Cellular kinetics in growth anomalies of the scleractinian corals *Porites australiensis* and *Montipora informis*

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ABSTRACT: Growth anomalies (GAs) in corals are characterized by morphological abnormalities of the skeleton as well as polyps and coenosarcs. GAs commonly appear as protuberances with fewer polyps and are paler in color due to decreased zooxanthellae density. To test the hypothesis that morphological anomalies in GAs may be caused by unregulated cellular kinetics, the relative abundances of apoptotic cells and proliferating cells were compared between GAs and apparently healthy regions in 2 corals, *Porites australiensis* and *Montipora informis*. Apoptotic cells and proliferating cells were detected using TUNEL assays and BrdU incorporation assays, respectively. The labeling indices for apoptotic nuclei and BrdU-labeled nuclei were measured in the epidermis, oral gastrodermis, aboral gastrodermis, and calicodermis. The labeling index for apoptotic nuclei in the oral gastrodermis and the calicodermis was significantly lower in GAs than in healthy regions in both coral species. The index for BrdU-labeled cells in the calicodermis was significantly higher in GAs than in healthy regions in both coral species. When GA regions partially died, the GA tissues directly adjacent to the dead areas exhibited signs of necrosis, although some apoptotic cells were also present. Healthy oral gastrodermis adjacent to the border between the healthy and GA regions exhibited higher frequencies of apoptotic cells. These results suggest that apoptotic pathways were suppressed and cell proliferation was promoted in GA regions, although cells in GAs may die through both necrosis and apoptosis.

KEY WORDS: Apoptosis · Necrosis · Cell proliferation · Coral disease · Growth anomaly · *Porites australiensis* · *Montipora informis*

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INTRODUCTION

Growth anomaly (GA) is a common and easily identifiable disease that has been reported for hydrozoans, gorgonians, and scleractinian corals (reviewed by Sutherland et al. 2004). GAs in scleractinian corals have been observed on reefs throughout the Caribbean Sea and the Indo-Pacific Ocean (e.g. Sutherland et al. 2004, Aeby et al. 2011) and from shallow waters (0.5 m; Aeby et al. 2011) to the deep sea (635 m; Squires 1965). GAs in scleractinian corals commonly manifest as protuberant shapes with few or no corallites/polyps and abnormal corallite/polyp structures (Yamashiro et al. 2000, Work et al. 2008,

Yasuda et al. 2012), and in most cases, the zooxanthellae density is low compared to healthy regions of host colonies (Domart-Coulon et al. 2006, Work et al. 2008, Yasuda et al. 2012).

Histological studies have described several remarkable structural anomalies of GA tissues, including degenerated polyps or complete lack of polyp structures (Yamashiro et al. 2000, Work et al. 2008, Burns & Takabayashi 2011), no or reduced mesenterial filaments (Peters et al. 1986, Work & Rameyer 2005, Burns & Takabayashi 2011), and a hyperplastic basal body wall (Work et al. 2008, Burns & Takabayashi 2011, Williams et al. 2011). Furthermore, Domart-Coulon et al. (2006) demonstrated that the proteins

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MutY, Hsp90a1, GRP75, and metallothionein, which are hyperplasia-associated proteins, were upregulated in GAs in *Porites compressa*. Their results also indicate hyper-proliferation of cells in GA tissues. However, the mechanism and the process of cellular kinetics in GA regions are not yet understood.

The etiology of GA is still unclear, although relationships between GA prevalence and several factors have been suggested, including UVB radiation (Peters et al. 1986, Coles & Seapy 1998), nutrients and organic carbon (Kaczmarek & Richardson 2010), water temperatures and photosynthetically active radiation (Stimson 2010), water turbidity and depth (Williams et al. 2010), host density and human population size (Aeby et al. 2011), and aging (Irikawa et al. 2011). GA regions are frequently associated with microorganisms in the tissue as well as skeletal space (Coles & Seapy 1998, Work & Rameyer 2005, Work et al. 2008), and the microorganisms have been suggested to be involved in GAs (Work & Rameyer 2005, Domart-Coulon et al. 2006). However, microorganisms are not always detected in GAs (Yamashiro et al. 2000, Burns & Takabayashi 2011), and the linkages between their presence and GA development are currently unknown.

