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Evolutionary biology

Programmed death in a unicellular organism has species-specific fitness effects

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Programmed cell death (PCD) is an ancient phenomenon and its origin and maintenance in unicellular life is unclear. We report that programmed death provides differential fitness effects that are species specific in the model organism *Chlamydomonas reinhardtii*. Remarkably, PCD in this organism not only benefits others of the same species, but also has an inhibitory effect on the growth of other species. These data reveal that the fitness effects of PCD can depend upon genetic relatedness.

1. Introduction

Unlike necrosis, which is passive and uncoordinated, programmed cell death (PCD) [1] comprises diverse, genetically controlled cell suicide mechanisms. Hallmarks of PCD including DNA fragmentation and the externalization of phosphatidylserine (PS) on the cellular membrane [2] have been observed in multicellular and unicellular organisms. The finding that diverse unicellular organisms are capable of undergoing PCD suggests that PCD emerged early in life's evolution [3]. In multicellular organisms, PCD makes evolutionary sense: cells cooperate to increase organismal fitness. In unicellular organisms, the cell *is* the organism and death cannot benefit the individual [4].

In the unicellular chlorophyte *Chlamydomonas reinhardtii* (electronic supplementary material, figure S1), we reported that heat-induced PCD is associated with positive fitness effects on neighbours [5]. However, do these benefits extend to other species? We used the same model system and examined the effects of PCD materials from *C. reinhardtii* strain CC125 on another strain (*C. reinhardtii* UTEX89) and two other species (*Chlamydomonas moewusii* UTEX9 and *Chlamydomonas debaryana* UTEX1344) and discovered species-specific fitness effects. PCD benefits others of the same strain but, surprisingly, inhibits the growth of two other species. There was no significant effect on the growth of *C. reinhardtii* strain UTEX89. These data indicate that the fitness effects of programmed death in unicells can depend upon the genetic relatedness between individuals.

2. Material and methods

(a) Cell culture and treatments

Three species of the unicellular chlorophyte *Chlamydomonas* (*C. reinhardtii* strains CC125 and UTEX89, *C. moewusii* UTEX9 and *C. debaryana* UTEX1344) were conditioned and maintained in *tris*-acetate-phosphate (TAP) medium [6] at 26°C on a 12 L : 12 D cycle with constant shaking. TAP medium was used for all organisms for consistency. Prior to PCD induction, late-log/early stationary phase cells were

washed after centrifugation at 5000 r.p.m. (Eppendorf5702) and re-suspended in fresh TAP medium to a cell density of 10^7 cells ml^{-1}.

(b) Programmed cell death induction

Ten millilitres of *C. reinhardtii* CC125 cell culture was heated at 42°C for 1 h (a more severe heat stimulus, 50°C for 10 min, produces similar results) and maintained under standard conditions for 14–16 h. Control cultures were untreated (no heating). An additional control where control medium was heated once cells were removed was performed. This excluded the unlikely possibility that heating *per se* of cellular waste products had an effect (electronic supplementary material, figure S2). Quadruplicate biological (independent cultures) and technical (independent readings per culture) replicates were performed. *Chlamydomonas* culture collections are not always axenic, and bacterial contamination may have initially impacted our findings. Experiments were therefore performed after antibiotic decontamination. No bacterial growth occurred in liquid culture or after plating on antibiotic-free TAP agar.

(c) Collection of supernatants and growth measurements

Supernatants from log-linear/early stationary phase *C. reinhardtii* strain CC125 cell culture were obtained by centrifugation (5000 r.p.m., 10 min, Eppendorf 5702) after induction of PCD. Supernatant obtained prior to PCD induction was used as a control and, in the case of heated control medium, after removal of cells. Ten millilitres of TAP medium supplemented with PCD or control supernatant (ratio of 2 : 1, TAP : supernatant) was inoculated with cells from early stationary phase cultures of one of the species/strains to a starting density of 10–30 cells μl^{-1}. Cell suspensions were cultured in 15 ml tubes, placed in a rack on a shaker at 100 r.p.m. at a distance of approximately 15 cm from a horizontal light source. Cell growth was measured daily by direct counts using a haemocytometer (average count of four squares with the counter blind to samples) and spectrophotometrically at 665 nm (Thermo, Biomate5). Counts and absorbance reflect fitness, the former determining offspring number and the latter number and size.

