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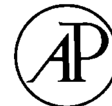

CELL RENEWAL AND APOPTOSIS IN *MACROSTOMUM* SP. [*LIGNANO*]

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In platyhelminths, all cell renewal is accomplished by totipotent stem cells (neoblasts). Tissue maintenance is achieved in a balance between cell proliferation and apoptosis. It is known that in *Macrostomum* sp. the epidermis undergoes extensive cell renewal. Here we show that parenchymal cells also exhibit a high rate of cell turnover. We demonstrate cell renewal using continuous 5′bromo-2-deoxyuridine (BrdU) exposure. About one-third of all cells are replaced after 14 days. The high level of replacement requires an equivalent removal of cells by apoptosis. Cell death is characterized using a combination of three methods: (1) terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), (2) specific binding of phosphatidylserine to fluorescent-labelled annexin V and (3) identification of apoptotic stages by ultrastructure. The number of cells observed in apoptosis is insufficient to explain the homeostasis of tissues in *Macrostomum*. Apoptosis-independent mechanisms may play an additional role in tissue dynamics. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: apoptosis; BrdU; cell cycle; *Macrostomum*; platyhelminths; programmed cell death; stem cells; ultrastructure; TUNEL.

INTRODUCTION

Programmed cell death is known to play an important role in the development of all organisms that have been studied to date (Kerr *et al.*, 1972; Farber, 1994; Jacobson *et al.*, 1997; Vaux and Korsmeyer, 1999; Meier *et al.*, 2000; Ranganath and Nagashree, 2001). Much progress has been made in elucidating the molecular mechanisms and proteins involved (Hale *et al.*, 1996; Green, 1998; Bergmann *et al.*, 1998). Among invertebrates, the genetic aspects of programmed cell death are well known from studies on *Caenorhabditis elegans* (Metzstein *et al.*, 1998; Fraser, 1999; Liu and Hengartner, 1999), *Drosophila* (Vernooy *et al.*, 2000; Bangs and White, 2000), leech (Tsubokawa and Wedeen, 1999) and *Hydra* (Cikala *et al.*, 1999). Nothing is known about apoptosis in free-living or parasitic platyhelminths. They possess a unique, probably totipotent stem cell system. Neoblasts are small basophilic cells with a high nucleocytoplasmic ratio. They show the capacity for permanent renewal and are able to differentiate into most, if not all, cell types (Baguñà, 1981; Ladurner *et al.*, 2000).

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Investigations on regeneration in planarians have shown that neoblasts are totipotent and migratory cells (Baguñà *et al.*, 1989; Cebrià *et al.*, 1997). In macrostomids, neoblasts are known to play a crucial role in development of the musculature (Rieger *et al.*, 1994; Ladurner and Rieger, 2000) and in regeneration (Salvenmoser *et al.*, 2001). As neoblasts are the only cells in *Macrostomum* sp. that can divide and differentiate (Ladurner *et al.*, 2000), they can be identified by labelling mitotic and S-phase cells by incorporation of the thymidine analogue BrdU (Ladurner *et al.*, 2000; Newmark and Sanchez Alvarado, 2000). In *Macrostomum* sp. S-phase cells are distributed in lateral bands along the longitudinal nerve cords (see Ladurner *et al.*, 1997 for nervous system of macrostomids). No S-phase cells are found anterior to the eyes after a 30 min BrdU pulse (Ladurner *et al.*, 2000). In contrast, there seems to be no correlation between the distribution of S-phase cells and the nervous system in the acoel

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Convolutriloba longifissura, probably due to the special mode of asexual reproduction in this species (Gschwentner *et al.*, 2001).

In various taxa such as planarians, macrostomids and acoels, neoblasts have been shown to migrate and differentiate (Ladurner *et al.*, 2000; Newmark and Sanchez Alvarado, 2000; Gschwentner *et al.*, 2001). Extensive cell renewal has been observed in all these species. *Macrostomum* sp. (Ladurner *et al.*, 2000) has about 25,000 cells, of which up to 20% can be considered to be neoblasts or neoblasts in differentiation. Approximately 500 S-phase cells are found in two bands along the lateral sides of the animal after a 30 min BrdU pulse. No proliferating cells are found in the region anterior to the eyes (the rostrum). Pulse chase experiments show migration towards the central body region and into the rostrum. Approximately one-third to one quarter of epidermal cells are renewed after 14 days. This high cell turnover requires a complementary loss of cells, either by programmed cell death or apoptosis-independent mechanisms.