GA-affected corals show deteriorated physiological states, such as low fecundity in *Montipora* spp. (Yamashiro et al. 2000, Burns & Takabayashi 2011), *Acropora* spp. (Irikawa et al. 2011), and *Porites* spp. (Hunter & Field 1997, Domart-Coulon et al. 2006, Yasuda et al. 2012), low lipid storage in *M. informis* (Yamashiro et al. 2001), and low photosynthetic productivity in *A. cytherea* and *P. australiensis* due to reduced numbers of zooxanthellae in the gastrodermis (Irikawa et al. 2011, Yasuda et al. 2012). However, previous field investigations demonstrated that GAs progressively spread toward healthy tissue on the host colonies (Cheney 1975, Bak 1983, Irikawa et al. 2011, Yasuda et al. 2012). Healthy fragments grafted to GA regions or fused with GA fragments exhibited reduced growth rates compared with non-grafted healthy fragments (Stimson 2010, Yasuda et al. 2012). These findings suggest that GAs grow utilizing nutrient supplies from surrounding healthy regions (Yamashiro 2001 et al., Domart-Coulon et al. 2006, Yasuda et al. 2012). However, the processes leading to GA progression, i.e. how GA tissues invade surrounding healthy regions, have never been investigated.

The partial death of GAs on coral colonies has been reported at gross and microscopic levels (Work et al. 2008, Irikawa et al. 2011, Williams et al. 2011, Yasuda et al. 2012). The microscopic studies revealed that tissue necrosis of GAs are often, though not always,

associated with infiltrating microorganisms such as algae and fungi (Work & Rameyer 2005, Work et al. 2008, Williams et al. 2011). However, the cause of the necrosis in GA is not yet fully understood.

In this study, we histologically assessed cellular kinetics in apparently healthy and GA tissues in 2 scleractinian corals, *Porites australiensis* and *Montipora informis*, whose GAs have been well studied morphologically and physiologically (e.g. Yamashiro et al. 2000, 2001, Yasuda et al. 2012). We compared the relative abundances of apoptotic cells and proliferating cells between healthy and GA tissues using the terminal deoxynucleotidyl transferase dUTP-biotin nick-end labeling (TUNEL) and 5-bromo-2-deoxyuridine (BrdU) incorporation assays, respectively. We also made histological observations of the interface between healthy and GA regions, and GAs directly adjacent to dead areas, to clarify the processes of progression of GAs and cause of necrosis in GAs.

MATERIALS AND METHODS

Sample collection

Pieces of 3 to 10 cm square (9 to 100 cm²) were collected from each of the following regions: apparently healthy, GAs, interface between healthy and GA regions, and GAs directly adjacent to the dead area of each of 4 *Porites australiensis* colonies (n = 4 for each region). Two of the 4 colonies were manifesting multiple GAs and an additional 2 to 3 pieces of interface region and GA adjacent to the dead area were sampled from different GA regions and used as supplementary materials for histology and the TUNEL assay. These additional samples were used only for observation and were not included in the statistical analyses. Pieces of similar size were collected from apparently healthy, GA, and interface regions of each of 3 *Montipora informis* colonies (n = 3 for each region). The pieces were collected using a hammer and chisel at a depth of 2 to 3 m in the back reef moats of Kayo (26° 33' N, 128° 08' E) and Odo (26° 05' N, 127° 42' E) on Okinawa Island, Japan. Collected samples of *P. australiensis* from Kayo and samples of *P. australiensis* and *M. informis* from Odo were brought to the laboratory and fixed for histological observations and TUNEL assays within 3 h. The samples were cut into <1 cm² pieces and fixed in 10% neutral buffered formalin (pH 7.4). The remaining samples for BrdU incorporation assays were transported to a flow-through water tank at Sesoko Station on the same day.

Histological observations

Fixed specimens of both corals were trimmed into 5 × 5 mm square pieces after decalcification in 10% EDTA in phosphate buffered saline (pH 7.8). They were dehydrated in a graded series of ethanol and embedded in Paraplast plus (Sigma P3683). Serial longitudinal sections 5 µm thick were cut and stained with hematoxylin and eosin.