(d) Programmed cell death detection

The regulated fragmentation of genomic DNA is a diagnostic feature of PCD. Control and PCD-induced cultures were centrifuged as above and the pellets lysed in 0.5% sodium-dodecyl-sulfate and proteinase K (10 mg ml^{-1}) and treated with RNase A (final concentration 1 mg ml^{-1}) for 10 min at 65°C. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) and electrophoresed in 1% agarose gel (45 min, 80 V). This provides a qualitative result, because not all cells in *C. reinhardtii* populations undergo PCD [7]. For confirmation and quantification of PCD, flow cytometric detection of PS exposure was performed. The flow cytometry TUNEL assay was intentionally avoided because it is also a measure of DNA fragmentation and not independent. Its sensitivity and specificity has been questioned [8].

In healthy cells, plasma membrane phospholipids are distributed asymmetrically and PS is confined to the cytoplasmic surface. During early PCD, cell membrane integrity is maintained despite PS exposure on the outer surface [9]. This can be detected by annexin V (binds PS reversibly) conjugated to a FITC fluorochrome. Propidium iodide (PI) intercalates into DNA and detects membrane disruption, which occurs during non-PCD death or late PCD. PCD^+ cells are $FITC^+$ and PI^- while healthy cells are negative for both fluorochromes. Necrotic cells, where the plasma membrane is disrupted, are PI^+.

PCD^+ cells were detected and analysed according to our previously described methods [10]. Briefly, cells were harvested and stained with FITC-conjugated annexin V and PI for 15 min (Apoptosis detection kit, BD Pharmingen). Annexin V positivity was analysed by a FACS Calibre cell sorter (Becton-Dickinson, San Jose, CA, USA) using standard FITC (525 nm emission) and PI filter sets (617 nm emission). An analysis of excitation and emission spectra of FITC and PI were performed prior to experiments to ensure that there was no autofluorescence spill over in the detection channels.

(e) Statistical analyses

The test statistic (mean t) used is a two-sample t-statistic comparing cell count (or absorbance) between the two groups at each time point averaged over the course of the experiment. The null hypothesis is that there is no significant difference between control and experiment growth; any difference arises by chance alone. The data were re-sampled multiple times (10 000 events for each comparison) consistent with the null hypothesis to calculate the sampling (permutation) distribution of the test statistic. The data (as a sequence of counts or absorbances in the form a time series) in each experiment are randomly allocated to each of the two groups (control versus experiment) and the mean t is recalculated for 10 000 sample sets. Once an experiment is assigned to a specific group, it carries all of its data values over to that group to avoid an influence of the time-dependence of data which could otherwise occur by swapping values at individual (rather than all) time points. The p-value is the proportion of permutations in which mean t is greater in absolute value than mean t for the original dataset. In other words, the original mean t is located on the permutation distribution of the mean t to assess the probability that the original mean t occurs by chance alone. A potential source of error in this procedure occurs when the curve for one group goes above the other group in some intervals and below that in other intervals. This is because the test measures means over time and can be insensitive to the way the groups compare when the difference changes in sign across different time segments. Such segmental behaviour was not observed in our analysis. Statistical significance was assessed using the statmod software package (http://bioinf.wehi.edu.au/software) with the 'compareGrowthCurves' function.

3. Results and discussion

(a) Induction and detection of programmed cell death

Agarose gel electrophoresis of genomic DNA isolated from control and heat-stressed *C. reinhardtii* CC125 cells was performed. The heat stimulus occurs in natural environments inhabited by *Chlamydomonas* species. Heat-induced PCD caused ordered fragmentation of DNA compared with the control (figure 1*a*). Flow cytometric analyses of PS exposure confirmed the PCD phenotype (figure 1*b*). A small number of cells in culture are always non-viable and possibly dying by PCD and we noted that 8.99% of the untreated control cells were $FITC^+$ and PI^- (I). In contrast, 26.41% of the heat treated (II) underwent PCD. These results confirmed that just under a third of cells were PCD^+.

