QUANTITATIVE IMMUNOFLUORESCENCE ANALYSIS OF NUCLEAR LAMIN BREAKDOWN DURING ECHINODERM (*STRONGYLOCENTROTUS DROEBACHIENSIS* AND *CUCUMARIA FRONDOSA*) COELOMOCYTE CELL DEATH

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One of the hallmark features of apoptosis, or programmed cell death, is the disassembly of the nucleus. This appears to be largely accomplished by members of the ICE cysteine protease family, that a) activate nucleases that cleave DNA into internucleosomal fragments, and b) degrade lamins, which are members of the intermediate filament protein superfamily that underlie and support the nuclear envelope. DNA fragmentation and lamin proteolysis are carried out by different members of the ICE protease family, and both pathways are necessary for the normal progression of apoptosis: inhibiting either event blocks complete nuclear disassembly (Lazebnik et al., Proc. Natl. Acad. Sci. USA 92, 9042-9046, 1995). DNA fragmentation during apoptosis can be detected by the appearance of a "DNA ladder" on agarose gels; however, the specific features of DNA cleavage may vary from cell type to cell type, thereby affecting the presence and appearance of a "ladder" (Eastman, Methods Cell Biol. 46, 41-55, 1995). Although lamin degradation during apoptosis is also considered to be a diagnostic feature of apoptosis, very little is known regarding possible variations in lamin proteolytic patterns among different cell types. In addition, most of the work determining which factors are effective in triggering apoptosis has been conducted using mammalian cells, and very little information exists regarding the cellular responses of marine species to environmental toxicants implicated in initiating the cell death pathway. To address these questions, nuclei of coelomocytes from two echinoderm species were examined for apoptotic changes in response to Cadmium, Copper, Mercury, and hydrogen peroxide treatment.

Petalloid coelomocytes from the sea urchin *Strongylocentrotus droebachiensis* and the sea cucumber *Cucumaria frondosa* were collected and cultured essentially as described by Edds (J. Cell Biol., 73, 479-491, 1977). Coelomocytes adhering to glass coverslips were cultured in the presence of 100 μM $CdCl_2$, 25 μM $CuCl_2$, 5 μM $HgCl_2$, or 0.06% hydrogen peroxide for 8 or 20 hours, fixed in ice-cold methanol, and immunolabeled with antibodies to lamin. Immunolabeling and Hoechst-staining of DNA was conducted as described by Holy and Schatten (J. Cell Sci., 98, 423-431, 1991), using a polyclonal antibody raised against recombinant *Strongylocentrotus purpuratus* lamin (Holy et al., Dev. Biol. 168, 464-478, 1995). For quantitative measurements of lamin immunoreactivity, five random fields of coelomocytes from each preparation were photographed at x40 magnification using identical exposure times. All of the film was developed together in the same run, and subsequently scanned under identical settings into a Power MacIntosh 7100/80AV computer with a Polaroid SprintScan 35. NIH Image (the public domain program written by Wayne Rasband, NIH) was used to measure the brightest areas of nuclear envelopes at standard "12:00," "3:00," "6:00," and "9:00" positions. All intact nuclei completely contained within each field were measured; the numbers of nuclei counted for each timepoint/treatment ranged from 33 to 69.

Examination of Hoechst-labeled nuclei reveals that a number of coelomocytes from both *S. droebachiensis* and *C. frondosa* become apoptotic after a few hours in culture, presumably reflecting the stress of isolation and their explanted state. Obvious differences exist in the

chromatin patterns of the two species; nuclei of *S. droebachiensis* coelomocytes (SDCs) progressively shrink and become more strongly and homogeneously stained with Hoechst as they undergo apoptosis (Fig. 1a). By contrast, *C. frondosa* coelomocytes (CFCs) exhibit a more "classic" pattern of clumped or reticular chromatin condensation as they undergo apoptosis (Fig 1c). That these cells are in fact dying is supported by tubulin labeling experiments, which show that microtubule patterns in cells exhibiting these types of nuclei are extensively disrupted (data not shown). Remarkably, striking differences in lamin labeling patterns are evident between SDCs and CFCs; nuclear envelopes of apoptotic SDCs exhibit a range of labeling intensities, with many actually appearing to be more strongly-labeled than non-apoptotic cells (Fig. 1a,b). By contrast, nuclear envelopes of apoptotic CFCs are routinely more weakly-labeled with the lamin antibody than are those of healthy cells (Fig. 1c,d). These differences are reflected in the fact that although a continuum of nuclear envelope immunoreactivities are present in both living and apoptotic SDCs, it is possible to readily categorize CFC lamin immunoreactivity into three classes: strong, medium, and weak labeling.