Detection of apoptotic cells

To detect free 3'-ends of DNA fragments, histological sections from each paraffin-embedded specimen were processed for TUNEL assays using an ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection kit (Chemicon S7111). The sections were also stained with 4', 6-diamidino-2-phenylindole (DAPI) to visualize all nuclei in the histological sections. The sections were observed under a fluorescent microscope (Nikon Optiphot-2) using a blue excitation filter (wave length: 495 nm) for anti-digoxigenin-fluorescein-labeled (TUNEL-positive) nuclei or an ultraviolet filter (wave length: 365 nm) for nuclei counterstained with DAPI. Photomicrographs of the same areas were taken under blue and then UV excitation using a digital camera (Nikon Digital Sight DS-L1).

In order to quantitate the percentage of apoptotic cells in each region of 2 coral species, the labeling index (LI) for TUNEL-positive cells was calculated by dividing the number of the fluorescein-labeled nuclei by the total number of nuclei stained with DAPI. Three histological sections were assayed for each specimen. Every third section was selected from serial sections of the specimen so that the 3 sections assayed were distant from each other by at least 15 µm. Three photographs were taken for each of the oral and aboral regions per histological section. Areas mostly occupied by the oral or aboral regions were photographed without overlap. In total, 18 photographs (each photographed area $5.1 \times 10^4 \mu\text{m}^2$) were used for measurements of the number of TUNEL-positive cells for each specimen, and average LI was calculated for each of epidermis, oral gastrodermis, aboral gastrodermis, and calicodermis. Dense apoptotic signals were occasionally observed in some regions. In such cases, the signals appeared as smears over a small area, and counting the number of TUNEL-positive nuclei was difficult. Therefore, dense signal areas were not included in the LI measurements.

Detection of proliferating cells

A thymidine analog, BrdU incorporated cells were immunohistologically detected as proliferating cells in each specimen. Samples of apparently healthy regions and samples containing both apparently healthy and GA regions of *Porites australiensis* and *Montipora informis* (n = 3 each for healthy and GA regions) were trimmed into approximately 1 × 1 cm square pieces and acclimatized for 1 wk in an indoor tank with running seawater that was exposed to natural sunlight. The samples were then placed individually in closed chambers filled with 300 ml of 100 µM BrdU in filtered seawater, and incubated with aeration for 3 d. These chambers were placed in a thermostatic bath at 25°C under light (approximately 400 µmol m⁻² s⁻¹) from a metal halide lamp (Dupla Electra) for 16 h (8:00–24:00 h) per day. The BrdU solution was changed daily, and the chamber positions were changed randomly every day. After incubation, the specimens were rinsed 3 times in filtered seawater with aeration each for 10 min. The specimens were fixed, decalcified, and processed for histology as described above.

Histological sections were processed for the incorporated BrdU assay using a BrdU *In Situ* Detection kit (BD Pharmingen™) and a Streptavidin/Biotin blocking kit (Vector Laboratories). Hematoxylin was used to stain all nuclei in the histological sections.

In order to quantitate proliferating cells in each region of the 2 coral species, photomicrographs were taken using a digital camera (Nikon Digital Sight DS-L1), and the LI for BrdU-labeled cells was calculated by dividing the number of BrdU-labeled cells that were stained with diaminobenzidine (DAB) by the total number of nuclei stained with hematoxylin. Three histological sections were assayed for each specimen as described for the TUNEL assay. Three photographs were taken for each of the oral and aboral regions per histological section. In total, 18 photographs (each photographed area $5.5 \times 10^4 \mu\text{m}^2$) were used for measurements of each specimen, and the average LI was calculated for each of epidermis, oral gastrodermis, aboral gastrodermis, and calicodermis, as well as for zooxanthellae.

Statistical analysis

The mean LI (percent data) of TUNEL-positive cells and BrdU incorporated cells in each tissue were compared between the apparently healthy and GA regions in both coral species. As the data did not

meet parametric requirements (Shapiro-Wilk test and F -test), Welch's t -test, which does not require equal variance, or the non-parametric Mann-Whitney U -test were conducted. All statistical analyses were performed using R Ver. 2.9.1.

