Author's response to reviews

Title: Elevated PDGF-BB Concentrations in Premature Neonates Who Develop Chronic Lung Disease

Authors:

Kim G Adcock (kadcock@pharmacy.umsmed.edu)
John Loggins (jloggi@lsuhsc.edu)
Thomas E Kruger (dkruer1@kc.rr.net)
Dr R John Baier (jbaier@lsuhsc.edu)
Jeremy Martin (JMarti9@lsuhsc.edu)

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PDF covering letter
Responses to reviewers

Reviewer 1

General:
1) The authors need to clearly define exclusion criteria and whether infants of mothers with presence of PROM /Chorioamnionitis or infants with early sepsis were included. These factors may independently affect the levels of PDGF-BB and incidence of CLD and introduce bias during analysis.

Infants whose mothers have either PROM or chorioamnionitis were not excluded as these infants comprise an important proportion of infants who subsequently develop CLD. None of the infants had documented positive blood cultures at birth, however many were treated as possible sepsis.

2) It would be useful if the authors could comment during their discussion on findings by Currie AE et al (Arch Dis Child Fetal Neonatal Ed2002;86:F193-97)(Ref28) in which they concluded that growth factors other than PDGF-BB were responsible for fibroblast mitogenic activity and that mitogenic activity of BALF was similar in RDS, Control and CLD groups.

An expanded discussion in this paper has been added.

3) 36/50 (72%) babies were treated with postnatal steroids!! The authors need to state in their methodology regarding indications for postnatal steroid usage and duration of therapy. Could the decrease in PDGF-BB levels from day 11 onwards be accounted for by anti-inflammatory effects of steroids which were started on approx day 10.

The use of postnatal steroids was an individual decision made by one of 6 attending physicians. The indications for steroid use as well as the duration and dose employed varied. In general the use of steroids was considered in infants who were ventilator dependent after 7-10 days of age. During the time when the infants were admitted the dose of steroids (Dexamethasone) used generally decreased from 0.5 mg/kg/day to 0.2 mg/kg/day. Because of this, we do not feel that details about steroid use which was not standardized adds to the paper. It is possible that the decrease in PDGF-BB concentrations may be related to postnatal steroid administration. However the decline in concentrations tended to occur before the initiation of steroids. Mean time of peak concentrations of PDGF-BB was 5 days and mean time of steroid administration was 11 days. The following sentence was modified in the revised manuscript “PDGF-BB significantly increased until days 4-6 (mean peak at day 5) and then declined to baseline levels between 12 and 14 days of life (p<0.001, ANOVA repeated measures)”.

4) Clinical data on duration of mechanical ventilation was one of the data collected but there is no mention of the findings of this data in the results. It is important to know the no of babies still ventilated and sampled at different times during the study period.
Information on the duration of ventilation (and oxygen therapy) has been added to Tables 1 and 2. This was not originally added as it is expected that infants with BPD are usually ventilated longer. The number of samples assayed at each point has been added to the figures where appropriate (See comment below). This resulted in increase complexity of the figures so that Figure 2 has now been split into 2 figures (2 and 3) and figures 3 and 4 relabeled 4 and 5.

5) Have the authors looked into the possibility of the concentration of PDGF-BB being independently related to gestational age in an inverse ratio.

This is an interesting idea. We did not look at this originally. We re-examined this issue and found no relationship between PDGF-BB concentrations and gestation (either positively or negatively related).

6) Are there any postmortem findings available on the 6 babies which can support the increased fibrosis presumably resulting from the raised PDGF-BB levels.

This would have been ideal; unfortunately none of the 6 infants had postmortem examinations.

Reviewer
1). In the materials and methods section it is mentioned that, after collection, TA’s were centrifuged at 14,000 g for 10 minutes. This methodology might introduce a very important problem resulting in meaningless PDGF levels in TA and in such makes the whole study irrelevant. From the materials and methods section it appears that inflammatory cells (which are present in high numbers in TA of babies suffering from RDS/CLD) were still present in TA at the time of centrifugation (14,000 g for 10 minutes). Inflammatory cells are well known to contribute to increased PDGF levels in various fibrotic lung diseases. By centrifugation of the TA’s (14,000 g) that still contain inflammatory cells PDGF might have been artificially released from the cells into the TA fluid as cells will collapse when spun down with such a force. If this is the case this influenced the PDGF levels in the TA’s making the PDGF measurements unsuitable for drawing conclusions. The authors should comment on this topic.

PDGF-BB is stored in alpha granules of platelets but not stored to any appreciable amounts in other cell types that secrete PDGF-BB. Since platelet activation occurs in the lung in HMD most (if not all of the PDGF) should have been released. Admittedly, there may be small differences (ie increased) in PDGF-BB levels by higher speed centrifugation, we feel these differences do not alter the overall results and conclusions of this paper.

