Mitochondria as integrators of information in an early-evolving animal: insights from a triterpenoid metabolite

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Mitochondria have the capacity to integrate environmental signals and, in animals with active stem cell populations, trigger responses in terms of growth and growth form. Colonial hydroids, which consist of feeding polyps connected by tube-like stolons, were treated with avicins, triterpenoid electrophiles whose anti-cancer properties in human cells are mediated in part by mitochondria. In treated hydroids, both oxygen uptake and mitochondrial reactive oxygen species were diminished relative to controls, similar to that observed in human cells exposed to avicins. While untreated colonies exhibit more stolon branches and connections in the centre of the colony than at the periphery, treated colonies exhibit the opposite: fewer stolon branches in the centre of the colony than at the periphery. The resulting growth form suggests an inversion of the normal pattern of colony development mediated by mitochondrial and redox-related perturbations. An as-yet-uncharacterized gradient within the colony may determine the ultimate phenotypic effect of avicin perturbation.

Keywords: avicins; cancer; hydroids; *Podocoryna*; reactive oxygen species; redox state

1. INTRODUCTION

An increasing number of roles for redox state in general, and mitochondria in particular, have been found in cell differentiation and death (e.g. Coffman *et al.* 2004; Green & Kroemer 2004; Irwin *et al.* 2003; Wood *et al.* 2003). Mitochondria may integrate environmental information and, depending on the circumstances, trigger differential cellular responses. Avicins, derived from *Acacia victoria* plants, are a case in point. This newly discovered family of triterpenoid electrophiles has been found to regulate the innate stress response in human cells (Haridas *et al.* 2004, submitted). For example, in cancer cells the avicins induce apoptosis by direct perturbation of the mitochondria, activating the intrinsic caspase pathway (Haridas *et al.* 2001, submitted), while in normal cells a minimal effect is seen (Mujoo *et al.* 2001). In addition, avicins induce a cytoprotective mechanism by the redox regulation of Nrf2, resulting in the enhancement of cell detoxification and antioxidant effects (Haridas *et al.* 2004, submitted). Generally, the avicins represent a new class of metabolites that regulate redox balance in human and mammalian cells through a coordinated system's response to stress, and much of this response involves mitochondria or related redox pathways. Such mitochondrion-related signalling pathways are arguably an ancient component of eukaryotic cells (Blackstone & Kirkwood 2003; Nedelcu *et al.* 2004). Here we examine the effects of avicin-related perturbations in an early-evolving animal, the colonial marine hydroid *Podocoryna carnea*. As with many simple animals, these hydroids have active stem cell populations at all stages of the life cycle (Holstein *et al.* 2003). Mitochondrion-related responses can thus have broad effects on cell proliferation and differentiation, and such effects are of clear interest with regard to anti-cancer selection (Galis & Metz 2003).

2. MATERIAL AND METHODS

(a) *Study animals and culture conditions*

Colonial hydroids encrust a surface with branching and anastomosing stolons that connect feeding polyps. Variation in branching patterns characterizes both between-species and within-species differences (Buss & Blackstone 1991). 'Sheet-like' species show closely spaced polyps with short stolonal connections; 'runner-like' species exhibit widely spaced polyps with long stolonal connections. Colonies of *P. carnea* and other species shift from a runner-like growth form to a sheet-like one as they encrust and cover a surface. Thus, the central region of a large colony will show relatively closely spaced polyps and short stolon connections as compared with peripheral regions of the same colony. A number of mitochondrial perturbations result in clear effects on branching patterns that are uniform throughout the colony, e.g. stolons are more closely branched throughout the entire colony or more widely branched throughout the entire colony (Blackstone 2003).

