Evidence for macromolecular crowding as a direct apoptotic stimulus

Priyanka S. Rana, Manabu Kurokawa and Michael A. Model
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Original submission

First decision letter

MS ID#: JOCES/2020/243931

MS TITLE: Evidence for macromolecular crowding as a direct apoptotic stimulus

AUTHORS: Priyanka S. Rana, Manabu Kurokawa, and Michael A. Model
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is a nice study which clearly establishes that the well-known loss of K+ accompanying cell shrinkage is not integral to the process of apoptosis. This significantly advances the field as K+ loss is still regarded as a main contributor to the triggering of the apoptotic process.

Comments for the author

Major:

1. Elimination of intracellular ions and cytoskeleton does not by itself support the idea of the importance of macromolecular crowding. There is still the possibility that cell shrinkage triggers a signaling cascade (e.g. coming from a receptor at the membrane) that would trigger apoptosis. I would then be careful to state as in the abstract that the data suggests "that an increase in protein density…" and in the discussion "appears to be most plausible". Yes, molecular crowding is a possibility, but the study only eliminates alternatives. This can easily be fixed by just stating that the data suggests alternative mechanisms, e.g. signaling, macromolecular crowing, or others.

2. All Figures should have proper statistical analyses. Figure 1A present means +/- SEM (per methods) but we do not know how many data points. Differences between bars look like statistically significant in Figures 2 and 3. Is it relevant?

3. Was Figure 5 done only once? The bars have no errors. There is no way to assess variability of the data.

4. Table 1. Is it important to present the percentage of caspase-positive cells with 2 decimal points? I would also add % after the values: 1%, 1.6%, 63%, etc.

5. The manuscript refers to a supplementary Figure and a supplementary Table. Why not add them to the main manuscript?

Minor:
1. It is always a good idea to add page numbers to the text.
2. When referring to the Na-K-2Cl cotransporter, please use hyphens instead of dashes as those are typically used for exchangers. Adding the valence is great.
3. Bottom of page 4, superscript the plus signs after Na and K.
4. On page 8, refer first to Figure 1A then Figure 1B (end of first paragraph)

Reviewer 2

Advance summary and potential significance to field

This manuscript explores the role of ionic homeostasis and apoptotic mechanisms, through a process termed macromolecular crowding. However the recombinant and in vitro nature of the study limits its impact and any advancement to the field.

Comments for the author

This is an interesting study but lacks robust interrogation of the research question posed to merit consideration for publication.
Major comments
(i) The use of recombinant cells limits the impact of the study- the authors do not provide an rationale as to why HeLa/MDCK cells were used.
(ii) There is a disconnect between the data presented and the conclusions- for example the authors do not provide evidence for macromolecular crowding as a direct apoptotic stimulus. The evidence presented pertains more to dynamic regulation of K+ homeostasis.
(iii) There is no control over the total concentration of macromolecules throughout the experimental design (only presented for the sucrose expts)- the authors conclude that crowding is involved but presenting negative data for potassium and cytoskeletal rearrgangements. In order to support their conclusion data on the macromolecular composition must be presented. This could be presented in cell fractionation experiments.
(v) Can the authors exclude changes in protein conformation as a result of their stimulus? Have they looked at the ratio of unfolded proteins?

(iv) The data analytics presented in the methods section is not sufficient to interpret the data presented. For example there is a lack of statistical approaches to comment fully on the data presented. There is also a lack of appropriate datasets included in the results section.

First revision

Author response to reviewers' comments

Dear Dr. Heath,

We appreciate the reviewers' constructive critique. We have edited the text, included more experimental results, and added statistical analysis. The most significant changes are marked red.

The abstract was shortened to fit the word limit.

Reviewer 1

This is a nice study which clearly establishes that the well-known loss of K+ accompanying cell shrinkage is not integral to the process of apoptosis. This significantly advances the field as K+ loss is still regarded as a main contributor to the triggering of the apoptotic process.

Reviewer 1 Comments for the Author:
Major:

1. Elimination of intracellular ions and cytoskeleton does not by itself support the idea of the importance of macromolecular crowding. There is still the possibility that cell shrinkage triggers a signaling cascade (e.g. coming from a receptor at the membrane) that would trigger apoptosis. I would then be careful to state as in the abstract that the data suggests "that an increase in protein density…" and in the discussion "appears to be most plausible". Yes, molecular crowding is a possibility, but the study only eliminates alternatives. This can easily be fixed by just stating that the data suggests alternative mechanisms, e.g. signaling, macromolecular crowing, or others.

MM: We have slightly tempered our assertions. At the same time, we rewrote the second paragraph of the Introduction where we tried to better explain our reasoning and the difference between primary and secondary responses: "…The initial sensor of cell volume must be some aspect of the volume itself: a change in size, area, concentration, proximity between molecules or mechanical forces directly resulting from changes in cell size". By contrast, receptors or traditional signaling must be turned on by something else, and that "something else" has ultimately to be the volume itself or an equivalent factor. To further clarify our approach we made more additions and rearrangements in the beginning of the Discussion: "Unfortunately, since protein density cannot be manipulated by genetic engineering or by specific inhibitors, many of the standard biological approaches cannot be applied to investigate the effects of crowding; they have to be surmised by exclusion of other possible explanations or by simulating similar effects in a test tube. While the latter remains a task for the future, in this work, we have adopted the "proof by contradiction" approach. It is certainly impossible to eliminate every alternative, and we have focused on the two other aspects of cell volume that might in principle initiate downstream signaling for apoptosis: potassium depletion and cytoskeleton-mediated effects (#3 and #4 of the mechanisms listed in the Introduction)."