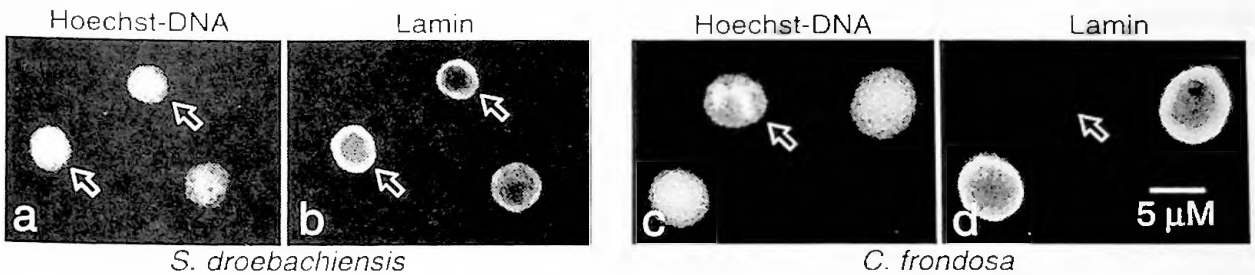

Figure 1. Comparison of chromatin patterns and lamin labeling in apoptotic and healthy SDCs and CFCs. a, b: SDC nuclei double-labeled for DNA and lamin, showing two apoptotic nuclei (arrows) and one healthy nucleus. b, c: CFC nuclei double-labeled for DNA and lamin, showing one apoptotic nucleus (arrow) and two healthy nuclei. a - d are the same magnification.

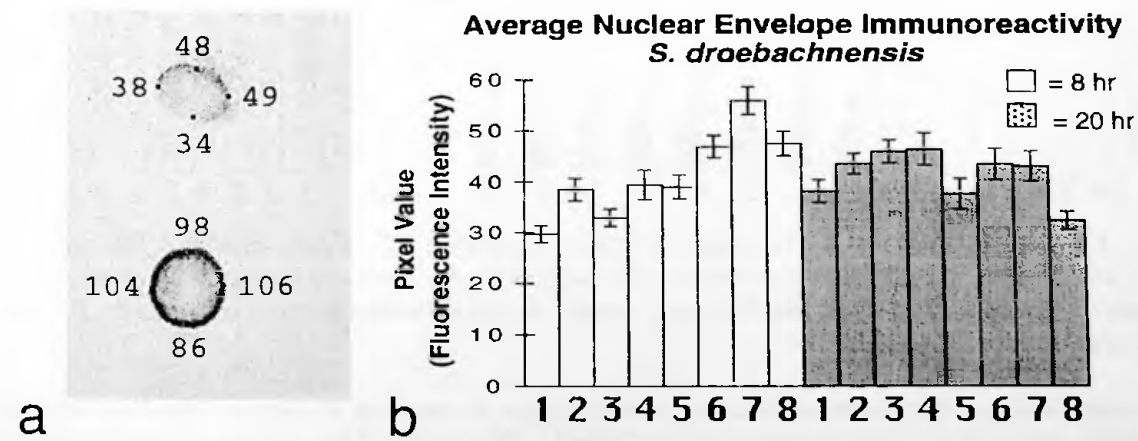

Figure 2. a: Example of lamin measurements using NIH image (contrast has been inverted to more clearly visualize lightly-labeled nuclear envelopes). b: Graph of the average SDC nuclear envelope labeling intensities after 8 and 20 hours in culture. Key: 1, Control; 2, Cd; 3, Cu; 4, Hg; 5, H_2O_2; 6, Cd/H_2O_2 ; 7, Cu/H_2O_2 ; 8, Hg/H_2O_2

Measurements of lamin immunoreactivity in SDCs exposed to different metals and oxidative stress conditions reveal distinct nuclear responses to these treatments (Fig. 2a,b). Subjectively, most of the apoptotic SDCs appear to exhibit more strongly-labeled nuclear

envelopes than non-apototic cells, regardless of the treatment. Statistical analyses verify that at the eight-hour timepoint, all treatments - with the exception of Cu - result in a significant *increase* in lamin immunoreactivity ($P < 0.05$). At the 20-hour timepoint, these differences are attenuated, and only SDCs treated with Cu, Hg, or Hg/H_2O_2 contain nuclei with lamins labeled significantly more strongly than the 20 hour control. Comparing the two timepoints indicates that differences also exist in the progression of nuclear envelope changes among cells in the different experimental groups. Control and Cu-treated SDCs both show a significant increase in lamin immunoreactivity between eight and 20 hours of culture; conversely, Cu/H_2O_2- and Cd/H_2O_2 treated SDCs display a *decrease* in lamin immunoreactivity after 20 hours.