In this study, we demonstrate cell renewal using BrdU labelling of S-phase neoblasts and screening with the TUNEL technique, annexin labelling and electron microscopy, in order to provide evidence for apoptosis and thus give a better understanding of the cell dynamics in *Macrostomum* sp. Representatives of basal free-living platyhelminths have proven to be particularly convenient organisms for studying cell differentiation and the development and organization of muscle (Rieger *et al.*, 1994; Ladurner and Rieger, 2000; Salvenmoser *et al.*, 2001) and nervous systems (Reuter *et al.*, 1995; Ladurner *et al.*, 1997; Nallur *et al.*, 2001) in intact and regenerating organisms (Cebrià *et al.*, 1997).

Tremendous progress has been made in the past few years in demonstrating mechanisms by which cells undergo programmed cell death (Hale *et al.*, 1996; Bergmann *et al.*, 1998). One of the most widely used histochemical methods to detect apoptotic cells is the TUNEL technique. However, critical interpretation of the results obtained is necessary because it has limited sensitivity and specificity (Willingham, 1999). Several groups have succeeded in improving the TUNEL method by applying different fixatives or pre-treatments (Negoescu *et al.*, 1996; Labat-Moleur *et al.*, 1998; Cuello-Carrion and Ciocca, 1999). Here we used a modified protocol after Gavrieli *et al.* (1992) to demonstrate apoptotic cells. In addition, we used annexin V binding to demonstrate externalization of phosphatidyl-serine as a relatively early assay for apoptotic cells. Annexin V labelling was combined with propidium iodide staining to distinguish apoptotic from necrotic cells. We are aware that not all apoptotic cells can be identified with this method (see Willingham, 1999). Finally, we used an ultrastructural approach to follow different morphological stages of cells undergoing apoptosis. In summary, apoptosis was demonstrated for the first time in platyhelminths. This indicates the role of programmed cell death in organisms with a rapid cell turnover based on a potent stem cell system.

MATERIALS AND METHODS

Animals

Cultures of the platyhelminth *Macrostomum* sp. (the description of this species is presently being prepared; Rieger, Salvenmoser, Ladurner manuscript in prep.) were reared in Petri dishes with the diatom *Nitzschia curvilineata* according to Rieger *et al.* (1988). Cultures were maintained in a temperature-controlled chamber at 20°C and a humidity of 60% with a photoperiod of 16 h light and 8 h dark. Animals for experiments were placed on new Petri dishes with diatoms for 7 days and then starved for two days ('standard animals').

BrdU-labelling

The specimens were labelled with BrdU (SIGMA) by incubation in 50 μM BrdU in artificial sea water (ASW) for 14 days. Animals were relaxed in $MgCl_2 6H_2O$ (7.14%) isotonic with sea water for 20 min, fixed in 4% paraformaldehyde (PFA) for 60 min, washed in phosphate buffered saline (PBS, containing 0.8% NaCl, pH 7.4) 2 × 5 min and incubated in PBS-T (PBS with 0.1% Triton X-100) for 1 h. Specimens were treated with 0.15 μg/ml Protease XIV (SIGMA) in 7.6 M Tris HCl at 37°C followed by an incubation in 0.1 N HCl for 10 min on ice. They were subsequently transferred to 2 N HCl at 37°C for 60 min to denature DNA. After washing 3 × 10 min in PBS, they were incubated in the blocking solution 'BSA-T' (PBS with 0.1% Triton X-100 and 1% bovine serum albumin) for 30 min.

Visualization of incorporated BrdU was performed by incubation with a monoclonal antibody to BrdU (Sigma, 1:1000 in BSA-T) at 4°C overnight, washing in PBS-T 3 × 10 min, followed by incubation in secondary FITC-conjugated goat-anti-mouse antibody (DAKO, 1:100 in BSA-T) for

60 min at room temperature and washing in PBS 3 × 10 min. Animals were mounted using Vectashield (Vector Laboratories) and observed with a Reichert POLYVAR epifluorescence microscope or a confocal ZEISS LSM 510 microscope.

