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LETTERS TO THE EDITOR

Cell death, DNA breaks and possible rearrangements: an alternative view

I thank Gilmore *et al.*[1] for their interest in the work of my colleagues and I on programmed cell death (PCD) and DNA double stranded breaks (DSBs), and in our ideas on neural DNA rearrangements[1]. This made for entertaining reading combined with the exciting implications raised by mutations of genes encoding nonhomologous end-joining (NHEJ) proteins. New data on mice with mutations in other genes for NHEJs (Ref. 2) strengthen the already compelling case for the involvement of these proteins in neurogenesis. However, perhaps because these topics are far removed from the published expertise of Gilmore *et al.*, several fundamental misconceptions are propagated in their article. In particular, their belief that neuroblast PCD is insignificant and that DSBs are unrelated to PCD mechanisms, and their proposal that a V(D)J-like mechanism could account for our detected DSBs is at odds with the published, experimental literature. Their conclusions are based on several lines of evidence.

Histology

A relative lack of pyknotic nuclei is cited as proof that PCD is not significantly occurring. However, in some of the best examples of PCD, pyknoses are not an accurate measure of the extent of PCD taking place: sparse pkynosis is evident in the thymus[3], where over 95% of thymocytes die[4], and also in small intestinal villi, where all cells die every few days[5].

Neuronogenetic models

Gilmore *et al.* make numerical arguments against PCD that rely on the validity of the models used[6,7]. If these models are inaccurate then conclusions based on them are uncertain at best. It would seem that the model of Caviness *et al.*[7] is certainly inaccurate as it does not account for neuronal contributions from noncortical regions like the ganglionic eminence (subcortical telencephalon), let alone even consider the operation of PCD. In addition, the problems of drawing temporally valid, mechanistic conclusions about the cell cycle and cell proliferation by comparing different populations of cells in different animals, increases the level of uncertainty. Furthermore, because bromo-deoxyuridine (BrdU) incorporation is not specific for cell proliferation (for example, during DNA repair), the primary data cited by Gilmore *et al.*[1] (from models of Caviness *et al.*[7]) are yet again uncertain. One is left with uncertainties about uncertainties about uncertainties. The numerical arguments raised by Gilmore *et al.* can easily be addressed. For example, 50% PCD is no problem if death affects subpopulations of cells and thus maintains a sufficient number of proliferative blasts to allow for brain growth. Finally, the studies on which the inaccurate models of Caviness *et al.* are based do not directly address the operation of PCD, and thus, any conclusions drawn are based on indirect inferences.

ISEL+

We have developed and used two independent approaches to study PCD: *in situ* end-labeling plus, ISEL+ and 'ligation mediated PCR' or LMPCR, which identifies nucleosomal ladders associated with apoptosis. Both techniques have been used in our studies and accurately identify dying cells in every tissue known to

undergo PCD that we examined (these techniques tell us nothing about the clearance time of dead cells). Examples include normal embryo, normal thymus, death-induced thymus, postnatal retina, Schwann cells in culture and induced death in tissue culture cells, among others[5,8–14]. Quantitative estimates of the number of DSBs required for detection by ISEL+ is of the order of thousands per cell, based on published calculations[11]. Even if this is an overestimation by a factor of 10, this still represents hundreds of DSBs per cell. Could a V(D)J-type DNA rearrangement, as proposed by Gilmore *et al.* to account for ISEL+ labeling, create this many DSBs? There is no evidence to support this, as V(D)J recombination is thought to generate ~four DSBs that involve two coding and two signal ends[15]. Moreover, it is probable that these ends are not normally accessible to terminal deoxynucleotidyl transferase polymerase used in ISEL+, because (1) the coding ends are covalently hairpinned, (2) the signal ends are rapidly joined to form DNA circles, and (3) the ends are probably protected by RAG (recombination-activating gene) and NHEJ proteins, or other molecules such as p53. The additional belief of Gilmore *et al.* that the less sensitive TUNEL [terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling] technique[16] might not be specific for PCD further ignores a rather substantial literature that uses this approach.

Mouse mutants

A major prediction from our studies using both ISEL+ and LMPCR was that if one could block neuroblast PCD, more neuroblasts should be present. Independent data from several laboratories in which caspase 3, caspase 9 or Apaf1 were deleted all show this increase in neuroblast number. The statement by Gilmore *et al.* that it is cell proliferation, independent of PCD, that accounts for the increase in cell numbers has no experimental support from the prior literature on caspase and Apaf1 function, nor from the knockout mouse reports. Gilmore *et al.* further dismiss the conclusions from all the authors of these seminal knockout reports[17–20] that loss of caspase or Apaf1 function reduces PCD, and they distort published data. For example, Gilmore *et al.* cite early increased cell proliferation [at embryonic-day (E) 10] as evidence against our cell ISEL+ data. By contrast, both reports of Apaf1 knockouts[17,18] showed no effects on cell number until around E12.5, and one study did not even examine ages under E10.5 (Ref. 18). Similar data are shown in the curiously omitted but essential report of caspase-3 knockouts, which also shows increased cell number around E12.5 (Ref. 19). The caspase-9 knockout might have an earlier phenotype; however, there is no quantitation of PCD until E12.5 (Ref. 20), when it is also reduced. Perhaps this upstream caspase precedes ISEL+-detectable DNA fragmentation in cells committed to dying, consistent with most views on PCD in which DNA fragmentation occurs downstream of caspase activation. Clearly, however, direct experimental ISEL+ analysis should be carried out in these mutants; we have recently reported this for cortical tissue from caspase-3 knockouts and, as expected, a significant decrease in ISEL+-labeled neuroblasts is observed[10].

If Gilmore *et al.* truly believe that there is no significant neuroblast PCD as they have stated, they should show this by direct experimentation. I continue to favor some form of neural DNA rearrangements; however, based on available experimental data, it would have to be distinct from V(D)J forms. My views on the role of NHEJ, PCD and possible DNA rearrangements are discussed elsewhere[21,22].

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Reply

The goal of our article[1] was to draw attention to the XRCC4 and ligase IV mutant mice as providing evidence for the possible existence of DNA double strand breaks (DSBs), and to the possibility of DNA recombination in proliferating cells in the developing brain[2–4]. We also suggested, as a secondary point, that the existence of larger numbers of DSBs than previously suspected might explain the incongruity between different methods of estimating the levels of cell death in the proliferative neuroepithelium.

Chun maintains that his studies using the ISEL+ (*in situ* end labeling plus) method establish levels of cell death in the