Podocoryna (= *Podocoryne*) *carnea* colonies of a single clone were cultured using standard methods (e.g. Blackstone 1999; the same clone, P3, has been used extensively in previous investigations). For measures of oxygen uptake, colonies were grown from single-polyp explants on 12 mm diameter round glass cover-slips; for various measures of polyp and stolon development, explants were grown on 18 mm diameter round

glass cover-slips; for measures of reactive oxygen species (ROS), explants were grown on 15 mm diameter round glass cover-slips. Growth of the colonies was confined to one side of the cover-slips by daily scraping with a razor blade. All experiments were carried out at 20.5 °C.

(b) Measures of oxygen uptake

Five treated and five control colonies were assayed using standard methods (Blackstone 2003). For each of these assays, a *P. carnea* colony on a 12 mm diameter cover-slip was attached with a drop of silicone grease to a cover-slip cemented to a small magnet. This assembly was contained in a 13 mm diameter sealed glass chamber (Strathkelvin RC300) with 0.7 ml of seawater (filtered to 0.2 μm). Chamber temperature was held constant (20.5 ± 0.02 °C) using an external circulation water bath (Neslab RTE-100D), and the rate of decline in oxygen concentration over a 30 min period was measured (using a Strathkelvin 1302 electrode and a 781 oxygen metre) with stirring (by slowly spinning the magnet, cover-slips and colony). The chamber was then opened, a small volume of seawater removed, an equivalent amount of the stock solution of avicin extracts in dimethyl sulfoxide (DMSO) was added to achieve the target concentration of $10 \, \mu g \, ml^{-1}$, the solution mixed and aerated thoroughly with a small pipette, and the chamber resealed (this procedure took approximately ~ 7 min). For the controls, DMSO was added to the same concentration as used in the avicin treatments. The rate of decline in oxygen concentration was then measured over another 30 min period. These assays were performed 3–5 h after the feeding of the subject colony as part of the normal feeding schedule. For each colony, the before/after difference in the rate of decline in oxygen concentration over a 30 min period was calculated, where this decline was measured by the least-squared slope of oxygen concentration versus time. An overall trend in these differences for the five colonies was analysed using a paired-comparison *t*-test.

(c) Comparisons of ROS

Hydrogen peroxide represents a major component of ROS under physiological conditions (Chance *et al*. 1979), and measures of H_2O_2 were taken using $2',7'$-dichlorofluorescin diacetate (H$_2$DCFDA; Nishikawa *et al*. 2000; Pei *et al*. 2000). This non-fluorescent dye is freely permeable to living cells. Once inside a cell, the acetate groups are removed by intracellular esterases. In turn, H$_2$DCF is oxidized by peroxides, usually in the presence of peroxidase, cytochrome c, or Fe^{2+}, to form $2',7'$-dichlorofluorescein, which can then be visualized with fluorescent microscopy. The activation of H$_2$DCF may be relatively specific for the detection of H_2O_2 as well as secondary and tertiary peroxides; alternatively, this may be regarded as a general, semi-quantitative assay of ROS (Finkel 2001). Stock solutions of H$_2$DCFDA and avicin extracts were prepared in anhydrous DMSO. Six hydroid colonies (24 h after feeding) were incubated in $10 \, \mu g \, ml^{-1}$ avicins. Within 1 h, H$_2$DCFDA was added to a concentration of $10 \, \mu mol^{-1}$ and colonies were incubated for an additional hour in the dark prior to measurement. Six control colonies were similarly treated, except only DMSO to the same concentration was added. Using a Hamamatsu Orca-100 cooled-CCD camera and a Zeiss Axiovert 135, ROS (as indicated by H$_2$DCFDA-derived

$2',7'$-dichlorofluorescein) were imaged for a $\sim 50 \times 150 \, \mu m$ contractile region at the base of three polyps per colony (excitation 450–490 nm, emission 515–565 nm). At these wavelengths, negative controls show that there is little native fluorescence. These regions were also imaged for native fluorescence of NAD(P)H (excitation 365 nm, emission > 420 nm), which is a general measure of redox state (Chance 1991). These contractile regions contain the mitochondrion-rich epitheliomuscular cells (EMCs) of the colony (Blackstone *et al*. 2004).

