did not inhibit IGF-mediated caspase activation and conclude that MAPK is not involved. However, all IGF-induced caspase activities were increased in SCM and caspase 3 increased in SFM, suggesting that p38 activity may inhibit IGF-induced-apoptosis.

Same as Akt signalling we also left MAPK pathway signalling out as we believed these two pathways need more work to clarify and we are currently carrying more studies on them now.

MINOR COMPULSORY REVISIONS

There are many instances of imprecise language and typographical errors that should be corrected. For example:

1. p. 3, para 2, line 3: ‘the mortality has not…’.
2. p. 7, para 1, line 3: ‘media’ not ‘mediums’.
3. p. 15, para 2, line 2: what is ‘SM’; should it be ‘SEM’ or ‘SD’?
4. Ref 9 is incomplete.

All these errors have been corrected.
DISCRETIONARY REVISIONS
1. In the introduction (p. 3), the authors state ‘the role of IGF-I in cellular apoptosis … remains controversial’. The overwhelming evidence is that IGF-I is antiapoptotic as published in hundreds of papers. A much smaller number of papers (perhaps 10) show proapoptotic actions of IGF-I, so it is perhaps misleading to say the role is ‘controversial’. It might be better to provide this perspective.

Some changes have been made to mirror this.

2. The paper would be strengthened by data showing that the pathway inhibitors were active in the cell lines at the concentrations used.

Akt and MAPK pathway signalling has been left out in the new version of the paper. We believed these two pathways need more work and we are currently carrying more studies on them now.

3. Chemical inhibitors are not entirely specific. The results with these inhibitors would be strengthened by confirmation with at least one other inhibitor or siRNA.

Akt and MAPK pathway signalling has been left out in the new version of the paper. We believed these two pathways need more work and we are currently carrying more studies on them now.

Reviewer’s report
Title: Insulin-like growth factor I activates apoptotic pathways in colorectal cancer cells
Version: 2 Date: 12 November 2008
Reviewer: Alexandre Arcaro
Reviewer’s report:
Major Compulsory Revisions

1) In its current state, the manuscript only relies on caspase activity measurements, which is problematic when trying to make firm conclusions on apoptosis induction. The authors need to perform additional work to firmly prove apoptosis induction in the colon cancer cells upon IGF stimulation. DNA fragmentation and PARP cleavage must be analysed to conclude that apoptosis is indeed triggered by IGF in the different cell lines under study. Moreover, a quantification of the number of cells undergoing apoptosis must be provided by performing Annexin V staining and FACS analysis. Without these data it is not possible to conclude that colon cancer cells do indeed significantly undergo apoptosis in response to IGF.

We agree with reviewer’s comments and have employed a previous reported cell death assessment method (Remacle-Bonnet MM 2005) to examine cell death following IGF-I treatment. And it was found that exogenous IGF-I
actually not cause cell death even it activates caspases 3/7, 8 and 9. We also induced the cancer cell (HT-29 and SW620) to apoptotic death with a known apoptotic inducer (5-FU) and the dead cells were then examined with the cell death assessment method. In this way we validate our cell death assay method. According to the new finding we have re-written the paper. In the new version of paper all the new data and new validated methods have been included. A cell proliferation assay was also carried out and the new data was included in the new version of the paper.

2) If IGF triggers caspase-dependent apoptosis in the cells this should also be assessed by performing cell viability assays (MTT) in the presence or absence of caspase inhibitors (ZVAD).

In the new version of the paper, cell viability assay have been included.

3) The authors have used pharmacological inhibitors of the PI3K and MAPK pathway in the caspase assays. However, they have not provided any evidence that IGF stimulates the Akt and/or the MAPK pathway under these conditions. Western blot analysis using phospho-specific antibodies must be performed to address this issue.

Akt and MAPK pathway signalling has been left out in the new version of the paper. We believed these two pathways need more work and we are currently carrying more studies on them now.