2. All Figures should have proper statistical analyses. Figure 1A present means +/- SEM (per methods) but we do not know how many data points. Differences between bars look like statistically significant in Figures 2 and 3. Is it relevant?

MM: P values and additional statistical information has been added to those and other figures.

3. Was Figure 5 done only once? The bars have no errors. There is no way to assess variability of the data.

MM: Three experiments have been performed, but only mentioned. In the revision, we included all the data. We also included additional data in Table 1.

4. Table 1. Is it important to present the percentage of caspase-positive cells with 2 decimal points? I would also add % after the values: 1%, 1.6%, 63%, etc.

MM: Done

5. The manuscript refers to a supplementary Figure and a supplementary Table. Why not add them to the main manuscript?

MM: Fig. S1 is now a new Fig. 3 and the conclusions from Table S1 are mentioned in the text as *Not shown* (top of p. 12). (Inclusion of that table in the main text would have exceeded the maximum allowed number of figures and tables). The former Table 1 has been converted into a graph and has become part of Fig. 7.

Minor:

1. It is always a good idea to add page numbers to the text.

MM: Done

2. When referring to the Na-K-2Cl cotransporter, please use hyphens instead of dashes as those are typically used for exchangers. Adding the valence is great.

MM: Done

3. Bottom of page 4, superscript the plus signs after Na and K.

MM: Done

4. On page 8, refer first to Figure 1A then Figure 1B (end of first paragraph)

MM: Done

Reviewer 2

This manuscript explores the role of ionic homeostasis and apoptotic mechanisms, through a process termed macromolecular crowding. However the recombinant and in vitro nature of the study limits its impact and any advancement to the field.

Reviewer 2 Comments for the Author:
This is an interesting study but lacks robust interrogation of the research question posed to merit consideration for publication.

Major comments
(i) The use of recombinant cells limits the impact of the study- the authors do not provide an rationale as to why HeLa/MDCK cells were used.

MM: Both HeLa and MDCK cells are widely used in apoptosis research (440,000 results on Google scholar on “HeLa” AND “apoptosis” and 33,000 on “MDCK” AND “apoptosis”), and we have previously characterized the volume behavior and apoptosis in these cell lines. Any choice of cell line would have its limitations. We are not claiming to investigate any particular physiological

condition, but rather exploring the possibilities of how macromolecular crowding may manifest itself in a living cell.
Recently, we have obtained similar results in mouse fibroblasts, but we have not enough data yet to include them in this paper.

(ii) There is a disconnect between the data presented and the conclusions- for example the authors do not provide evidence for macromolecular crowding as a direct apoptotic stimulus. The evidence presented pertains more to dynamic regulation of K+ homeostasis.

MM: We did show changes in macromolecular density in Fig. 1B. Also, please see our response to comment #1 of Reviewer 1.

(iii) There is no control over the total concentration of macromolecules throughout the experimental design (only presented for the sucrose expts)- the authors conclude that crowding is involved but presenting negative data for potassium and cytoskeletal rearrgangements. In order to support their conclusion data on the macromolecular composition must be presented. This could be presented in cell fractionation experiments.

MM: We understand the reviewer's comment in the sense that more quantification of protein density is needed. We, therefore, added 6 h data to Fig. 1B that show persistent swelling in the absence of sucrose and persistent shrinkage in its presence. Macromolecular crowding refers to the total amount of macromolecules, and the TIE/TTD technique specifically quantifies this parameter.

(v) Can the authors exclude changes in protein conformation as a result of their stimulus? Have they looked at the ratio of unfolded proteins?

MM: This question may have been provoked by our poorly thought-out sentence, "protein conformation or protein density seem better suited for the role of shrinkage sensor" (2nd paragraph before Materials and Methods). What we meant was that an increase in macromolecular density may stimulate apoptosis not only through the volume exclusion effect but also through changes in protein aggregation and possibly in protein conformation. To avoid confusion, we deleted "protein conformation" from that sentence. As regards the possibility of accumulation of misfolded proteins and ER stress **as a result of compression**, it cannot be excluded. However, we believe that exploring such a mechanism would be beyond the scope of the present manuscript. We are planning to study the molecular mechanisms that link cell compression (i.e., macromolecular crowding) and apoptosis, and the ER stress due to accumulation of misfolded proteins would be one interesting possibility to look into.

(iv) The data analytics presented in the methods section is not sufficient to interpret the data presented. For example there is a lack of statistical approaches to comment fully on the data presented. There is also a lack of appropriate datasets included in the results section.

MM: We added statistical data (see above). If the reviewer would specify which datasets are missing, we would be happy to provide them.

Second decision letter

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AUTHORS: Priyanka S Rana, Manabu Kurokawa, and Michael A Model
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Many studies have suggested that the loss of K+ that is observed as an early event of apoptosis is a signal that likely triggers the cascade leading to cell death. This studies clearly demonstrates that it is not the case, the apoptotic process can still occur if K+ is clamped and maintained at its regular intracellular concentration.

Comments for the author

The authors have responded appropriately to all of my concerns.