(b) Effect of programmed cell death materials on other *Chlamydomonas* strains/species

Our previous work showed that heat-induced PCD can be beneficial to others of the same strain [5] and was repeated to serve

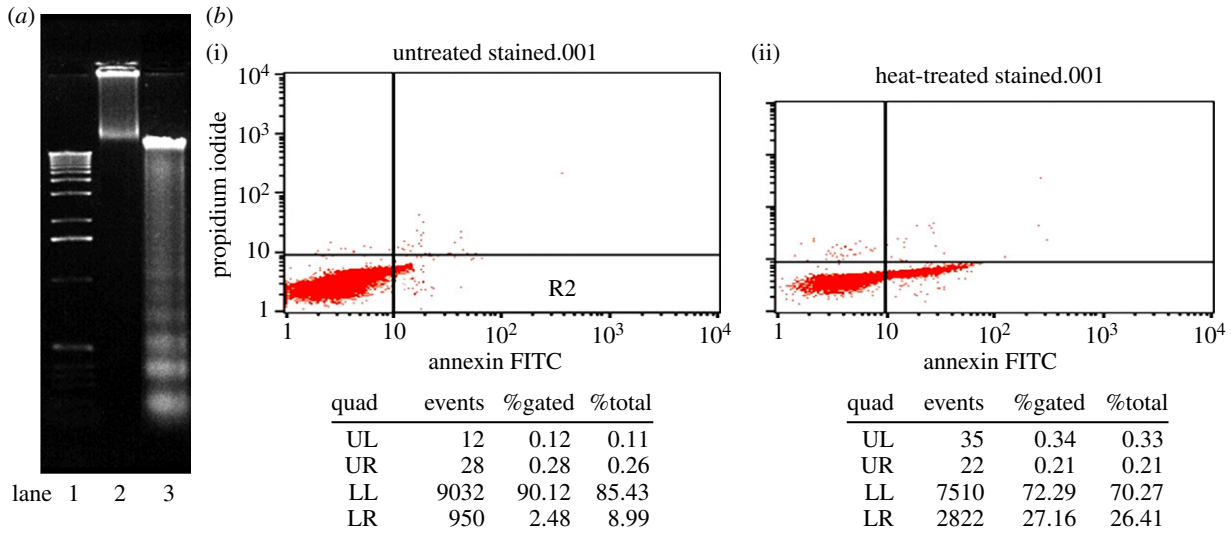

Figure 1. PCD induction and detection. PCD was induced in *C. reinhardtii* CC125 using heat and confirmed using DNA fragmentation patterns and PS exposure. (*a*) Genomic DNA fragmentation profiles in *C. reinhardtii* CC125. Lane 1: 100 bp DNA ladder; lane 2: untreated control; lane 3: heat-induced PCD. (*b*) Flow cytometric analysis. I and II are dot-plots of a sample (30 000 cells) of the population of control and heat-induced PCD cells, respectively. Axes are: (*x*) AV FITC fluorescence and (*y*) PI fluorescence. The four quadrants are as follows: (i) lower left comprises normal, healthy cells (isotype control, AV^- and PI^-); (ii) lower right comprises early PCD cells (AV^+ and PI^-); (iii) upper right comprises late PCD (AV^+ and PI^+) and (iv) upper left comprises necrotic cells (AV^- and PI^+).