RESULTS

Histological observations

Polyps in the apparently healthy region of *Porites australiensis* exhibited normal columnar tentacles (Fig. 1A), with abundant zooxanthellae in the gastrodermis. Mesenterial filaments and the gastrovascular canals were longitudinally arranged in the aboral region (Fig. 1B). Filamentous algae or fungal hyphae were observed in the skeletal space, but were mostly

limited to under the basal floor (Fig. 1B). In contrast, polyps in the GA region of *P. australiensis* exhibited swollen tentacles. The epidermis of some neighboring tentacles fused to form a flattened surface at the tip (Fig. 1C). When 2 neighboring tentacles fused, the epidermis of the column region disappeared at the interface, leaving the gastrodermis shared by 2 tentacles. In such cases, the tentacular gastrodermis contained swollen and hyperplastic mucocytes, and fused tentacles appeared as one large tentacle. In some cases, the epidermis of several tentacles became partly continuous. When 2 neighboring tentacles touched each other but did not fuse, aggregations of spirocytes and other epidermal cells were observed at the surface of the tentacles (Fig. 1D). The oral gastrodermis contained fewer zooxanthellae compared with healthy polyps. Mesenterial filaments were less developed and sporadically distributed in

Fig. 1. *Porites australiensis*. Photomicrographs of hematoxylin and eosin stained longitudinal sections of healthy and growth anomaly (GA) regions. (A) Columnar tentacles of a healthy polyp. (B) Aboral tissue in healthy region; note mesenterial filaments (arrows). Filamentous algae/fungi (arrowhead) infiltrated in the skeletal space beneath the basal floor. (C) Fused tentacles of a polyp in GA region; the epidermis of the neighboring tentacles is fused and forms a flattened surface. (D) Tips of 2 tentacles touching each other; note aggregates of spirocytes containing eosinophilic spirocysts (stained pink) and proliferated epidermal cells beneath them. (E) Aboral tissue in GA region; note sporadic distribution of less developed mesenterial filaments (arrow), enlarged and disorganized gastrovascular canals (*), and dense infiltration of filamentous algae/fungi (arrowhead) in the skeletal space. Ep: epidermis; Sp: spirocytes. Scale bar = 100 μ m in A and C; 50 μ m in D; 500 μ m in B and E

the aboral region of the GA (Fig. 1E). The aboral region was mainly composed of disorganized gastrovascular canals (Fig. 1E). The coenosarc in the GA region swelled and protruded to almost the same level as polyps (data not shown). Dense filamentous algae or fungi were observed throughout the skeletal space in the GA region, and they sometimes infiltrated even into the oral regions of the GA. Necrotic tissue which manifested as clusters of lysed cells without intact plasma membranes were observed adjacent to the accumulation of organic matrix components in both oral and aboral regions at the interface between GA and dead regions of *P. australiensis* (data not shown). The occurrence of necrotic tissue was often associated with filamentous algae/fungi.

Detection of apoptotic cells

Apoptotic nuclei appeared as bright green dots against the yellow autofluorescence of the host tissue under blue excitation (wave length: 495 nm), while all nuclei stained with DAPI exhibited blue fluorescence under UV excitation (Fig. 2A,B). Symbionts appeared bright to dull orange or were non-fluorescent and showed no apoptotic signals in the present assay (Fig. 2A, C, D). No TUNEL-positive signal was detected in the negative control slides that had been incubated with deionized water instead of TdT enzyme (image not shown).

TUNEL-positive nuclei were few in both oral and aboral regions of apparently healthy tissues of *Porites australiensis* (Fig. 2A, C). However, TUNEL-positive nuclei were occasionally observed in the oral gastrodermis in the tentacles (Fig. 2D) or mesenterial filaments of apparently healthy polyps at the interface between healthy and GA regions (5 of 7 interface regions observed). In contrast, they were rarely detected in the aboral regions of polyps at the interface.

TUNEL-positive nuclei were also rare in the oral and aboral regions in the GA regions of *Porites australiensis*. However, many labeled nuclei were observed in the cells exhibiting a fibrous structure near the site where numerous spirocytes aggregated in the tentacles of GA polyps (Fig. 2E; 4 of 4 specimens). GAs facing dead areas generally exhibited no apoptotic signals (Fig. 2F; 5 of 6 regions), although in one case, small numbers of apoptotic signals were detected both in the surface and basal body walls. The apoptotic cells were associated with dense infiltration of filamentous algae/fungi. Chromophore cells, which have been described in GAs of *P. compressa* (Domart-Coulon et al. 2006), were occasion-

ally observed as amoeboid or round cells with bright yellow autofluorescence in both apparently healthy and GA regions (Fig. 2A,E,F).

