Author's response to reviews

Title: Heparin (GAG-hed) inhibits LCR activity of Human Papillomavirus type 18 by decreasing AP1 binding

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Version: 2 Date: 25 June 2006

Author's response to reviews: see over
June 24th, 2006

Iratxe Puebla  
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MS: 1883308808103186

Dear Prof. Puebla,

Please find enclosed the revised version of the manuscript entitled “Heparin (GAG-hed) inhibits LCR activity of Human Papillomavirus type 18 by decreasing AP1 binding”.

In the revised version, we have taken into consideration the valuable comments of the referees and have made the pertinent experiments and modifications to the original manuscript. A detailed description of our answers to the referees is listed below:

**Reviewer 1: Carlos Ciudad**

Major points

The main criticism is a lack of consistency in the report of the EMSA in Materials and Methods with regard to which is described later on in the legend to Figure 5. The authors state that they end-label the ds-oligonucleotides with alpha-32P-dATP or dCTP and Klenow. This can be done, but then the oligonucleotides have to have sticky ends to allow for the labelling. However, in Figure 5, they say the oligomers were labelled with 32P-ATP or CTP, probably meaning gamma-32P-ATP or CTP. My guess is that a mistake have been made in the description in either one place or the other. Most likely, the authors labelled the ds-oligonucleotides with gamma-32P-ATP and polynucleotide kinase. The authors must clarify this apparent conflict before publication.

*Answer:* We are sorry for lack of consistency about EMSA. We end-label all ds-oligonucleotides with alpha-32P-dATP or alpha-32P-dCTP and Klenow enzyme. In this
corrected version, we stated clearly the oligonucleotide’s sequences, (Materials and Methods), indicating bases added for sticky ends. We also corrected Figure 5 legend.

Minor Essential Revisions

1) I wonder if the authors did ever experiment the possibility of treating with GAG-hed at the same time that when injecting with HeLa cells (together). That might increase even further the observed effects.

Answer: We did not perform this kind of experiments. At that point we preferred to have an evident solid tumour before starting treatment. We believed that the total inhibition we observed was a good starting point for our further observations. In our hands, some other experiments with cultured cells are indicative that, as the reviewer states, GAG-hed effects would be more evident when treatment is applied together with cells.

2) In Figure 5, panel E, it would be nice to show the effects on Sp1 binding (as a negative controls) as the authors did for panels C and D. Also a supershift with specific antibodies against AP1 could be used to ensure the nature of the band that is decreased by GAG-hed treatment in panel B.

Answer: Figure 5, panel E contains indeed the SP-1 binding negative control. However we modified panel assignation to clarify this point and accordingly, we labelled all sections in the figure equally. Figure 5, panel F, shows the unaffected SP-1 complexes.

Answer: In previous work published for us (Aguirre et al., 2000; Lopez-Bayghen et al., 1996), we had performed extensive characterization of the very same oligonucleotide that we use here for AP1 detection. This why, we are confident we are detecting AP1 and we did not considered necessary to repeat this control once more.
And, there is one experiment that requires more careful observation and documentation on the result. Heparin is known to induce cancer cell death through inhibition of transcription factors and causing apoptosis (for example, Berry D, Lynn DM, Sasisekharan R, Langer R., 2004). Therefore, GAG-hed used in this work is expected to induce cell death independently of the inhibition on HPV E6/E7 expression. On page 12, authors described the effect of GAG-hed on HeLa cell viability. Authors documented at the same suppressive effects of GAG-hed--- can be translated into a significative decrease in viability and growth for HeLa cell cultures. However, authors carried out MTT assay which provides only the relative numbers of live cells. The result presented in Fig.4, does not provide any information on whether the treatment caused only cell growth arrest (usual outcome of E6/E7 inhibition is G1 arrest followed by a state of cellular senescence) or also induced cell death. The cell morphology presented in B does not provide any information on the effect of the treatment, and the text neither described anything on this observation. The authors may also want to observe a longer effect on SW480 cells.

