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The death receptor CD95 activates the cofilin pathway to stimulate tumor cell invasion

Ernst J.A. Steller, Laila Ritsma, Danielle A.E. Raats, Frederik J.H. Hoogwater, Benjamin L. Emmink, Klaas M. Govaert, Jamila Laoukili, Inne H.M. Borel Rinkes, Jacco van Rheenen, Onno Kranenburg

Corresponding author: Onno Kranenburg, UMC Utrecht

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 February 2011

Many thanks for your patience while we have peer-reviewed the manuscript you submitted to EMBO reports. I have now received the comments of both referees that we asked to assess your work. I would like to ask you to undertake a major revision of your manuscript with a view to having it reviewed a second time prior to potential publication in EMBO reports.

Both referees agree that your results are novel, important and of broad interest. However, both have serious concerns about the strength of the conclusions you are able to draw from your current experiments and ask that you undertake work to improve the evidence presented. Both agree that your final conclusion, that CD95 stimulation activates PLCgamma1 via PDGFR, is not yet well supported by the data.

To address this problem, both referees make a number of suggestions, all of which I think should be addressed.

Most importantly, both referees indicate that you should undertake RNA interference experiments to reduce the expression of PLCgamma1 and PDGFR to present more convincing evidence of their involvement than is currently offered using pharmacological inhibitors (see Referee #1 point 1 and 3, and Referee #2 point 2). Essentially, the evidence presented using pharmacological inhibitors they both find unconvincing.

Referee #1 also asks that you use these KD experiments to show the absence of CD95-mediated phosphorylation of PLCgamma1/migration in the absence of PDGFR. He or she also asks that you demonstrate Tyr phosphorylation of PDGFR upon CD95 stimulation. Referee #2 requests that you repeat your basic findings in at least a second cell line to demonstrate that your results are of general relevance. All of these points should be addressed.

Both referees are also interested in how CD95 might signal via PDGFR. Although referee #1 proposes that you investigate the physical interaction between PDGFR and PLCgamma1 in the context of the CD95 ligand, I do not find this experiment fundamental to your manuscript. I think it is sufficient to show by RNAi of PDGFR and PLCgamma1 that in its absence, CD95 is unable to stimulate PLCgamma1 phosphorylation, and that in the absence of PLCgamma1 and/or PDGFR there is no CD95-induced phenotype. However, I do agree with the referees that it would be worthwhile to discuss the possibilities in your Discussion.

Overall I believe that addressing the referees' criticisms will significantly enhance the data presented in your manuscript and will provide compelling evidence for your observation that CD95 stimulation activates PLCgamma1 and invasion via PDGFR. We allow three months for the revision of a manuscript, starting from the date of this letter.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In the submitted manuscript Steller et al. describe the molecular events required for CD95-mediated migration of murine colorectal cancer cell line C26. They described for the first time a link between CD95-driven phosphorylation of PLCγ1 Y783 and reorganization of actin cytoskeleton, ultimately leading to formation of cell membrane protrusions and invasion of C26 cells. They identify PDGFR as the possible kinase linking CD95 and PLCγ1 but this conclusion is not substantiated by the data. As I already mentioned, the strongest point of the work is the identification of important players conducting the invasive signal originating from the stimulated CD95 receptor. Even though the single steps of the cascade (CD95-driven phosphorylation of PLCγ1 Y783, PIP2-dependent regulation of cofilin) have been already described in the literature, their combination provides a novel mechanism of CD95-dependent cancer cell migration. The authors used appropriate methods to support the activation of PLCγ1 Y783 as well as formation of motile cell membrane structures. The experimental data in this part is also of sufficient quality. However, the final conclusion that CD95 stimulation activates PLCγ1 via PDGFR is not supported by the data.
Major concerns are as follows:
1. The data presented in Fig. 4 is not sufficient to prove the specific role of PDGFR in the presented mechanism. First of all, it is confusing for the reader that Sorafenib, a PDGFR/VEGFR inhibitor, but not AG1296, an inhibitor more specific to PDGFR, was used for the invasion experiments. Secondly, as authors note, Sorafenib inhibits the basal migration already. However, there is still significant increase of migrating cell number after CD95L stimulation. Therefore, it is difficult to argue that PDGFR is indispensable for CD95-driven invasion. To claim that PDGFR links CD95 to PLCγ1 the authors should at least show Tyr phosphorylation of PDGFR upon CD95 stimulation and, preferably, absence of CD95-mediated phosphorylation of PLCγ1/migration in absence of PDGFR with siRNA knock down experiments in addition.
2. Along the same line, and in order to extend the experimental data on this subject, it would be worth investigating the physical interaction between PDGFR and PLCγ1 in the context of CD95 ligand. The authors do not discuss the possibilities of how CD95 itself could signal via PDGFR.

