Author's response to reviews

Title: Increase in intracellular PGE2 induces apoptosis in Bax-expressing colon cancer cells.

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

Please find enclosed the revised version of our manuscript entitled “Increase in intracellular PGE$_2$ induces apoptosis in Bax-expressing colon cancer cells”. We took into account the many remarks of the reviewers. We answered to their questions as good as possible and we hope they will find this new version satisfying. Please find afterwards the detailed answers to both reviewers.

We expect our revised version of the manuscript will be suitable for publication in BMC Cancer.

Sincerely yours,
Lisenn Lalier, PhD
Answer to Young Jung

The reviewer raises the question if our results are representative of what occurs in whole tumours. We agree with him that this point is questionable.

As suggested by the reviewer, the appropriate validation would be to establish a correlation between the intracellular concentration of PGE$_2$ in the colon cancer cells and apoptosis. Unfortunately, we failed in imaging PGE$_2$ with anti-PGE$_2$ antibodies by immunohistochemistry, probably due to the pleiotropic distribution of PGE$_2$.

Several studies have been published studying COX-2 expression in colon tumours. In a large dataset of tumours, no significant correlation was established between COX-2 expression and apoptosis or patient survival (Zhang et al, Am J Gastroenterol 2002). In contrast, COX-2 expression was rather associated to proliferation (Zhang 2002) and related to stroma (Hull et al, Brit J Cancer 1999; Fujimori et al, Gastroenterol 2000). No correlation could be observed between COX-2 and mPGES-1 expression (Yoshimatsu et al, Clin Cancer Res 2001 and our results). In addition, it would also be necessary to correlate COX-2 and mPGES-1 expression with MRP4 and 15-PGDH, and even maybe with the other enzymes implicated in prostaglandins synthesis (L-PGDS for example).

The use of cell lines enabled us to focus on one pathway involved in PGE$_2$ signalling in colon cancer cells. With the care to be as representative as possible for colon cancers, we confirmed our results with two additional cell lines. Our in vitro work was thus realised with four colon cancer cell lines, two of which presented LOH (SW1116 and HT29) whereas the other two were classified MSI-positive (HCT-116 and HCT-8) (Gayet et al, Oncogene 2001 and Li et al, Cancer Res 2004). We hope the reviewer will find this satisfying giving the technical limitations.

Minor revisions:

1. The reviewer is right and we added data showing that the cell death observed is correlated to caspase 3 activation.

2. We did not observe any modification in Bax expression induced by the PGE$_2$ treatments. Concerning Bax activation, we demonstrated the ability of PGE$_2$ to activate Bax directly, independent from any cellular model, in a very recently published article (Lalier et al., Cell Death Diff, 2010). By contrast, Bax expression level definitely conditions the apoptotic response of HCT-116 cells to PGE$_2$ as described in the figures 3B and 4D.

3. CAY alone induced a marginal cell death. We added the data on the graph.
Answer to Ivonne Loeffler

Major compulsory revisions:

1. To answer to the reviewer’s question, we induced mPGES-1 overexpression in two additional colon cancer cell lines and measured intracellular PGE$_2$.

2. The reviewer is right that we did not explore the paracrine effects of mPGES-1 overexpression in this work. Our point was to demonstrate that mPGES-1 also had a pro-apoptotic activity in Bax-expressing colon cancer cells through direct activity of intracellular PGE$_2$ mediated by Bax, as we have previously demonstrated in glioma cells. We believe that this demonstration could be of interest in colon cancer cells, since extensive work has already been published on the deleterious effect of (extracellular) PGE$_2$ in colon cancer through paracrine effect. We do not cast doubt on the paracrine effect of PGE$_2$ (and consequently also of mPGES-1 overexpression) on colon cancer cells and we certainly could have studied them in the conditions we used. Nevertheless, the most striking point for us was that mPGES-1 overexpression was associated to a decrease in tumour growth (Elander et al., BBRC, 2008) whereas extracellular PGE$_2$ had the opposite effect in the same model (Wang et al., Cancer Cell, 2004). Besides, following to the reviewer’s comments, we treated the four colon cell lines with extracellular PGE$_2$ and did not observe the cell death inducing effect. Even if not excluding the existence of paracrine effects induced by PGE$_2$, this indicates to our mind that the death-inducing activity of PGE$_2$ is not linked to EP signalling but rather to an intracellular target.

3. The reviewer points out the fact that we detect COX-2 expression at the mRNA and protein levels in HCT-116 cells, even if we show that the protein expression is much fainter than in the SW1116 cell line. We agree with the reviewer that the HCT-116 cell line has often been used in the literature as a COX-2 deficient cell line. Yet, some of the data presented in the literature clearly show that COX-2 is constitutively expressed in HCT-116 cells. We would just point out the fact that it is sometimes difficult to ascertain the lack of expression of a protein by western blot, all the more than we compare several cell lines which expression levels are very different. Considering the work by Banu et al. for example, it seems that a faint band corresponding to COX-2 might be seen on the gel presented, even if the authors conclude that the cells do not express the protein. On the other hand, we do not forget that HCT-116 cells are MSI-positive, suggesting that their genetic instability may result in several laboratories working on HCT-116 cells phenotypically slightly different. In our case anyway, we also verified that the constitutively expressed COX-2 was functionally active, even if faintly expressed, since the amount of PGE$_2$ secreted by HCT-116 cells was almost 40 times higher than by rat fibroblasts (382.6 pg/mg protein vs 11.2 pg/mg protein).

4. We tested the cell viability following to PGE$_2$ treatment alone with four cell lines and a range of 0.1-100µM. We observed no significant cell death induction in three out of four cell lines. Only HCT-116 cells were affected by extracellular PGE$_2$ even if the difference between control and treated cells did not exceed 10%. Of note, we measured that PGE$_2$ intracellular concentration increased in PGE$_2$-treated HCT-116 cells, contrary to the other cell lines, suggesting a missing compensatory phenomenon. This might be linked to the faint 15-PGDH expression in these cells (figure 4A) but we did not further investigate this difference.
5. The reviewer is right that we did not investigate the effect of all the substrates of 15-PGDH and MRP4. Nevertheless our purpose was to document the apoptotic activity of PGE\(_2\). To this aim, we focused on the effect induced by PGE\(_2\) by comparing the viability of the cells submitted to 15-PGDH and MRP4 inhibitors either alone or with extracellular PGE\(_2\) added.

6. The reviewer is right and we added the results of the experiments on the four cell lines as supplementary data.

7. We confirmed that mPGES-1 transfection in the four cell lines and that the treatment of SW1116 cells by PGE\(_2\) and pharmacological inhibitors induced a raise in DEVDase activity, confirming that the cell death observed is an apoptotic cell death.

8. We agree with the reviewer that the study of the EP-receptors-linked signalling pathways activated by PGE\(_2\) in the conditions we used would be of interest. Nevertheless, we believe that our data demonstrate that the apoptotic cell death induced by PGE\(_2\) in colon cancer cells is due, at least in part, to the cytoplasmic accumulation of PGE\(_2\) and is Bax-dependent. This is consistent with what we have previously published on the direct regulation of prostaglandins on Bax activity (Lalier et al, Oncogene 2007 and Lalier et al, Cell Death Diff 2010). We therefore think that this could be the purpose of another study.

Minor revisions:

1. We added a few references.

2. We added clinical information in supplementary data.

3. We added some details in the methodologies section when commercial kits or reagents were not used. Unless otherwise stated, we followed the manufacturer’s instructions.

4. We regret that we have not been able to obtain high quality blots with tumour samples, especially for COX-2. The clinical samples we obtained in the first set were very little tumour fragments which only enabled us to perform three western blots and we could not obtain the corresponding control tissue. The same membranes were probed with anti-mPGES-1 antibody, then the membranes were stripped prior to the sequential hybridization with anti-COX-2 and anti-actin antibodies. We added the mean +/- sd quantification.

5. The numbers in figure 1B are the identifiers of the second set of patients we have obtained. They are different from figure 1A. The mean values presented here are the mean of three independent normalized gene expression determination as described in the Methods section (\(\Delta\Delta CT\) methodology). We added a reference for more detailed description of this methodology.

6. COX-2, actin and mPGES-1 were blotted on the same membranes. Statistical significance was added.

7. We removed the figure.

8. We added a conclusion chapter.