Answer: The microtiter plate assay which uses the tetrazolium salt MTT is now widely used to quantify cell proliferation and cytotoxicity (for example see Oram et al 2006, BMC Cancer 6:154). Since proliferating cells are metabolically more active than non-proliferating resting or death cells, the assays are suitable not only for the determination of cell viability and factor mediated cytotoxicity but also for the determination of cell proliferation. We agree, heparinoids and glucosaminosacarides induced apoptosis and cell death (Bennett et al., 1994; Berry et al., 2004; Linhardt, 2004), and it is beyond the scope of this work to explore exact mechanisms for apoptosis. However, in this new version, we have performed additional experiments in order to record more carefully cell proliferation along time (as reported elsewhere). We seeded cells and apply treatment three hours later, and promptly started (12 h) a more detailed recording of GAG-hed effects on cell proliferation along time (data provided in Figure 4B). Longer effects produced by GAG-hed on SW480 cells
were also tested and data added. As stated in panel B of Figure 4, no further effects were recorded for this particular cell type.

**Major review**

Secondly, in Fig 6, authors provided a model for the effect of GAG-hed on HPV LCR. According to the cartoon in the proposed model and its legend, GAG-hed and AP1 form a stable complex that is incapable of binding to DNA. Is there any evidence for the existence of this complex either from any published report or from the work of the authors’ own? If not, the proposed model should be retracted. If yes, it should be mentioned and discussed.

*Answer:* We totally agree and now, we correctly stated that the proposed model is based in previous published work together with our observations. Berry et al., 2004 have shown important changes in the levels of “free” transcription factors, after heparin internalization and originally proposed this effect may result from their bond-off from DNA. We also based our model in work by Busch et al., 1992 on AP1 effect, group that showed that $^{125}$I-labeled-heparin binds directly to Fos and Jun peptides. Our model attempts only to particularized our main observation: the HPV18 regulatory region is downregulated by heparin through a very probable AP1-heparin interaction that blocks AP1-binding to DNA, affecting the overall transcription outcome, and particularly E6/E7 synthesis.

**Reviewer: Werner Zwerschke**

Minor Essential Revisions:

1. The author should specific the terms cell proliferation and cell growth.

*Answer:* In revised version we stated clearly we are determination of cell viability and proliferation. We apologize for misuse in terms.

2. Although the presented findings suggest that GAG-hed inhibits the transcription of HPV-18 E6/E7 by an AP1-dependent mechanism, the underlying mechanism remains largely unknown. The authors should discuss how GAG-hed could
function. For example, does it bind to specific receptors at the cytoplasm membrane? How blocks GAG-hed AP1 binding to its DNA-binding side?

**Answer:** There are direct evidence for heparin incorporation into cell cytoplasm and its presence in the nuclei (Berry et al., 2004; Busch et al., 1992). There is also evidence that heparan sulfate and glycosaminoglycans in the Extracellular Matrix and on the cell surface can be internalized by cells while bound to receptors, and up taking heparin involves complexation and internalization with fibroblast growth factor and fibroblast growth factor receptor (Schlessinger et al., 2000; Sperinde and Nugent, 2000). Once in nuclei, important changes in the levels of “free” transcription factors, after heparin internalization and originally proposed this may result from bound-off them from DNA (Berry et al., 2004).

GAGs are acidic and highly negative charged molecules, which interact with a large number of proteins and other basic molecules through ionic and hydrogen bonding interactions (Capila and Linhardt, 2002). Busch, et. al in 1992, explored heparin effect over AP1 showing that $^{125}$I-labeled-heparin binds directly to Fos and Jun peptides, in line with reported data. In this corrected version, we properly added this to discussion section. Complete additional references are included below and in the appropriated section in the paper.

3. One major problem is that the manuscript has a lot of grammatical errors and English should be improved

We did our very best in this regard getting help from a native English speaking colleague and we did our very best. I hope you find this version acceptable. We hope all mistakes are now properly corrected.

We hopefully wish that all the performed changes in the manuscript account adequately referees suggestions and our work is now suitable for publication in *BMC Cancer*

Sincerely,

[Signature]
Additional complete references:


