Author's response to reviews

**Title:** Induction of plasminogen activator inhibitor type-1 (PAI-1) by hypoxia and irradiation in human head and neck carcinoma cell lines

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**Author's response to reviews:** see over
Dear Dr. Edmunds,

Thank you for giving us the opportunity to submit a revised version of our manuscript entitled „Induction of plasminogen activator inhibitor type-1 (PAI-1) by hypoxia and irradiation in human head and neck carcinoma cell lines“ to BMC Cancer.

Attached you will find:
1. Our point-by-point response to the concerns of the reviewers.
2. Four supplementary figures to support our responses – these are meant purely for the reviewers and the editor and are not supposed to be included in the manuscript.
3. Our revised manuscript, in which we addressed the reviewers' comments.

We hope our revisions address the majority of the referees’ concerns and our revised version is now acceptable for publication.

Thank you again for the opportunity to respond to the helpful comments of each referee.

Yours sincerely,
Daniela Schilling

Reviewer 1: Vladimir Zachar

1/ The experiment employing desferroxamin to induce hypoxia is not described in the Methods section.

This has been added to the Methods section in the part, “Western Blot”.

2/ In the Results section, it is stated that the hypoxia has no significant influence on cell viability inferring from the fact that 5% of BHY cells were dead in comparison to 1% at ambient air. At first glance this difference seems substantial. On what basis was the conclusion about non significance between 1% and 5% determined?

We apologize for this inaccuracy. After 24 h of hypoxia 2.9 % of the adherent BHY cells were dead (= 97.1% viable) and this was not significant compared to 99.1 % viability of normoxic grown cells. Significance between 24 h N and 24 h H was calculated by the t-test.

This has been corrected and redrafted (viability instead of the number of dead cells) in the Results section (page 8) and a table was added including the mean values, SEM and associated significance (see revised manuscript Table 1).

3/ In conclusion sections, the authors claim that both short- and long-term hypoxia can induce PAI-1. This statement should be more specific. The duration of hypoxic experiments was 24 hrs. Should that be understood as short-term or long-term exposure? It might be conceivable that if the hypoxic experiments were extended to up to three days, under the given conditions, the levels of PAI-1 could even decrease.

We defined short-term as ~ 4 h to 8 h and long-term as ~ 20 h to 24 h. This was included in the conclusions section. We agree that extended treatment longer than 24 h could possibly lead to adaptation of cells to hypoxic conditions and decreased PAI-1 levels.

4/ It is not immediately clear what was the purpose of experiments on the stability of PAI-1. Interpretation of these experiments should be included in the Discussion section.
The purpose was to investigate whether secreted PAI-1 in the medium is detectable by ELISA after longer incubation periods at 37°C in the absence of PAI-1 secreting cells. This is important for later reoxygenation studies, in which we intended to measure (by the same ELISA assay) only PAI-1 that was secreted during the 24 h reoxygenation period and not during the preceding 24 h hypoxic exposure. Because we showed unchanged levels of PAI-1 in the medium at 37°C over 7 days, we decided to change the medium before reoxygenation. If we had not changed the medium, we would have detected the accumulated level of PAI-1 after both 24 h hypoxia and 24 h reoxygenation. In order to make this clearer, we moved the paragraph “Stability of PAI-1 in cell culture supernatants” to the Results section “Effect of reoxygenation on PAI-1 expression and secretion” and explained its importance for the medium change. Since this experiment was only performed in order to decide whether a medium change was necessary or not, we did not include it in the discussion section.

5/ It is not clear where the correlation between samples was statistically evaluated although it is stated that this was one of the statistical methods used.

The correlation between samples was evaluated between the two cell lines and between intracellular and secreted PAI-1 levels (page 10). This was added to the Methods section in the part “Statistics”.

**Reviewer 2: Mike Robbins**

**Major Compulsory Revisions:**

**Methods:**

1. The reoxygenation experiments are interesting, but it is unclear as to why the authors chose to change the medium after 24h of hypoxia. This does not appear to have any physiological relevance, and needs to be justified if this is to be continued.

We showed that secreted PAI-1 in the medium (in the absence of cells) is detectable by ELISA at constant levels for up to 7 days at 37°C (see revised manuscript Table 2). In reoxygenation experiments, we wanted to compare a 24 h period of normoxia, hypoxia and reoxygenation. Therefore, the medium was changed so that only newly secreted PAI-1 protein during the 24 h reoxygenation period was measured and not PAI-1 secreted during the preceding 24 h hypoxic exposure.

Additionally, in vivo hypoxia is not a static process. Due to structural abnormalities in tumour blood vessels, blood flow can change suddenly and lead to acute hypoxia. This is often temporary because the flow can subsequently increase again and lead to reoxygenation (Janssen et al., Head Neck 2005, 27(7):622-38).

2. In the Western Blot analyses, the authors state that they use only 5 ?g of cell lysate to detect HIF-1?. This seems surprising, since in the literature it seems more common to use in the order of 30 ?g of nuclear protein.

