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Location, Location, Location: Appraising the Pleiotropic Function of HMGB1 in Fetal Brain

We read with interest the paper by Zhang et al entitled "HMGB1 translocation after ischemia in the ovine fetal brain" (1). High-mobility group box 1 protein (HMGB1) is a fascinating protein with "many faces," yet we are only beginning to uncover its roles in the developing brain (2). The findings of Zhang et al add to the much-needed body of literature in the field and validate our work in the same animal model and at the same near-term gestational age. Under intermittent umbilical cord occlusions (UCOs) insult with 3–4 hours of worsening acidemia and pH $<$7.00, we found an intracellular translocation of HMGB1 in neurons (3, 4), astrocytes (4), and microglia (5, 6) measured 24 hours post-insult. Interestingly, under control conditions, we found the dominant localization of HMGB1 to cytosol in neurons and astrocytes but not in microglia. Acidemic insult due to UCOs caused subtle and brain region-specific reverse shifts in neuronal HMGB1 patterns of cortical gray matter (3).

Our findings seem to stand in contrast to those by Zhang et al. A number of methodological differences in quantification of HMGB1 translocation distinguish the present paper by Zhang et al from our approach and may explain the contrasting findings in the dominant neuronal HMGB1 localization.

A stronger scenario of 30 min cerebral ischemia was modeled by Zhang et al with immunohistochemical (IHC) analyses performed at 48 and 72 hours post-insult whereas we attempted to mimic the generic human labor process with multiple repetitive, intermittent 1-min lasting global ischemia episodes and IHC at 24 hours post-insult. It is possible that with the more severe brain ischemia apoptosis pathways have been triggered and the nuclear HMGB1 translocated to the cytosol en route to act as the extracellular cytokine for microglia (7).

With the DAB chromogen stain used, the delineation between nucleus and cytosol without a counterstain is less clear than with fluorescence or dual/triple stains. It is not clear how the authors determined where to draw these lines except by visually recognizing the translocation. Rather than contouring around specific brain regions, the authors drew around where they thought they saw positive translocation, and then measured the percentage size of that outline relative to the whole brain contour. Next, the authors deliberately selected only cells showing evidence of HMGB1 translocation. An additional selection bias may have been difficult to avoid as the authors chose the already larger pyramidal cortical gray matter cells as "translocated" because they have large nuclei, in which they could see the translocation better. We suggest that an additional systematic approach of all layers needs to be undertaken with quantification of cytosol and nuclear HMGB1 signals. This may reveal additional translocated cells being the granular cells, which may seem as non-translocated because of their tight cytosol and dark nuclei. Lastly, formalin immersion fixation only penetrates tissue at a rate of approximately 1.6 mm/hour and after penetration it still needs to create crosslinks to fix proteins. We and others perfuse and then immerse. The fixation is then coming from two directions at once (i.e. inside vessels and outside brain) and reaches the tissues faster to stabilize them. The areas the authors outlined as having heightened HMGB1 cytosolic signal seem close to a ventricle where formalin would be entering from, and their high signal may be reflective of good perfusion there vs. deep within the tissue.

In light of the strong focus on the inflammatory roles of HMGB1 in the extracellular space in the last decade of research it is worth remembering that HGMB1 is also essential for life per se and for tissue regeneration processes (8). Perhaps in line with the many faces of HMGB1 during development and in various cell types in health and disease, HMGB1 nucleo-cytosolic distribution patterns are remarkably tissue- and time-specific (8–10). A predominantly cytosolic location of HMGB1 has been reported in developing brain, promoting neurite outgrowth and implicated in early neuronal development *in vitro* and *in vivo* (8,9,11). Thus, alterations in nucleo-cytosolic predominance of HMGB1 in developing brain may not only result in pro-inflammatory and neurodestructive effects due to its extracellular functions but also in disruption of physiological neurodevelopmental programs due to its intracellular, notably also cytosolic, functions, which are yet to be well defined in mammals.

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Letter to the Editor

The Authors' Reply

To the Editor,

We appreciate the comments by M.G. Frasch and K. Nygard and their interest in our work. We fully agree that the High-Mobility Group Box 1 (HMGB1) has very important roles for the normal developing brain as well as contributing to the pathogenesis of prenatal and perinatal brain injury (1,2). We are also pleased to learn that they found intracellular translocation of HMGB1 in neurons, astrocytes, and microglia after intermittent umbilical cord occlusions (UCO).