3. Concerning the inhibition of PLCγ1, there are several papers reporting nonspecific targets of U73122 (Bosch et al. (1998) PMID: 9652379, Cenni & Picard (1999) PMID: 10425187, Muto et al. (1997) PMID: 9316850, Walker et al. (1998) PMID: 9827577, Hughes et al. (2000) PMID: 11138848, Meyer et al. (2001) PMID: 11104770 and Cho et al. (2001) PMID: 11682455). In addition, U73343 does affect the migration to some extent since the difference between the control and CD95L-treated cells is not significant anymore. More convincing data could be acquired by repeating the experiments with PLCγ1 KD.
Minor concerns:
4. On page 4 it is written: "These effects were completely abrogated upon knockdown of the endogenous mutant Kras allele..."
There is still a significant increase in the number of migrating cells through the transwell chamber as well and increase in the surface area of stimulated C26 Ras KD cells. Therefore, one cannot speak of complete abrogation
5. On Page 6: "PIP5 kinase"
The subscript is confusing; the enzyme is described in the literature as PIP5 or PIP-5 kinase.

Referee #2:

Steller et al. show that in K-ras expressing C26 colon carcinoma cells CD95 ligation potently induces tumor cell migration via mechanisms involving PDGFR-dependent PLC-gamma-1 phosphorylation and subsequent cofilin activation. This manuscript represents an interesting follow-up study of the work published by the same group last year (Hoogwater et al. Gastroenterology 2010; 138:2357) and I believe that it is of interest to a broad audience. Nevertheless, there are some points, which in my mind could strengthen the manuscript and the authors therefore might want to address.

Major:
1. All the experiments were performed with a single cell line. The basic findings should be repeated with at least one more cell line (for instance DLD-1 or HCT-116) to demonstrate that they are of general relevance.
2. The specificity of pharmacological inhibitors is well-known to be limited. Hence, the involvement of PLC-gamma-1 and PDFG-R (in addition to the inhibitor experiments shown in Figs. 3 and 4) should be investigated by RNA interference targeting PLC-gamma-1 and PDGF-R
3. In the invasion assays, CD95L was added to the lower compartment of the double chamber raising the question if CD95-stimulated migration of tumor cells is due to directional chemotaxis or rather chemokinesis. This point can be easily addressed by checkerboard analyses.
4. How does CD95 activate PDGF-R? I am aware that addressing this question experimentally would be beyond the scope of this report. But the authors could present a hypothesis.

Minor:
1. To render the manuscript more comprehensible, it would be helpful for the reader to include primary microscopical data into Fig. 1 illustrating the number of protrusions per cell and the area covered by each cell.
2. The y-axis labels of the invasion assays say 'Invasion % of control'. This is somehow misleading. In my mind 'x-fold of control' would be more appropriate.
3. Throughout the manuscript there are some typographical errors that should be corrected.

1st Revision - authors' response 29 April 2011

Point by point response to the reviewers' comments

REVIEWER 1:

Point 1:
"Sunitinib, but not AG1296, an inhibitor more specific for PDGFR, was used in the invasion experiments."

We now also used AG1296 to assess the role of PDGFR-β in CD95L-stimulated invasion in both C26 and MC38 cells. In line with our previous results, AG1296 completely blocked (MC38) or strongly reduced (C26) CD95L-stimulated tumor cell invasion. These data are now shown in Figure 4E of the revised manuscript.