Images with 12-bit depth (4096 grey levels) were thus obtained and were analysed using IMAGE-PRO PLUS software. In such images, fluorescence is visible from many $\sim 10 \, \mu m^2$ sized clusters of mitochondria from EMCs at polyp–stolon junctions (Blackstone *et al*. 2004). The luminance and area for each of these fluorescent objects was measured by: (i) selecting the object and an equivalent area of its immediate surroundings (background) as a circular region of interest, (ii) allowing the software to identify the area and luminance of the foreground 'bright' region (i.e. the area of fluorescent signal), (iii) exporting these measures to file, (iv) automatically identifying the area and luminance of the complementary background 'dark' region and exporting these measures to file. The area of each cluster was thus calculated, and the luminance of the cluster was adjusted for the background luminance by subtraction. These measures were analysed by a nested ANOVA: clusters nested within polyps, polyps nested within clonal replicates, and replicates within treatments.

(d) Comparisons of colony growth and development

Stock solutions of avicin extracts were prepared in anhydrous DMSO. Seven colonies treated with avicin extracts at $10 \, \mu g \, ml^{-1}$ and seven control colonies, treated with DMSO to the same concentration, were used. Each group was treated with the appropriate solution for approximately ~ 4–$6 \, h \, d^{-1}$. As each colony was nearly covering the surface of the 18 mm cover-slip (up to 60 days after explanting), that colony was imaged. Images were processed to facilitate automatic measurement using IMAGE-PRO PLUS software. The grey level of some image objects (i.e. background, stolons or polyps) was adjusted using COREL PHOTO-PAINT software (background grey level = 10, stolon = 201, polyp = 255). Processed images were checked against the original images to ensure accuracy. Each colony was divided up into a central region, arbitrarily defined as 30% of the total area (range 29.8–30.4%) centred on the original explant; and a peripheral region, defined as the remaining 70% of the colony. The central and peripheral regions of each processed image were measured in IMAGE-PRO PLUS software for areas of unencrusted cover-slip. These unencrusted areas provide an indication of stolon branching and anastomosis—large areas indicate a low rate of stolon branching and anastomosis, while small areas indicate a high rate. The boundary between the central and peripheral regions was used to assign unencrusted areas to the respective regions. The perimeter of the colony was used to distinguish unencrusted areas within the peripheral region from unencrusted areas outside the colony. Total colony area was measured as well. Using PC-SAS software, natural log-transformed data for unencrusted areas in the central region and in the peripheral region were compared between treated colonies and controls using
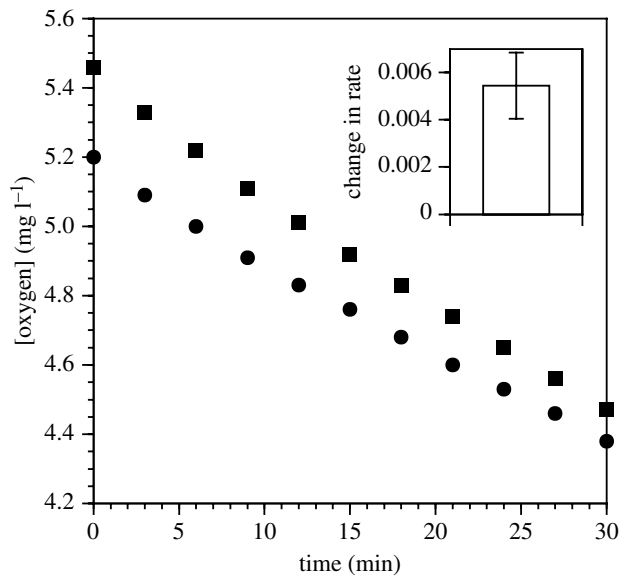
Figure 1. Rate of decline in oxygen concentration for a *P. carnea* colony before (squares) and after (circles) treatment with avicin extracts ($10 \, \mu g \, ml^{-1}$). For five colonies of each treatment, inset plot shows the mean$\pm$s.e.m. of the before/after difference in the rate of decline in oxygen concentration, where this decline is measured by the least-squares slope of oxygen concentration versus time. This difference in rate was significantly positive, i.e. the oxygen uptake decreased after treatment.

univariate (ANOVA) and multivariate analysis of variance (MANOVA).