Annexin V labelling

Animals were washed in calcium and magnesium free medium (CMF; 0.4 mg/ml PO_4H_2Na, 0.8 mg/ml NaCl, 1.2 mg/ml KCl, 0.8 mg/ml $NaCO_3H$, 270 µg/ml glucose, 1% BSA, 3.75 mg/ml hepes buffer) and macerated for 1 h in 100 µl CMF with 0.1% trypsin (Sigma, T-7409). Cell suspensions were spun down (5 min at 325 × *g*), resuspended in Hepes-buffer and incubated in annexin V Alexa 586 (Boehringer Cat. No. 1 985 485) for 10–15 min at room temperature. Slides were mounted in Vectashield with a dimeric cyanin nucleic acid stain (Y-3601, YO YO-1 iodide, Molecular Probes).

TUNEL labelling

Fluorescent TUNEL labelling of macrostomum cell suspension and paraffin sections. Animals were washed in CMF, macerated for 1 h in 100 µl CMF with 0.1% trypsin and fixed for 15 min in 4% PFA. Fixed cells were centrifuged (5 min at 325 × *g*) onto poly-L-lysine (PLL) coated slides and dried for 1 h at 37°C. The cells were washed in 0.1 M PBS and permeabilized in PBS-TSC (PBS with 0.1% Triton X-100 and 0.1% sodium citrate) on ice for 2 min. Slides were then washed in PBS and incubated in TUNEL-reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, 1684795, Roche Diagnostics) for 1 h in a humid chamber at 37°C. After washing in PBS they were mounted in antifade mounting medium with 1 ng/ml 4,6-Diamidin-2-phenylindol-dihydrochloride (DAPI; Molecular Probes) or 20 ng/ml propidium iodide (PI; Molecular Probes).

Both negative and positive controls were included. For positive controls slides were treated with 50 µg/ml DNAse I (SIGMA, D-4263) in DNAse-buffer for 10 min at room temperature. For negative controls either dUTP or the TdT enzyme were omitted from the reaction mixture. In positive controls every nucleus was stained, in negative controls no staining was observed.

For paraffin sections animals were relaxed in aqueous magnesium chloride solution isotonic to sea water (7.14% $MgCl_26H_2O$) and fixed in 4% PFA for 1 h. After washing in PBS, specimens were dehydrated in a graded series of ethanol, embedded in paraffin wax, and sectioned (7 µm). Sections were cleared in xylene, rehydrated through a graded series of ethanol, and immersed in distilled water. Slides were incubated in 20 µg/ml 10 mM proteinase K in Tris-HCl for 5 min at 37°C, washed in PBS 2 × 5 min, incubated in TUNEL-reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, 1684795, Roche Diagnostics) for 1 h in a humid chamber at 37°C, washed in PBS 3 × 5 min and mounted in antifade mounting medium with PI. Again, both negative and positive controls were performed as described above Observation was done with a Reichert POLYVAR epifluorescence microscope or a confocal ZEISS LSM 510 microscope.

TUNEL-biotin-labelling of whole mounts according to Gavrieli et al. *(1992).* Animals were relaxed in magnesium chloride ($MgCl_2$) as described above and fixed in 4% PFA for 1 h. Specimens/sections were washed three times in PBS for 5 min and then 4 × in double distilled water (DDW) for 2 min. Endogenous peroxidase was blocked by incubation in 2% H_2O_2 for 5 min. Animals were rinsed in DDW, and immersed in terminal deoxytransferase (TdT) buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). 0.25 U/µl TdT and 0.02 nmol/µl biotinylated dUTP in TdT buffer were added, and specimens were incubated in a humid chamber at 37°C for 60 min. The reaction was terminated by adding TB buffer (300 mM sodium chloride, 30 mM sodium citrate in DDW) and incubating for 15 min at RT. The samples were rinsed in DDW, incubated in 2% BSA in PBS for 10 min at RT, rinsed in DDW, and immersed in PBS for 5 min. The animals were treated with Extra-Avidin peroxidase (10 µl Streptavidin + 10 µl biotinylated peroxidase + 2 ml DDW, StreptABC Complex/HRP Duet, Mouse/Rabbit, K0492 DAKO) at 37°C for 30 min, washed in PBS and stained with diaminobenzidine (DAB; 20 µl DAB-chromogen + 1.5 ml buffered substrate DAKO-Liquid DAB, K3466, DAKO) for a few min at RT under visual control. Specimens were mounted in Histosafe (Cat. No. E6000 Histogel, Camon Labor Service, Wiesbaden, Germany).

As positive controls, slides or whole mounts were incubated in DNAse for 10 min at RT. For the negative controls either biotinylated dUTP or TdT were omitted from the reaction mixture. In positive controls every nucleus was stained, in negative controls no nucleus was stained.