Few TUNEL-positive nuclei were observed in both oral and aboral regions of apparently healthy regions of *Montipora informis* (Fig. 3A, B). TUNEL-positive cells were also rare in both oral and aboral regions of the GAs (Fig. 3C–E). In some oral regions of GAs, the surface of epidermis was fused leaving the epidermis laid between 2 layers of gastrodermis (Fig. 3C). This type of epidermis with oral gastrodermis on both sides was observed in all GA samples. In other oral regions in the GAs, fewer vacuolated mucus secretory cells were observed in the epidermis, and the gastrodermis contained orange autofluorescent granules but few zooxanthellae (Fig. 3E). In the aboral regions of GAs, thinner basal body walls containing numerous orange granules in the aboral gastrodermis were visible (Fig. 3D). Filamentous algae/fungi occasionally invaded the skeletal space of both apparently healthy and GA regions. Aggregations of labeled nuclei were occasionally seen in the mesenterial filaments of apparently healthy polyps at the interface between healthy and GA regions (Fig. 3F; 3 of 3 specimens). They were rare in the aboral region at the interface between apparently healthy and GA regions.

Significant differences in apoptotic cells were found between healthy and GA regions in some tissues but not in all 4 tissues ($p < 0.05$, Fig. 4). In *Porites australiensis*, the LI values for TUNEL-positive cells in the oral gastrodermis and the calicodermis in apparently healthy regions were significantly higher than those in the GAs (Mann-Whitney *U*-test, $p < 0.05$; Fig. 4A). In *Montipora informis*, the LI values of TUNEL-positive cells of the surface body wall (epidermis and gastrodermis) and the calicodermis in apparently healthy regions were significantly higher than those in the GAs (Welch's *t*-test, $p < 0.05$; Fig. 4B).

Detection of proliferating cells

Host and symbiont nuclei with incorporated BrdU were scattered in both healthy and GA tissues in *Porites australiensis* and *Montipora informis* (Fig. 5). BrdU incorporated chromophore cells were occasionally seen in both apparently healthy and GA regions of *P. australiensis*. Clusters of BrdU incorporated cells were not observed except in gonads in the apparently healthy tissue of *M. informis*. No BrdU incorporated cells were detected in the negative control

Fig. 2. *Porites australiensis*. Photomicrographs of TUNEL-assayed longitudinal sections of healthy, interface, and growth anomaly (GA) regions. Sections were observed under blue excitation (495 nm) except in B, where DAPI-stained nuclei under UV excitation are shown. Green fluorescence and blue fluorescence represent TUNEL-positive signals and DAPI-stained nuclei, respectively. (A) Oral tissue in healthy region, visualized by TUNEL assay. (B) DAPI-stained nuclei of the same area shown in A. (C) Aboral tissue in healthy region. (D) Tentacle of a polyp at the interface between healthy and GA regions; note many TUNEL-positive nuclei in the oral gastrodermis. (E) Tentacle tip of a polyp in the GA region; note the aggregation of TUNEL-positive nuclei in the epidermis under a cluster of spirocytes containing spirocysts (orange fluorescent ellipses). (F) Oral tissue in GA region facing a dead area; note lysed cells and organic matrix components associated with filamentous algae/fungi (arrowhead). Ch: chromophore cells; Ep: epidermis; Gvc: gastrovascular canal; Og: oral gastrodermis; Omc: organic matrix components; Sk: skeletal space; Sp: spirocytes; Z: zooxanthella. Scale bar = 50 µm

slides that had been incubated with phosphate-buffered saline instead of the anti-BrdU antibody (images not shown).