There are papers in which the authors used cell extracts for HIF-1a western blots (e.g. Cohen et al., Laryngoscope 2004, 114(3):418-23; Li et al., Kidney Int 2005, 68: 569-583, Chadderton et al., Int J Radiat Oncol Biol Phys 2005, 62: 213-222.). The reviewer is correct that normally larger amounts of cell extracts or nuclear extracts were used. However, for our cell lines, only 5 µg of cell extracts was sufficient. We added the original western blots as supplementary material (suppl. 1) to demonstrate that the HIF-1a band has the correct size and is specific. Both our negative (24 h N) and positive control lysates (DFO) also showed the expected results.
Results:

3. Influence of hypoxia on cell viability: The claim that prolonged hypoxic exposure leads to increased detachment without a significant increase in viability of the adherent cells does not make sense. It would be simpler to state that hypoxia caused 30% death in the BHY cells and 14% death in the FaDu cells.

We claim that prolonged hypoxic exposure leads to increased detachment of cells without a significant decrease in viability of the adherent cells.

Since we measured PAI-1 concentrations in the cell lysates of adherent cells, it was important to prove beforehand whether hypoxia had an effect on the viability of the adherent cells.

Therefore, we indicated the difference between viability of adherent cells and number of detached cells. To make this clearer we added a table (see revised manuscript table 1) and redrafted this part in the results section.

4. Figure 2: There are a number of concerns with this figure. The authors conclude that phalloidin staining was unchanged with hypoxia, and yet it appears as if the red staining decreases under hypoxia, particularly in the FaDu cells. Although the figure shows qualitatively a time-dependent increase in PAI-1 staining under hypoxia, it is not quantitative and should be accompanied by Western Blots to show increases in PAI-1 immunoreactive protein. Further, the API-1 staining appears to shift from cytoplasmic to nuclear, and yet there is no explanation of this in the text.

It only appears that the phalloidin staining is decreased under hypoxia due to the overlapped picture. However, after careful examination of the individual pictures (see suppl. 2 and 3), it is clear that this is not the case. The green PAI-1 staining is simply stronger and covers the red phalloidin staining.

We agree that immunofluorescence is not a quantitative method. We chose ELISA assays for an accurate quantification of PAI-1 (see figure 3) because it is a more sensitive method and can be quantified better than immunoblot. We additionally performed PAI-1 immunoblots which strongly support our data from ELISA measurements. We didn’t include the immunoblots in our manuscript because they are only semi-quantitative and we believe that the two different methods, immunofluorescence and ELISA, are sufficient to demonstrate hypoxia-induced PAI-1 expression. PAI-1 immunoblots of cell lysates have been added as supplementary material (suppl. 4).

Nuclear PAI-1 staining could be assumed from the overlapped 24 h H picture of BHY. However, the individual pictures (see suppl. 3) show PAI-1 staining very near to the nucleus (perinuclear region) and not primarily in the nucleus. As we didn’t perform confocal microscopy, the exact determination of intracellular localization is difficult and therefore we can not exclude nuclear PAI-1 staining. This is indeed very interesting, but was not the intent of this experiment.

Most importantly, the immunofluorescence data show that hypoxia induces PAI-1 expression in BHY and FaDu cells.

5. The use of ELISA to determine PAI-1 stability appears inappropriate. This should be repeated using 35S methionine-labeling.

The reviewer is right, we didn’t show real stability of PAI-1 by measuring PAI-1 levels with ELISA, but this was not our intention. We agree that the term “stability” is wrong and changed this in the manuscript. We also moved this section “Stability of PAI-1 in cell-culture supernatant” to the beginning of the section entitled “Effect of reoxygenation on PAI-1 expression and secretion” to make our intensions clearer.

Our purpose was to investigate whether secreted PAI-1 in the medium is detectable by ELISA after longer incubation periods at 37°C (see comment 1). This is important for later
reoxygenation studies, in which we intended to measure only PAI-1 that was secreted during the 24 h reoxygenation period and not during preceding hypoxic exposure.

6. In Figure 5, the authors show data suggesting that PAI-1 levels in the media decrease after reoxygenation, and yet in Table 1 the data indicate no change in PAI-1 levels 1 day after 24 h hypoxia. These findings appear contradictory and require explanation.

In reoxygenation experiments (figure 4, formerly figure 5), cells were kept for 24 h under hypoxic conditions. Afterwards the old medium was removed and fresh medium added. The cells were then incubated for 24 h under normoxia (=reoxygenation). This guarantees that only PAI-1 that is secreted during the following 24 h reoxygenation period is measured. Figure 4 shows that during 24 h reoxygenation less PAI-1 is secreted than during 24 h hypoxia.

Table 2 (formerly table 1) shows something different and is not in contrast to figure 4. In this experiment, cells were grown for 24 h under hypoxia. Afterwards cell-free supernatants were placed in fresh dishes and kept for up to 7 days at 37°C. After 1, 2, 3 and 7 days, samples were drawn and analyzed for PAI-1. Results in table 2 show constant PAI-1 levels during 7 days incubation at 37°C measured by ELISA.