Electron microscopy

For transmission electron microscopy (TEM) worms were relaxed in $MgCl_26H_2O$ (7.14%), fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 10% sucrose for 1 h and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Specimens were then dehydrated in a standard acetone series and embedded in Spurr's Low Viscosity Resin (Spurr, 1969). Ultra-thin sections (75 nm) were double stained with uranyl acetate and lead citrate and observed with a Zeiss EM 902 TEM.

Induction of apoptosis in Macrostomum sp.

Three methods were used to induce apoptosis: (1) actinomycin D (Roche) was dissolved in dimethyl sulfoxide (DMSO) and diluted in DDW to a concentration of 1 mg/ml. This solution was added to ASW of animal cultures for 1 to 64 h (final concentration: 5 µg/ml actinomycin D) or 41 h (100 µg/ml). (2) Colchicine (0.005%, Sigma, stored in aliquots of 0.05%) was added for 15 to 65 h. (3) Animals were starved in ASW without diatoms in darkness for up to 22 days.

RESULTS

Cell renewal

Approximately one-third of all nuclei were labelled after 14 days' continuous exposure to BrdU (Fig. 1a, n=8). Nuclei of different cell types could be identified by their morphology. Light- and electron microscopy showed that BrdU incorporation had no effect on the morphology of the cell nuclei compared to untreated animals. Epidermal cell nuclei showed a lobated appearance (Fig. 1b) that was also apparent using TEM (Fig. 4). BrdU labelled nuclei of epidermal cells were homogeneously distributed on the dorsal and ventral side. Larger roundish labelled nuclei were found in gastrodermal cells (Fig. 1d), while longitudinal muscle cell nuclei or differentiating neoblasts were slightly elongated (Fig. 1c).

Cell renewal progressed continuously with a bilateral distribution of gastrodermal, pharyngeal and frontal organ cells, while newly incorporated epidermal cells were distributed homogeneously. Adult *Macrostomum* sp. possess about 25,000 cells. According to the number of BrdU labelled cells after 14 days of continuous exposure, we estimate that about 7000 cells (compare Fig. 1a for cell renewal of a subadult animal) were replaced. This implies that about 500 cells (2% of total) were renewed every day, equivalent to 20.8 cells per hour. These data are supported by studies on the cell cycle length of neoblasts in *Macrostomum* sp. (Ladurner *et al.*, 2000, Nimeth and Ladurner, unpubl.).

Labelling of apoptotic cells

Three methods were used to identify apoptotic cells in *Macrostomum* sp. (1) TUNEL technique with fluorescent and peroxidase detection in whole mounts and tissue sections in the following specimens: (1a) standard animals (see Materials and Methods), (1b) long-term starved animals, (1c) animals treated with colchicine or actinomycin D to induce apoptosis, as well as DNAse I treated specimens as controls. (2) Specific binding of annexin V to apoptotic cells in macerated cell preparations and (3) morphology of apoptotic stages by their ultrastructure.

We found 8.13 ± 5.50 (SEM, n=101) apoptotic cells per individual in standard animals using the TUNEL method. The majority were located below the epidermal layer in the parenchyma (Fig. 2). Few apoptotic cells were found within the epidermis (Fig. 2b), while some cells were sloughed off the epidermal sheath (Fig. 2a). It has not been possible to determine the number of cells that were lost by sloughing off. In electron microscopic examinations, apoptotic figures were found in the parenchyma between the epidermis and gastrodermis (Fig. 4d). Their ultrastructure showed the beginnings of fragmentation of the nuclei, but always had intact mitochondria with a normal organization of membranes. A high percentage of these cells had secondary lysosomes containing degenerating microvilli and cilia, and we could therefore identify them as apoptotic epidermal cells.