3. RESULTS

(a) *Measures of oxygen uptake*
Treatment with avicins shows an immediate (within 10 min) inhibition of oxygen uptake (figure 1; paired comparison *t*-test of before and after slopes of oxygen concentration versus time, $t = 3.884$, $p < 0.02$, $n = 5$), compared to the controls in seawater and DMSO at the same concentration ($t = 0.080 \, 36$, $p > 0.40$, $n = 5$).

(b) *Comparisons of ROS*
Within 2 h, avicins show an effect on peroxide and perhaps other ROS, as indicated by H_2DCFDA-derived $2',7'$-dichlorofluorescein (figure 2; $F_{1,10} = 7.02$, $p < 0.05$, $n = 6$). Previous studies suggest that mitochondria are the principal source of these peroxides (Blackstone *et al.* 2004). Nevertheless, avicins do not seem to be acting as uncouplers or inhibitors of mitochondria, since no clear effect is observed on redox state as indicated by native fluorescence of NAD(P)H (figure 3; $F_{1,10} = 0.08$, $p < 0.70$). A more general cellular antioxidant response is thus suggested.

(c) *Comparisons of colony growth and development*
In typical growth of *P. carnea* colonies, stolons branch more in the centre of a colony, resulting in smaller mean sizes for unencrusted areas (figure 4). On the other hand, closer to the periphery of the colony, stolons branch less and mean sizes for unencrusted areas are larger. This pattern is nearly completely reversed in colonies treated
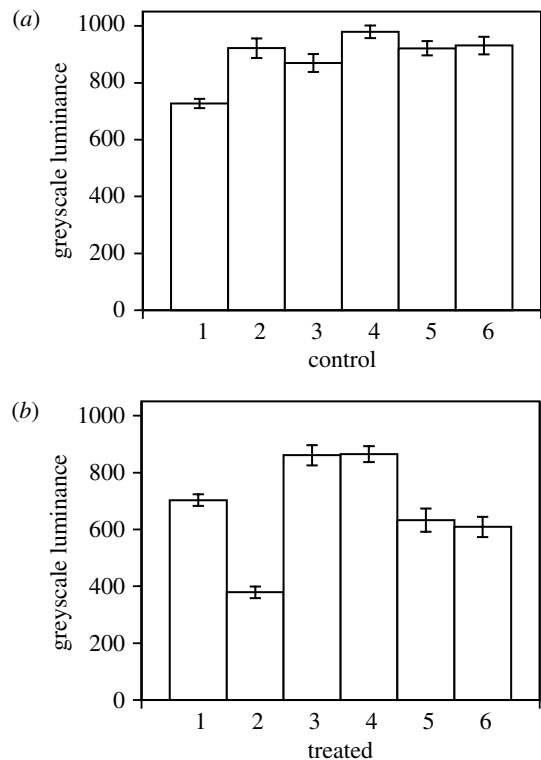

Figure 2. Mean$\pm$s.e.m. luminance (greyscale from 0 to 4095) for three polyps per replicate colony after incubation in H_2DCFDA. ROS are thus visualized. (*a*) Control, (*b*) colonies treated with avicin extracts ($10 \, \mu g \, ml^{-1}$).

with avicins (figure 4). Treated colonies tend to branch less in the centre of the colony and more on the periphery. There was no significant difference in total colony area ($F_{1,12} = 0.61$, $p > 0.45$). Differences between control and treated colonies were significant for central inner areas ($F_{1,12} = 36$, $p \ll 0.001$), for peripheral inner areas ($F_{1,12} = 5.3$, $p < 0.05$), and for both variables analysed simultaneously (MANOVA, $F_{2,11} = 23$, $p \ll 0.001$).