We rewrote this in the results section to make it easier to understand (see comment 5).

**Discussion:**

7. The data are limited to showing that, as in other cell lines, both hypoxia and radiation can upregulate PAI-1 gene expression and protein. Moreover, there are no studies aimed at testing the hypothesis that this radiation-induced increase in PAI-1 protein could play a role in the poor outcome for these patients.

The only publication investigating PAI-1 expression and secretion is by Fink *et al.* (Blood 2002, 99: 2077-2083) for a human hepatoma cell line. Moreover, they didn't investigate the influence of either reoxygenation or radiation on PAI-1 expression and secretion. Furthermore, no data exists showing the impact of reoxygenation (which is very important for radiotherapy) on PAI-1 expression and secretion in vitro.

Importantly, not much is known about radiation-induced PAI-1 expression/secretion. The reviewer's group (Zhao *et al.*, Cancer Res 2001, 61: 5537-5543; Int J Radiat Biol. 2000;76(3):391-402) has shown an irradiation-induced increase in PAI-1 transcription and secretion in rat kidney tubule epithelial cells in vitro (in context with fibrosis). However, we are interested in radiation induced changes in human tumour cells. To the best of our knowledge, the only in vitro study investigating the effect of radiation on PAI-1 in human tumour cells was on a single hepatoma cell line and on the transcriptional level (Hageman *et al.*, Clin Cancer Res 2005, 11: 5956-5964).

Therefore, our paper is the first describing the kinetics of PAI-1 expression and secretion simultaneously under hypoxia as well as the impact of reoxygenation and irradiation on PAI-1 expression and secretion in tumour cells (SCCHN).

The reviewer is correct that we didn’t investigate the influence of radiation-induced increase in PAI-1 protein on outcome. This is an assumption which we drew from our results. As PAI-1 promotes migration and angiogenesis and inhibits apoptosis of tumour cells (Bajou *et al.*, J Cell Biol 2001, 152: 777-784; Chen *et al.*, J Cell Biochem 2004, 92: 178-188; Kwaan *et al.*, Br J Cancer 2000, 82: 1702-1708.), PAI-1 could increase aggressiveness of the tumour. Therefore, radiation-induced increased PAI-1 levels could render tumour cells, which are not killed immediately, more aggressive and subsequently lead to increased metastasis.

We changed this in the conclusions section of the abstract and formulated it more carefully.

Further studies in our lab are under way investigating the influence of PAI-1 on radiosensitivity and outcome in vitro and in vivo.
Minor Essential Revisions:

8. Figure 1: It is surprising that the authors did not note a time-dependent increase in HIF-1α protein during hypoxia. Further, it is unclear as to the concentration of DFO used here; the increase in HIF-1α with DFO appears modest.

We agree with the reviewer that there is a time-dependent increase in HIF-1α, which we didn’t mention. Therefore, we revised the Results section “Hypoxia induces HIF-1α expression” to describe the time-dependent HIF-1α increase.

100 µM DFO was used. We included this in the Methods section. HIF-1α expression levels after the addition of 100 µM DFO and 24 h hypoxia are comparable. Data from the literature (Vordermark et al., Int J Radiat Oncol Biol Phys. 2004, 58(4):1242-50) also show comparable HIF1-a expression levels between 24 h hypoxia (1% O₂) and the addition of 100 µM DFO in FaDu cells.

9. Combine Fig 2, Fig 3 and Fig 4.

We rather prefer to show figure 2 in a separate figure. But we agree to combine figure 3 and figure 4.

Reviewer 3: Harun Said

No revisions demanded.

We thank Dr. Said for his kind comments.

Supplementary data:

1. Original HIF-1α Western Blot (see also fig.1)

2. Immunofluorescent staining with the PAI-1 antibody, PAb-Rb (1:125) (green), Phalloidin (red) and DAPI (blue) of FaDu cells grown for 24 h under normoxic (24h N) and for 8 (8hH), 16 (16hH) and 24 h (24h H) under hypoxic conditions.

3. Immunofluorescent staining with the PAI-1 antibody, PAb-Rb (1:250) (green), Phalloidin (red) and DAPI (blue) of BHY cells grown for 24 h under normoxic (24h N) and for 8 (8hH), 16 (16hH) and 24 h (24h H) under hypoxic conditions.

4. PAI-1 Western Blot

Cells were maintained under normoxia for 24 h (24hN) or hypoxia for 2 (2hH), 8 (8hH), 16 (16hH) and 24 h (24hH). 5 µg of each cell lysate was loaded per well and visualized on western blots using rabbit anti-PAI-1 (PAb-Rb) or β-actin antibodies as indicated.
HIF-1α Western blot

BHY

FaDu
PAI-1 Western Blot

**BHY**

- **PAI-1**
- **β-actin**

**FaDu**

- **PAI-1**
- **β-actin**