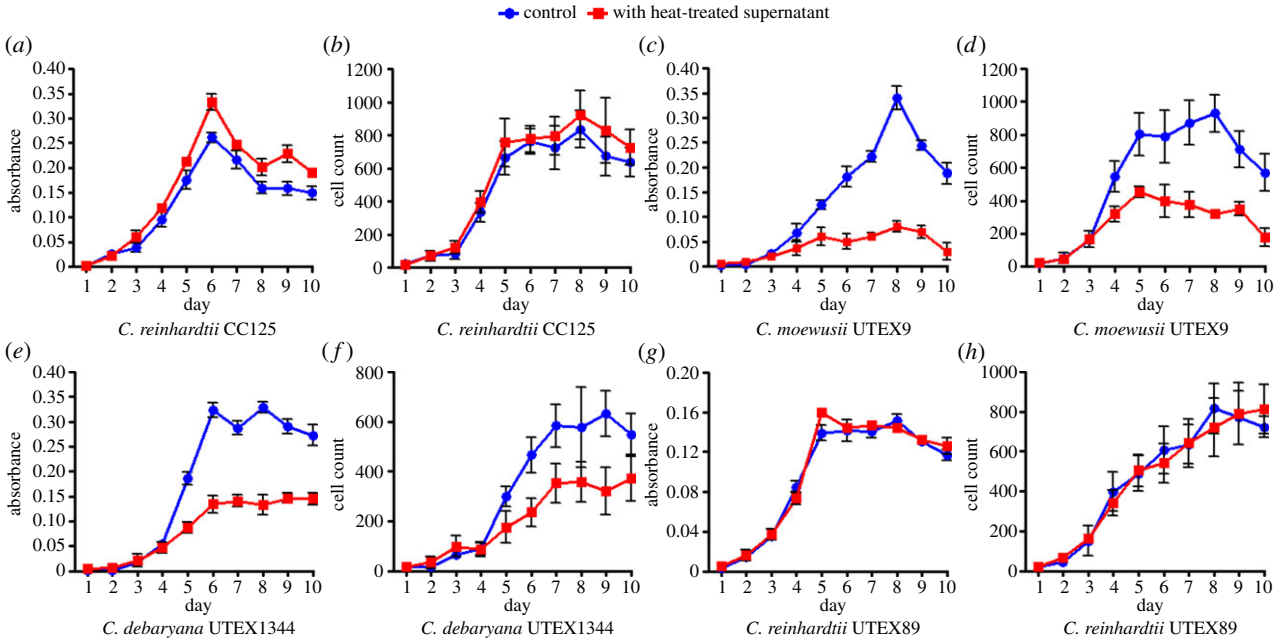

Figure 2. Differential fitness effects of PCD supernatant. Culture absorbances and cell counts are for two strains of *C. reinhardtii* (CC125 and UTEX89) and two other species *C. moewusii* and *C. debaryana*. PCD supernatant was obtained from *C. reinhardtii* CC125 cultures and supplemented with TAP medium (1 : 2). The fitness effects of PCD supernatant were determined spectrophotometrically (absorbance at 665 nm) and by direct cell counts (average of four readings). Supernatant from an untreated (non-PCD) *C. reinhardtii* CC125 culture was used as the control. PCD supernatant had a beneficial effect on the growth of others of the same species and strain according to (*a*) a statistically significant difference in absorbances ($p = 0.03$) and (*b*) an obvious, albeit not statistically significant, trend in count comparisons ($p = 0.39$), the latter possibly due to the relatively small sample size. Unexpectedly, PCD supernatant from *C. reinhardtii* CC125 inhibited the growth of two other species *C. moewusii* and *C. debaryana* for both absorbance ($p = 0.02$ in both species) and count comparisons $p = 0.03$ and $p = 0.02$, respectively (*c*–*f*). The effect on the same species, different strain was not significant (absorbances $p = 0.30$; counts $p = 0.71$, *g*,*h*). Error bars are ± 1 s.d. ($n = 4$).

as a positive control. In addition, the fitness effects of PCD materials released by one strain (*C. reinhardtii* strain CC125) on another strain *C. reinhardtii* UTEX89 and two other species (*C. moewusii* UTEX9 and *C. debaryana* UTEX1344) were examined. Growth dynamics were atypical due to the relatively nutrient-depleted media (PCD supernatant obtained after 1–2 days). The effect of PCD on growth of the same species was positive (figure 2*a*,*b*), consistent with previous reports [5,11]. Unexpectedly, PCD supernatant from *C. reinhardtii* CC125 inhibited the growth of two other species, *C. moewusii* (figure 2*c*,*d*) and *C. debaryana* (figure 2*e*,*f*). After a brief initial overlap for 3–4 days, the curves separated and remained so for the duration of the experiments. The growth curve comparison for a different strain (UTEX89) was not significant (figure 2*g*,*h*). The additional control comparing heat-treated supernatant after removing cells to standard medium demonstrated that the positive effects were not due to heating of the cellular waste products (electronic supplementary material, figure S2).

These data indicate that PCD in *Chlamydomonas* benefits others of the same species and strain but has negative inter-species fitness effects. The mechanism is unknown; however, in another chlorophyte, *Dunaliella salina*, PCD releases organic materials that are either used directly by *D. salina* cells not undergoing PCD or re-mineralized by a co-habiting archaeon [11]. A similar mechanism may be at work in *Chlamydomonas* and is supported by our previous findings that the active ingredients are simple heat-stable molecules [5].

4. Concluding remarks

Our results show that the fitness effects of PCD can depend upon genetic relatedness, suggesting that PCD may be maintained in unicells by kin selection despite being detrimental to the individual. Death is not a neutral event. It affects neighbouring organisms and the inter-species differential fitness effects may have driven the early evolution of PCD. Remarkably, an organism even *after* death continues to exert species-specific fitness effects on its neighbours.

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Data accessibility. Data available from the dryad digital repository: http://dx.doi.org/10.5061/dryad.jt2b5.

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