The percentage of proliferating host cells (BrdU incorporated host cells) was higher in GA regions than in apparently healthy regions, with significant differences in 2 of 4 tissue layers in *Porites australiensis* and 3 of 4 tissue layers in *Montipora informis* (Welch's *t*-test, $p < 0.05$, Fig. 6). The LI values for BrdU incorporated host nuclei in the basal body wall (aboral gastrodermis and calicodermis) in GA regions were significantly higher than those in apparently healthy regions of *P. australiensis* (Welch's *t*-test, $p < 0.05$; Fig. 6A). The LI values for BrdU incorporated nuclei of host cells of the surface body wall (epidermis and oral gastrodermis) and calicodermis were significantly higher in GA regions than those in apparently healthy regions of *M. informis* (Welch's *t*-test, $p < 0.05$; Fig. 6B). The LI values for BrdU-labeled nuclei of zooxanthellae were similar in both healthy and GA tissues in both *P. australiensis* and *M. informis*.

DISCUSSION

Anomalies of skeletal and polyp structures in GA regions

GAs in *Porites australiensis* and *Montipora informis* exhibited protuberant shapes due to newly-developed coenosteum/coenosarc tissue. While polyp structures were more or less retained in GAs in *P. australiensis*, they were apparently absent due to the newly-developed coenosteum/coenosarc tissue in GAs of *M. informis*. These findings are consistent with previous observations of GAs in other *Porites* spp. (Hunter & Field 1997, Domart-Coulon et al. 2006, McClanahan et al. 2009, Stimson 2010, Yasuda et al. 2012) and in other *Montipora* spp. (Yamashiro et al. 2000, Burns et al. 2011, Williams et al. 2011).

Our histological study of GAs in 2 coral species detected the common histopathological hallmarks in GA tissue across coral genera, viz. no or a reduced number of polyps, deformed polyp structure with disorganized mesenterial filaments, and hyperplastic

Fig. 3. *Montipora informis*. Photomicrographs of TUNEL-assayed longitudinal sections of healthy, growth anomaly (GA), and interface regions from sections under blue excitation (495 nm). Green fluorescence represents TUNEL-positive signal. (A) Oral tissue in healthy region. (B) Aboral tissue in healthy region. (C) Oral tissue in GA region; note the fused epidermis with few TUNEL-positive nuclei but with a number of vacuoles. (D) Aboral tissue in GA region; note aboral gastrodermis containing orange autofluorescent granules instead of zooxanthellae, and thinner basal body wall. (E) Oral tissue in GA region; note fewer vacuolated mucocytes in the epidermis and cells filled with autofluorescent granules (orange particles) in the oral gastrodermis. (F) Oral tissues at the interface between healthy and GA region; note many TUNEL-positive nuclei in the apparently healthy mesenterial filament. Ep: epidermis; Gvc: gastrovascular canal; Mf: mesenterial filament; Og: oral gastrodermis; Sk: skeletal space; Z: zooxanthella. Scale bars = 50 μ m

basal body walls and/or gastrovascular canals (Peters et al. 1986, Yamashiro et al. 2000, Work & Rameyer 2005, Domart-Coulon et al. 2006, Work et al. 2008, Burns & Takabayashi 2011, Williams et al. 2011). In addition, our study revealed the histological process leading to morphological abnormalities in GA regions. In oral regions of GAs in *Porites australiensis*, the epidermis of some neighboring tentacles fused to form a flat surface at the tips of the tentacles, while the epidermis of the column region of the tentacles at the interface disappeared, leaving the gastrodermis alone. This observation suggests that large tentacles characteristic of GAs in *P. australiensis* are formed by fusion of neighboring tentacles. Furthermore, aggregations of numerous spirocytes were observed at the surface of the tentacles that contacted other tentacles or the coenosarc. The spirocyst functions mainly for substrate attachment in cnidarians (Mariscal et al. 1977, Goldberg & Taylor 1996). Therefore, the proliferation and aggregation of spirocytes at the tentacles might imply enhanced adhesion of tentacles to other tentacles and coenosarc in the GA region. This might

be an initial step of fusion of the epidermis of neighboring tissues. Such aggregations of spirocytes in tentacles were not observed in either healthy or GA regions in *Montipora informis*. However, fusion of the epidermis of neighboring tentacles or fusion between tentacles and coenosarcs was frequently observed in *M. informis*, and eventually the whole GA-affected area was overlaid by the flat epidermal layer. Yamashiro et al. (2000) also observed that polyps in GA regions in *M. informis* became deteriorated and partially buried. It is likely that fusion of protruded coenosarc epidermis leads to the burial of polyps by the proliferated coenosarc tissue, and finally to atrophy or absorption of polyps.