There are infants included in this study that suffer from PH. It is not surprising at all that PH itself is associated with increased PDGF-BB levels in TA. However, at what time was PH diagnosed in these infants? In other words do the infants with PH cause the increase of PDGF-BB levels in the TA’s at days 4 and 6 as depicted in figure 1?
Infants with PH had a peak PDGF at about the same time or a little later than those without. So infants with PH do not cause the peak in PDGF concentrations seen in figure 1. See Figure.

The authors should state when PH was diagnosed and whether PH biased the result to increased PDGF-BB levels between at days 4 to 6 as presented in figure 1. If the patients with PH did bias the measurements at days 4 to 6 to increased PDGF-BB levels it is impossible to conclude that PDGF peaked between days 4 and 6 of life as most patients did not suffer from PH.

The authors state that TA PDGF-BB concentrations correlate with the development of BPD/CLD. However, the infants with PH are more likely to develop BPD/CLD. Again, do these infants account for the fact that in figure 2 A and B the levels of PDGF-BB are higher in the infants developing chronic lung disease compared to the ones recovering from RDS. This should be addressed otherwise the conclusion drawn from these data are not valid in my point of view. In addition to this the differences in PDGF between resolving disease and progression toward chronic lung disease are not significant.

The reviewer raises some good points. Platelets are a rich source of PDGF (mainly AB not BB) and undoubtedly some of the PDGF measured in infants who have a PH is from this source. When infants with PH are removed from analysis maximal PDGF-BB concentrations are still significantly greater in infants in infants who require oxygen at 28 days and a trend exists for oxygen dependency at 36 weeks PCA. This suggests that the increases in PDGF-BB are not entirely secondary to PH. The following has been added to the results section:

“When infants with PH were excluded from analysis maximum PDGF-BB concentrations remained significantly higher in infants who were oxygen dependent at 28 days”
A similar non significant trend was observed for oxygen dependency at 36 weeks PCA (3935±2403 pg/µg vs. 1076±253 pg/µg; p=0.090). Further neither the timing or pattern of PDGF-BB was different when cases of PH were eliminated. This information can be added as an additional figure (see figure) is required by the editors, but it adds little to the paper in our opinion. It could be added as a supplemental file.

From figures 1 and 2 it is unclear how many TA were analyzed per time point (I assume that only one sample per patient was examined per time point, is this the case?).

Yes only one sample per patient was examined per time point. The number of samples assayed at each point has been added to the figures where appropriate (See comment below).

The data in figures 1 and 2 should be replaced by dot blots depicting every TA sample (patient) per time point as a dot. In this figures median values should be given instead of mean values. Presenting data such way provides insight in the number of subjects represented by each time points as well as the variation of PDGF levels between the different patients per time point (this variation appears to be considerable regarding that SEM values are given in figures 1 and 2 from the current draft of the manuscript).

We agree that this is a way (and perhaps better) of presenting the data. However we feel that this leads to unacceptably complex figures that in the end do not aid in understanding the data. Data could be represented in this manner if absolutely required by the editors.

How did the authors determine the timing of the inflammatory process? Cell counts and differentials or cytokine measurements should be provided then.

We did not measure cell counts in these infants. Cytokine concentrations (MCP-1 and IL-8) in our NICU infants have shown differences in peak cytokine concentrations between infants. What was obvious was that the timing of the peak PDGF-BB concentrations varied quite significantly between patients. (See below)

Are max PDGF levels in most infants developing chronic lung disease detected at days 4 and 6? which is suggested by figure 2.

Yes The following was added to the results to clarify this: The peak PDGF-BB response occurred later in infants who required oxygen at 28 days compared to those who were weaned from oxygen by 28 days (2.9±0.5 days vs. 5.6±0.6 days; p=0.013).

In the discussion section the authors state that peak concentrations of PDGF occur within the first week of life when CLD developed. Then they mention that this is consistent with a study by Currie et al demonstrating mitogenic activity in BAL fluids...
from infants developing CLD and those resolving from RDS. However the study by Currie et al shows no difference in mitogenic activity of BAL fluid from RDS or CLD during the first week of life. Furthermore, Currie and colleagues were unable to demonstrate a role for PDGF-BB in this mitogenic activity. In my opinion the fact that Currie and colleagues were unable to block PDGF activity in BAL fluid was due to the fact that they used antibodies to block PDGF activity. We also performed these kind of studies and were also unable to block PDGF activity in BAL fluid by using antibodies. In a recent manuscript from our lab (Eur Respir J 2003; 21: 842-847) it was demonstrated that, using an alternative way of blocking PDGF activity, PDGF does contribute to the mitogenic activity of BAL fluid. Therefore, if the authors want to suggest a possible role for PDGF in stimulating fibroproliferation during CLD this reference should be added.

At the time this manuscript was prepared the interesting data (Eur Respir J 2003; 21: 842-847) was not available. We have added this reference and reworked the discussion.

The authors should state whether steroid treatment did influence PDGF levels.

“Postnatal steroids had an inconsistent effect on those infants in whom pre and post treatment PDGF-BB concentrations were available (17 infants). 7 infants had a decline in PDGF concentrations 48-72 hours after starting steroids, 5 infants had increased PDGF-BB concentrations and 5 infants had undetectable concentrations at both sampling times.” This has been added to the revised text. The figure could be added if desired by the editors, but we believe it adds little to the manuscript, particularly since assessing the effects of steroids was not part of the original study.