In our study, immunofluorescence staining of whole brain sections revealed that sham-operated fetal sheep exhibited HMGB1 staining that was mostly localized in the nuclear compartment in the cerebral cortex, white matter, and hippocampus (2). Interestingly, we also detected HMGB1 in the cytosolic compartment in some cerebral cortical regions. This finding is in contrast with reports in adult rodents (3,4). We considered that the differences between the rodents and our fetal sheep could be related to the comparative aspects of brain development, species differences, and/or that our fetal sheep had not undergone *in vivo* perfusion fixation, which had been performed in the adult rats (4,5).

It is highly improbable that the method of fixation affected our findings. We have confirmed that *in vivo* perfusion fixed brain segments from fetal sheep of the identical gestational age also showed cytosolic staining of HMGB1 in some neuronal cells in the brain sections of the pre-fixed brain samples and there were no major differences in the distribution of the cytosolic staining of HMGB1 between the *in vivo* perfused and non-perfused fetal sheep (2). This is consistent with our previous experience that immersion fixation did not significantly impact the outcomes of the studies (6–8). We are always careful to include a control group of sham operated identically treated fetal sheep. By using immersion fixation we can obtain fresh frozen tissue for other purposes such as Western immunoblot, in parallel with immunohistochemical analysis. Moreover, the biohazard committee at Brown University does not permit *in vivo* perfusion within the laboratory, necessitating a delay after the study completion to obtain the fetal tissues.

Similarly, our assessment protocol was developed to avoid selection bias by examining the entire cortex. HMGB1 staining was quantified in the fetal brain after ischemia by areas of cells within the cerebral cortex that had HMGB1 translocation using a contouring method, as described in our manuscript (2). Cells with HMGB1 translocation were identified with positive staining of HMGB1 in the cytosolic compartment; the areas of cells with HMGB1 translocation were compared to total area of the brain to determine percentage of cells with HMGB1 translocation. The study was conducted in a double-blind fashion and the brains exposed to ischemia were compared to brains from sham operated control fetal sheep, thereby eliminating potential bias. This approach enabled examination of the entire area of the large fetal sheep brain for changes in HMGB1 rather than in isolated areas. We found that the standard deviation of numbers of cells with HMGB1 translocation counted in random areas in the cerebral cortex was very high. Overall examination of entire coronal sections under the stereomicroscope revealed that cells with HMGB1 translocation were not evenly distributed over the entire cerebral cortex; rather, they were condensed within the deep sulci of the cerebral cortex. Therefore, after consultation and review by a neuropathologist (E.G.S.), we quantified HMGB1 translocation using the area fraction method. There was a clear border between the areas containing the cells exhibiting HMGB1 translocation and the surrounding brain areas containing the cells without HMGB1 translocation. These contours were easily defined under our stereomicroscope and were independently confirmed by Dr Stopa. This clearly significant difference between the contoured areas and the surrounding areas enabled us to trace the area of cells with HMGB1 translocation without difficulty, supporting the finding of increased area of translocation of HMGB1 into the cytosol in fetal sheep after brain ischemia vs. sham operated animals. This finding was corroborated by Western immunoblot data.

We are pleased to learn that our fetal ovine model shares some similarities as well as some notable differences. As discussed in their letter, we suspect that many of the differences observed relate to timing because they examined changes at 24 hours (i.e. when injury is still developing) compared with 48–72 hours after the ischemic insult, when bulk cell death is established. Further, our study employed a moderate to severe ischemic insult of 30 minutes duration whereas the study by Frasch and Nygard used the repeated intermittent UCO model which, has been associated with milder, more variable neuronal loss in previous studies (9,10). Not surprisingly, the neuropathological outcomes after carotid occlusion and repeated UCO differ greatly (10), and, hence it would be expected that their effects on HMGB1 should differ as well.

We wholly agree that current understanding of the different roles of HMGB1 protein is very limited. Many cytokines and growth factors such as basic fibroblast growth factor (FGF-2) exhibit diverse functions with varying subcellular distributions in nucleus or cytoplasm depending on both physiological state and differing isoform expression (11). We may reasonably suspect that HMGB1 has similarly diverse roles. Its distribution in neurons, astrocytes, and microglia as well as its subcellular distribution in nucleus or cytoplasm may be a function of physiological stimulus and post-translational modification.

Given the numerous unfolding roles that HMGB1 seems to play in early development, additional studies are needed to characterize better the physiological changes in HMGB1 that occur during both normal and pathological neurodevelopment.

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