The responses of CFC nuclei to metal poisoning and oxidative stress were clearly different from SDC nuclei. Compared to controls, total average lamin labeling values of CFC nuclei are significantly different only for the Hg, H_2O_2, and Hg/H_2O_2 treatments (Fig. 3a). Among these groups, overall lamin immunoreactivity increases following Hg and Hg/H_2O_2 treatment, and decreases in the presence of H_2O_2 alone. The ability to categorize CFCs into one of three groups based on lamin labeling intensity enables a number of further analyses to be carried out. First of all, the overall labeling intensity within any given group (i.e. strong, medium or weak) remains quite constant between the different treatments (there are significant differences between some of the groups - e.g., H_2O_2 vs. Cu/H_2O_2 - within the "strong" category, however) (Fig. 3b). Comparing the percentages of cells in each category between treatments reveals striking differences in the response of cells to the various culture conditions (Fig. 4). Notably, Cu and H_2O_2 treatments produce many more cells with weakly-labeled nuclear envelopes; by contrast, a relatively high proportion of CFCs cultured in Hg and Hg/H_2O_2 maintain a strong level of lamin labeling.

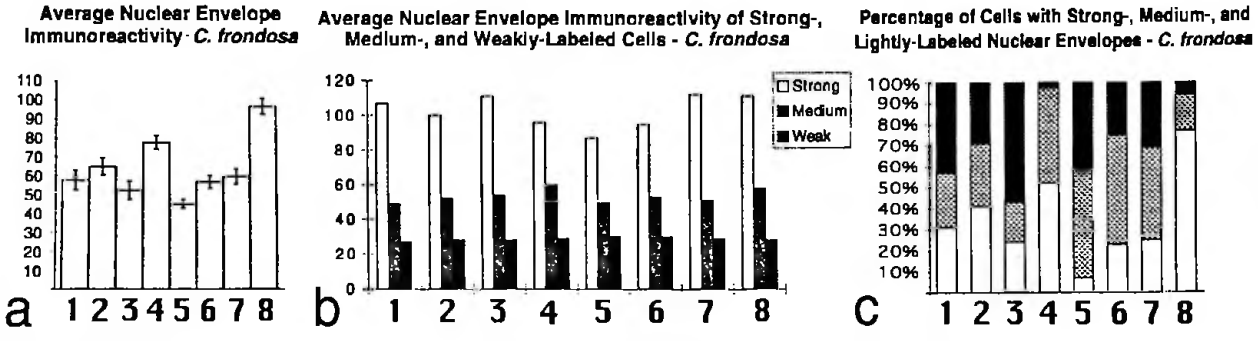

Figure 3. Graphs comparing CFC lamin immunoreactivity. a: Total average fluorescence intensity of all groups (strong, medium and weak) combined. b: Average intensity of each group. c: Percentage of cells with strong, medium, and weakly-labeled lamins in each treatment. For key to numbers (1 - 8) see Figure 2.

To test whether the immunofluorescence changes in nuclear envelope immunoreactivity reflected lamin proteolysis, immunoblots of SDCs and CFCs cultured in various concentrations of metals and H_2O_2, or subjected to UV irradiation, were probed with the anti-lamin antibody. Surprisingly, very little lamin degradation could be detected in either species under these conditions (data not shown). It remains to be determined whether the decrease in nuclear envelope labeling at the immunofluorescence level reflects some nonproteolytic-based change in lamin organization, or whether the antibody and blotting conditions used were sub-optimal for detecting lamin proteolysis.

These results demonstrate that clear differences exist between the responses of SDC and CFC nuclei during apoptosis. Similar to what has been shown to occur in mammalian cells, CFC nuclei exhibit an unequivocal and marked decrease in lamin labeling during apoptosis. Oxidative stress is a potent initiator of apoptosis in many cell types, and both H_2O_2 and Cu treatment were effective in increasing the number of CFCs with weakly-labeled lamins. It is curious that the combined Cu/H_2O_2 treatment was not as effective as either alone, since the two are capable of interacting to produce free radicals through Fenton-type reactions. By contrast, Hg and Hg/H_2O_2 reduced the number of CFCs with weakly-labeled nuclei, and increased the number containing strongly-labeled nuclei. Mercury is a potent inhibitor of enzyme activity in general, and it is possible that this shift reflects an inhibition of a presumptive lamin-directed protease in these cells. In contrast to the CFCs, the behavior of SDC lamin during apoptosis significantly diverges from that of other cells described to date. It is possible that the increase in lamin immunoreactivity in apoptotic SDCs reflects either physical changes in the nuclear envelope or post-translational modifications of lamins, thereby enhancing antibody binding. Alternatively, the timecourse encompassed in these experiments is sufficient to allow quantitative changes in lamins to occur via either increased protein synthesis or reduced turnover. The dissimilar lamin behavior occuring in SDCs and CDCs during apoptosis constitutes a compelling argument to exert caution in using this nuclear matrix protein as a benchmark to assess cell death. This is underscored by the similar morphology, function, and phylogenetic proximity of the cells used in this study. It will be of great interest to test which elements of the apoptotic pathway differ between these cells to produce the striking differences in lamin behavior reported here, and to determine whether these differences are important features in the ability of a given cell type to withstand a potentially apoptosis-triggering toxic insult.

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