Our study revealed that anomalies in the architecture of basal body walls and gastrovascular canals corresponded to the deformations in the skeletal architectures described in GAs of *Porites australiensis* (Yasuda et al. 2012). Hyperplasia of the basal body walls and gastrovascular canals manifest as common histopathological signs in *P. australiensis* and *Montipora informis* as in other Acroporidae corals (Peters

Fig. 4. (A) *Porites australiensis* and (B) *Montipora informis*. Labeling index (LI) of TUNEL-positive cells in healthy and growth anomaly (GA) regions. The LIs of TUNEL-positive cells in the epidermis, oral gastrodermis, aboral gastrodermis, and calicodermis are shown. Means $\pm$ SD ($n = 4$ in A and $n = 3$ in B). Asterisks indicate significant differences in A (Mann-Whitney U -test, $p < 0.05$) and B (Welch's t -test, $p < 0.05$). Black columns: LI value of the healthy region; white columns: LI value of the GA region

et al. 1986, Work & Rameyer 2005, Work et al. 2008, Burns & Takabayashi 2011, Williams et al. 2011) and *Porites* spp. (Domart-Coulon et al. 2006, Williams et al. 2011). These observations strongly suggest that hyperplasia of the basal body wall including the calicodermis and gastrovascular cavity may be related to skeletal anomalies such as porous skeletal structures, low skeletal densities, and protuberant shapes of GA regions.

Cellular kinetics in GA and healthy regions

The relative abundance of TUNEL-positive (apoptotic) cells was generally lower in GA regions than in apparently healthy regions in both *Porites australiensis* and *Montipora informis*. The difference was sig-

nificant in the oral gastrodermis and calicodermis in both species. In contrast, the percentage of proliferating cells, which was expressed by LI values of BrdU incorporated cells, was generally higher in GA regions than in apparently healthy regions in both species. Significant differences were found in the calicodermis in both species. These results suggest that apoptotic pathways are suppressed, whereas cell proliferation was promoted in GA regions, especially in the calicodermis. Reduced apoptosis and accelerated proliferation of calicodermal cells may cause the hyperplastic basal body walls and the skeletal anomalies. The epidermis in GAs of *M. informis*, which showed a remarkable decrease in apoptotic cells and accelerated cell proliferation, exhibited more compromised homeostatic cell turnover than that of *P. australiensis*. This might explain why GAs of *M. informis* exhibited more conspicuous changes in the gross morphology compared to the GAs of *P. australiensis*.

At the interface between healthy and GA regions, both oral and aboral body walls were continuous between the 2 regions, as reported previously (Peters et al. 1986, Yamashiro et al. 2000, Work et al. 2008). The oral gastrodermis and mesenterial filament of apparently healthy polyps located at the interface between healthy and GA regions showed higher frequencies of apoptotic cells in both coral species. This might indicate the initiation of degeneration of the polyps, and possibly the collapse of the symbiosis with zooxanthellae at the interface region. Whether apoptotic cell death in healthy regions is induced by the GA to facilitate invasion into the healthy region, or whether cells in the interface region undergo apoptotic death to protect the healthy tissue against invasion by the GA remains to be determined.

GA tissues adjacent to dead areas exhibited extensive necrosis in *Porites australiensis*. The necrotic tissue observed in the GA of *P. australiensis* was similar to diffuse necrosis described in *Acropora* spp. (Work et al. 2008). Initiation of necrosis has been reported to start at the region far from the GA progression area (Kaczmarek 2006, Irikawa et al. 2011, Yasuda et al. 2012). Even though GAs are supported by nutritional supplies from surrounding healthy tissues, the compromised gastrovascular canals of GAs and the increased distance from healthy tissues may cause malnutrition in a GA (Domart-Coulon et al. 2006, Work et al. 2008, Yasuda et al. 2012). Malnutrition in GAs has been supported by various observations such as low lipid storage (Yamashiro et al. 2001) and low productivity (Irikawa et al. 2011, Yasuda et al. 2012) in the GAs. Necrosis is caused via biochemical