"…To claim that PDGFR links CD95 to PLC-gamma the authors should at least show Tyr phosphorylation of PDGFR upon CD95 stimulation and absence of CD95-mediated PLC phosphorylation/migration in the absence of PDFGR with siRNA experiments."
Tyrosine 1021 (Y1021) in the PDGFR-β is responsible for binding to PLC-gamma1. Stimulation of C26 cells with CD95L rapidly promoted Y1021-PDGFR phosphorylation (Figure 4B), concomitantly with PLC-gamma1 tyrosine phosphorylation (Figure 4C). Furthermore, both pharmacological inhibition using AG1296 and sunitinib (Figure 4C), as well as RNA interference-mediated suppression of the PDGFR (Figure 4D) completely blocked CD95-ligand-induced PLC-gamma1 tyrosine phosphorylation. In addition, pharmacological or RNAi-mediated suppression of the PDGFR-β reduced CD95L-stimulated cell area enlargement (Figure 4B) as well as tumor cell invasion (Figure 4F). These new data are now shown in Figures 4B-F.

Point 2:
"The authors do not discuss the possibilities of how CD95 itself could signal via PDGFR."
We added a paragraph on page 7 in which we discuss how CD95 may stimulate PDGFR-β signaling.

Point 3:
"Concerning the inhibition of PLC-gamma….more convincing data could be acquired by repeating the experiments with PLC-gamma knockdown".
siRNA's directed at PLC-gamma1 significantly reduced CD95L-stimulated area enlargement (Figure 3B). Furthermore PLC-gamma1-targeted siRNA's completely blocked CD95L stimulated tumor cell invasion (Figure 3D).

Point 4:
On page 4 it is written: "These effects were completely abrogated upon knockdown of the endogenous mutant Kras allele..." There is still a significant increase in the number of migrating cells through the transwell chamber as well and increase in the surface area of stimulated C26 Ras KD cells. Therefore, one cannot speak of complete abrogation.
We adjusted the text by changing 'abrogated' into 'largely prevented' on page 4 of the revised manuscript.

Point 5:
PIP5 kinase is confusing: either PIP5 or PIP-5 kinase.
We changed PIP5 kinase into PIP-5 kinase.

REVIEWER 2:

Point 1:
The basic findings should be repeated with at least one extra cell line.
In addition to C26, we now also used MC38, a second murine colorectal cancer cell line. Stimulation of MC38 cells with CD95L failed to induce apoptosis but stimulated migration, increased the number of cell protrusions, and promoted tumor cell invasion (suppl. fig. 2). Furthermore, inhibition of PLC-y1 (Figure 3C) or the PDGFR-β (Figure 4E), and siRNA-mediated knockdown of PLC-y1 (Figure 3D) or the PDGFR-β (Figure 4F) all strongly reduced CD95-ligand stimulated tumor cell invasion. Finally, pharmacological inhibition of the PDGFR-β in MC38 cells using either sunitinib or AG1296 abrogated CD95L-induced tyrosine phosphorylation of PLC-gamma1.

Point 2:
"Specificity of pharmacological inhibitors is limited. Involvement of PLC-y1 and PDGFR-B should be investigated by RNA interference targeting PLC-y1 and PDGFR-B."
Please see our answers to points 1 & 3 of referee 1.

Point 3:
"Is CD95L-stimulated migration due to chemotaxis or chemokinesis? This can be easily addressed by checkerboard analysis."
As suggested by the reviewer, we have performed a checkerboard analysis to distinguish between these possibilities. The data presented in Figure 1B unequivocally show that CD95L stimulates directed migration (i.e.: chemotaxis). This is in line with our previous results in DLD1 cells (See Hoogwater et al, Gastroenterology 2010, Figure 3C).

Point 4:
"How does CD95 activate PDGF-R?"
We added a paragraph on page 7 in which we discuss how CD95 may stimulate PDGFR-β signaling.

Minor point 1:
"…include primary microscopical data to figure 1."
An example of primary microscopy data and how these were used to calculate the number of cell protrusions and cell area is now provided in Figure 1C.

Minor point 2
"The y-axis labels of the invasion assays say 'Invasion % of control'. This is somehow misleading. In my mind 'x-fold of control' would be more appropriate."
We changed all axes to 'fold of control'.

Minor point 3:
Typographical errors
We checked the entire manuscript and corrected all typos that were found.

2nd Editorial Decision 24 May 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have addressed each reviewer's concern. I would recommend the manuscript for publication in EMBO reports.

Referee #2:

There is no further revision required.