4. DISCUSSION
While the physiology and molecular biology of cancer are becoming well understood, the evolution of cancer remains less well known (Galis & Metz 2003). In this regard, studies of early-evolving animals can provide insight. A central innovation in the evolution of multicellularity is the appropriate downregulation of cell division (Szathmary & Wolpert 2003). Cancer is a general term for the abrogation of such downregulation. Nevertheless, animals such as cnidarians, which exhibit agametic, asexual reproduction and have active stem cells at all stages of the life cycle, show only modest endogenous downregulation of cell division. In this respect, are there fundamental differences between cnidarians and more derived animals?

Certainly, cnidarian cell division and differentiation seems highly responsive to environmental signals. A variety of environmental perturbations affect growth and the growth form of hydroids colonies (Dudgeon & Buss 1996; Buss 2001; Blackstone 2003). Aspects of the gastrovascular system may detect and integrate the environmental inputs and produce an altered pattern of system-level behaviour, ultimately transduced by local,
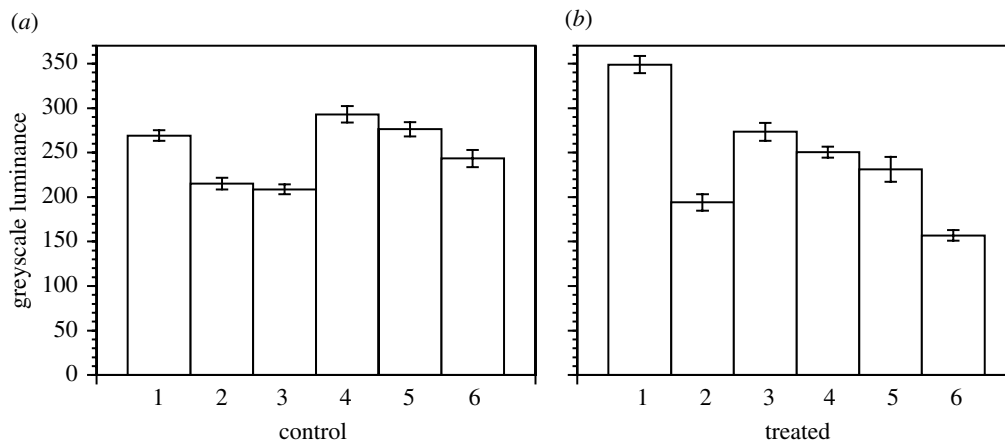
Figure 3. Mean $\pm$ s.e.m. luminance (greyscale from 0 to 4095) for three polyps per replicate colony. Native fluorescence of NAD(P)H is measured. (*a*) Control, (*b*) colonies treated with avicin extracts (10 μg ml^{-1}).