From which company was the recombinant PDGF used to generate the standard curve for the ELISA procedure?

This has been added in the revised manuscript. “Recombinant human PDGF was purchased from Pepro Tech (Rocky Hill, NJ).”

The authors describe that very low birth weight infants were used in their study. However they include infants with a weight of less then 1500 grams while real low birth weight infants are considered to have a weight of less than 1000 grams.

We used a standard definition of very low birth weight (ie <1500 grams) in this study. We agree however that most of the morbidity now occurs in extremely low birth
weight infants (ie <1000 grams at birth)

Reviewer

The manuscript reports the use of appropriate methods to tackle the problem. The methods are adequately detailed, although it is unclear whether all of the appropriate assay controls were performed.

The authors do not make clear whether or not some of their subjects have had results reported previously.

Some of the subjects have been reported in our other studies of TA cytokines and CLD. The following has been added the methods section: “Many of these subjects have been reported in our earlier studies of inflammatory mediators and the development of BPD.”

Figure 1. What post-hoc test was used to determine that days 4, 5 and 6 differed from the other days?

The Newman-Keuls test was used to assess differences. This has been added in the revised manuscript: “Post hoc analysis of daily TA cytokine concentrations was performed using the Newman-Keuls test”

Figures 1 and 2. The levels of PDGF-BB fall to baseline by day 10, even in the subjects who develop CLD. This is despite the fact that active fibrosis is occurring at this time and thereafter in infants who are developing CLD. The authors may wish to comment on this in the Discussion.

“Chronic lung disease” is misspelled on the abbreviation list.

Corrected

The description of the effects of PDGF-BB is incomplete. For what cells is it chemotactic and a “competence” growth factor?

This has been addressed in the revised manuscript. The following sentence has been changed in the introduction. “PDGF-BB is a potent chemotactic and a “competence” growth factor for fibroblasts and smooth muscle cells, and has been implicated in the pathogenesis of fibrotic lung conditions.”

The sampling scheme could result in not every intubated subject being sampled at every day. This could cause variations in results from day to day as differing subjects were sampled. How frequently did this occur?

This inevitably occurs quite frequently in this sort of study as infants are removed from mechanical ventilation and may explain the apparent drop of PDGF-BB
concentrations on day 5.

Did it occur often enough to invalidate the assumptions of a repeated measures ANOVA?

This is a difficult and may be impossible question to answer as I can find no straight answer to the question. One would think that if the assumptions of the ANOVA are violated that the results would be nonsignificant. Since this was not the case I can only assume that the number of missing data points was not large enough to invalidate the analysis. Apart from ANOVA if one using repeated t-test with Bonferonni correction you get a similar answer so I tend to believe the results.

Figure 2. There is an apparent drop in PDGF-BB levels at day 5 in subjects who later developed CLD. Are there factors (e.g. different subjects sampled) that may explain this?

This apparent difference may be due to different patients sampled but also could be due to variability in assay sample collection or other random events.

The authors have published similar results for other cytokines and growth factors previously. Do the subjects reported here overlap with those reported previously?

Yes some of the subjects were reported previously. This has been added to the revised manuscript. “Most of these subjects have been reported in our earlier studies of inflammatory mediators and the development of BPD”

The PDGF-BB ELISA described appears to be new. What steps did the authors take to document its sensitivity? Does the ELISA detect other PDGF isoforms?

According to the manufacturer of the reagents used, an immunoassay employing the PDGF-B receptor chimera and antibody the PDGF-BB shows less than 2% cross-reactivity with PDGF-AB and less than 0.6% cross-reactivity with PDGF-AA. This has been added to the methods section of the revised manuscript.

Is PDGF-BB detection ablated by pre-treatment of samples with a neutralizing antibody?

We did not do this. This is not really that useful, since it would depend on the epitope specificity of the neutralizing antibody as well as that of the detection system.

The authors report the incidence of detectable PDGF-BB concentrations in every group other than the healthiest group of children (survivors without CLD at 28 days). What proportion of these children had detectable PDGF-BB concentrations?

We thank the reviewer for pointing this out. In fact, PDGF-BB was less frequently detectable in infants who recovered. The following paragraph was modified in the
"The frequency of detectable PDGF-BB concentrations was significantly lower in infants who did not develop BPD (p=0.006): 37/100 (37%) in infants who recovered from RDS and were off oxygen at 28 days, 162/279 (58%) in infants who were O₂ dependent at 28 days of life, 28/52 (54%) in infants who died, 90/159 (56%) in infants who were O₂ dependent at 36 weeks PCA."

Figures. The authors should report the number of samples at each day in Figure 1, the number of samples per condition at each day in Figure 2, and the number of samples per condition in Figures 3 and 4.

This has been done for figures 1 and 2. This is not applicable for figures 3 and 4 since they refer to the maximum concentration detected.