Fig. 5. *Montipora informis*. Assay for BrdU incorporated nuclei in healthy and growth anomaly (GA) regions. Brown dots indicate BrdU incorporated nuclei, while nuclei counterstained by hematoxylin appear as blue-purple dots. (A) Oral tissue in healthy region. (B) Aboral tissue in healthy region. (C) Oral tissue in GA region. (D) Aboral tissue in GA region. Ep: epidermis; Gvc: gastrovascular canal; Og: oral gastrodermis; Sk: skeletal space; Z: zooxanthella. Scale bars = 50 µm

pathways activated by severe depletion of cell energy stores (Denecker et al. 2001, Rana et al. 2001). Thus, the lack of energy in GA regions might lead to spontaneous necrosis. Depletion of energy might deteriorate resistance of corals against stressors such as invasions by endolithic algae, fungi, and sponges. The necrotic tissue was frequently, but not always, accompanied by invasive microorganisms as in other GAs (Work & Rameyer 2005, Work et al. 2008, Williams et al. 2011). Further histological examination is required to understand whether the presence of invasive microorganisms is the cause or consequence of necrosis in GAs.

Cell types of some of the TUNEL- or BrdU-labeled cells were estimated based on the present observation. The epidermal cells located at the base of the spirocytes exhibited apoptotic signals in TUNEL assays of the GAs of *Porites australiensis*. Since the apoptotic cells that were in close proximity to aggregated spirocytes exhibited a fibrous structure, the apoptotic cells were presumed to be neurons connecting to spirocytes. The discharge of spirocysts is controlled by neuro-spirocyte synaptic pathways in sea anemones (e.g. Westfall et al. 1999). We also detected the BrdU incorporated cells, which were amoeboid or round and emitted bright yellow autofluorescence at 495 nm excitation, throughout apparently healthy and GA regions of *P. australiensis*. These morphological and optical features suggest that these BrdU-labeled cells correspond to the chromophore cells described in GAs of *Porites compressa*

(Domart-Coulon et al. 2006). Infiltration of the cells similar to the chromophore cells were also documented in non-normally pigmented regions and wound healing regions in massive *Porites* sp. (Palmer et al. 2008, 2011). Infiltration of chromophore cells or chromophore-like cells has been considered as part of the innate immune system in *Porites* (Domart-Coulon et al. 2006, Palmer et al. 2008, 2011). Similarly, in other corals, amoeboid cells termed amoebocytes have been suggested to be involved in the inflammatory response or wound healing response (e.g. Vargas-Ángel et al. 2007, Mydlarz et al. 2008). Cells containing orange autofluorescent granules under blue excitation were also observed in the gastrodermis of GAs in *Montipora informis* in this study, though their biological function was not clear.

CONCLUSIONS

Our results suggest that apoptotic pathways are suppressed while cell proliferation is stimulated in GAs of the 2 coral species. This anomalous pattern of homeostatic balance of cell death and proliferation was observed in the calicodermis in *Porites australiensis*, and in both the surface body wall and calicodermis in *Montipora informis*. The compromised homeostatic cell turnovers may lead to the common signs of GAs, that is, anomalous skeletal structures, degeneration of the polyps, hyperplasia of gastrovascular canals, and reduced algal densities. The fusion

Fig. 6. (A) *Porites australiensis* and (B) *Montipora informis*. Labeling index (LI) of BrdU incorporated cells in healthy and growth anomaly (GA) regions. The LIs of BrdU incorporated cells in the epidermis, oral gastrodermis, aboral gastrodermis, and calicodermis, and in zooxanthellae are shown. Means $\pm$ SD ($n = 3$ in both A and B). Asterisks indicate significant differences (Welch's *t*-test, $p < 0.05$). Black columns: LI value of the healthy region; white columns: LI value of the GA region

of the epidermis might lead to formation of large tentacles in *P. australiensis* and to burial of polyps within the coenosarc in *M. informis*. High frequencies of apoptotic cell death were observed in the oral gastrodermis and mesenterial filaments in the healthy regions adjacent to the GA region. This might indicate the initiation of atrophy of the polyps at the interface region, and might facilitate GA invasion into the healthy region. Extensive necrosis, which might be caused by nutrient depletion, was observed in areas of *P. australiensis* GAs that were distant from the progression region.

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