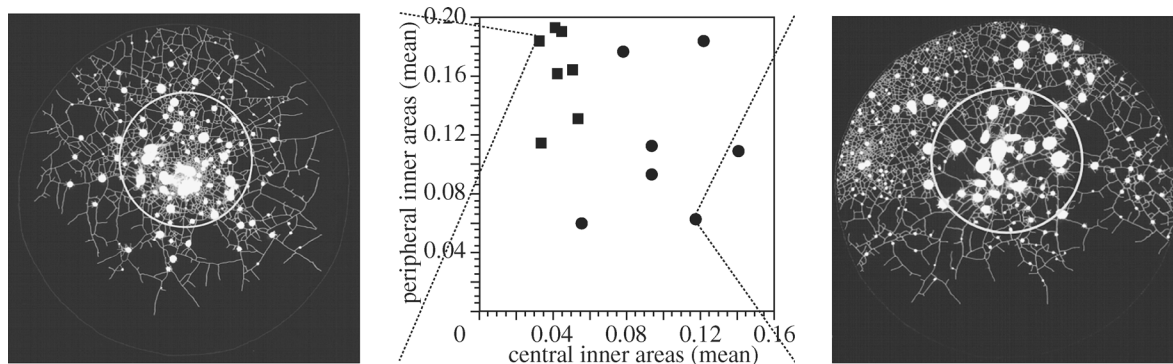

Figure 4. Each data point represents a colony of *P. carnea*; circles are treated colonies (avicin extracts at 10 μg ml^{-1}), and squares are the controls. Images of two highly divergent colonies are shown. Peripheral and central inner areas are distinguished by region of interest (shown on images by a circle) that was 30% of the total area.

cell-level receptors (Buss 2001). At least in some hydroids, regions of mitochondrion-rich EMCs may have a central role in such colony-wide responses (Blackstone *et al.* 2004). In the early-evolving animals, mitochondria may thus have a greater role as integrators of information and environmental signalling than in more derived animals. Nevertheless, the environmental perturbations that have been studied to date all show uniform effects, e.g. sheets are converted to runners or vice-versa, rather than differential effects, e.g. some regions of a colony become more sheet-like, while other regions become more runner-like.

Hydroids exhibit some interesting parallels to mammals in their response to avicins, a novel anti-cancer therapeutic treatment. In human cells, avicins cause a differential response, triggering apoptosis in cancer cells as well as activating a general stress response by redox regulation of Nrf2 (Haridas *et al.* 2004, submitted). In hydroid colonies, avicins diminish both oxygen uptake and ROS, the source of which appears to be mitochondrial (Blackstone *et al.* 2004). This is consistent with the decrease in both oxygen consumption and ROS (Haridas *et al.* 2004, submitted) seen in avicin-treated Jurkat cells, a human T-cell leukaemia line. Remarkably, the treatment of hydroid colonies with avicins causes a differential response between the central and peripheral regions of the colonies such that the central region becomes more runner-like, while the peripheral region becomes more

sheet-like. At this time, it is difficult to account for these differential morphological changes. Certainly, there are numerous central/peripheral differences in a hydroid colony, including among others age, density of endodermal vacuoles, cell division rates, elasticity, rate and direction of gastrovascular flow and density of mitochondrion-rich contractile regions.

Tissue morphogenesis is driven by integrating signals to determine cell fate and phenotype. Even cells close to each other can enter different mutually exclusive cellular states: proliferation, differentiation, quiescence or apoptosis. One or more of the above central/peripheral differences apparently contributes to spatial variation within a hydroid colony. For instance, the peripheral regions of a colony may be less metabolically active, since mitochondrion-rich EMCs are more abundant at the base of large polyps, which tend to be centrally located (figure 4). In peripheral regions, avicins may thus induce the regression of stolons via cell death, leading to greater branching and the conversion from a runner-like to a sheet-like morphology. On the other hand, in the metabolically active centre of the colony, containing many mitochondrion-rich EMCs, avicins may simulate low metabolic demand and cause mitochondria to enter the resting state. Such an effect has been shown to convert a sheet-like morphology to a runner-like one (Blackstone 2003). The data suggest that this differential effect is mediated by mitochondrial perturbations (as in human cells), and the avicins appear

to be acting as an oxidomimetic. Nevertheless, as with other patterning events (e.g. Coffman *et al.* 2004), the mechanisms by which mitochondria affect development remain obscure. Plausibly, both human cancer and normal cells differ in redox state, as do different regions in the hydroid colony. These differences in the initial conditions predicate the differential response to avicins, i.e. apoptosis versus cytoprotective effect on human cells, and branching or lack of branching in hydroid colony regions. These differential effects on growth may be hallmarks of effective anti-cancer therapeutics in general.

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