In colchicine-treated specimens, the number of apoptotic cells was increased. A group of apoptotic cells was detected in the brain, showing different stages of apoptosis-like disintegration of nuclear chromatin and fragmentation of the nuclei, but again intact mitochondria (Fig. 3). In the epidermis we found cells in retraction from the epithelium. We observed epidermal cells at different apoptotic stages. Some showed secondary lysosomes, containing microvilli and cilia. These cells always had a normally structured nucleus (Fig. 4a,b). Such cells may later have entered apoptosis and become dislocated into the parenchyma. In the epidermal layer, no cells in the later stages of apoptosis were found, either with light- or electron-microscopical methods. In one case, a migrating late stage

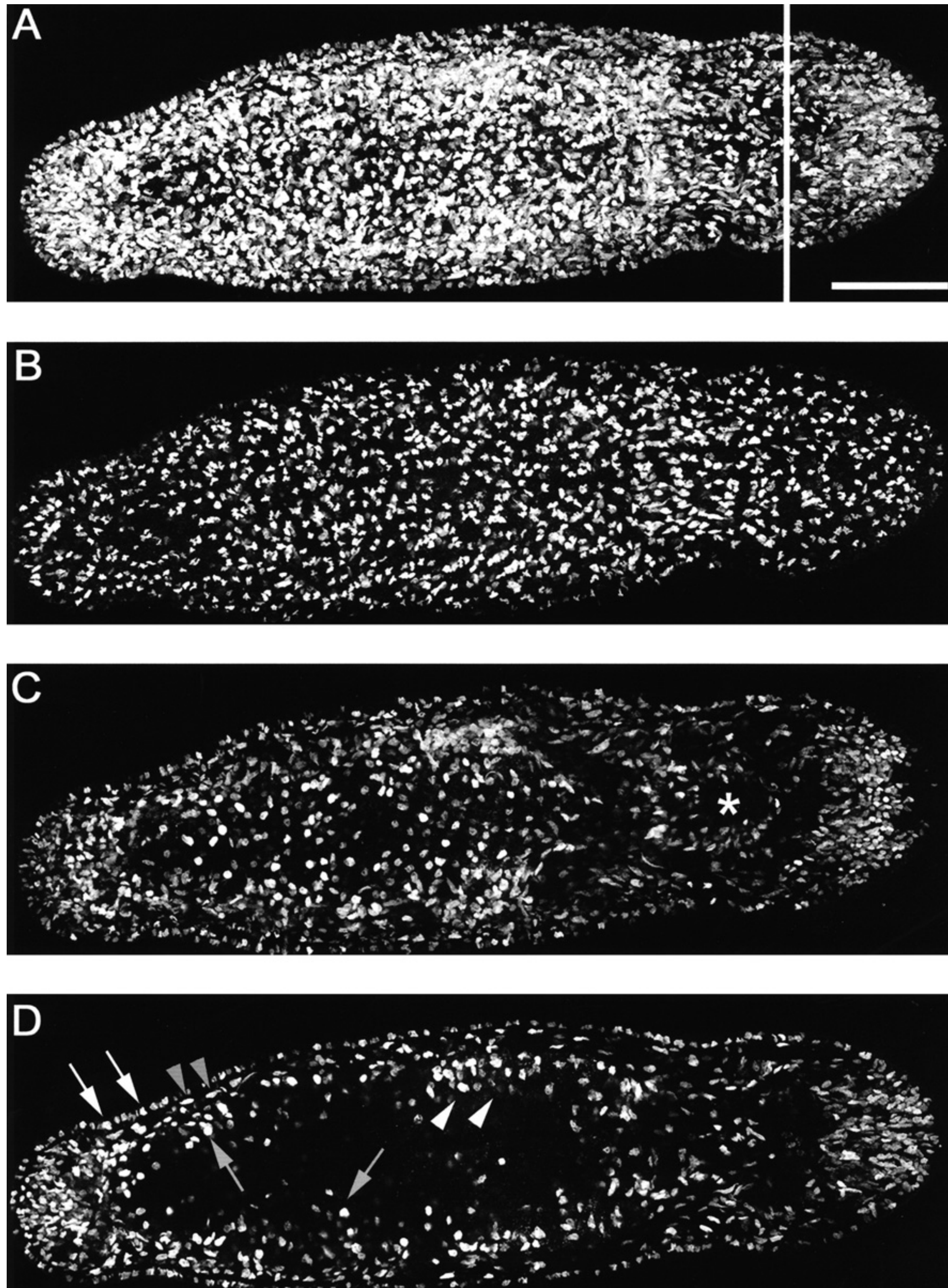

Fig. 1. Confocal projection of *Macrostomum* sp. after 14 days of continuous BrdU exposure. (A) Projection of all optical sections (white line marks the level of the eyes). (B) Confocal projection of dorsal epidermis. (C) Confocal projection at the level of the pharynx (asterisk indicates mouth area). (D) Confocal projection at the level of the gut. Few cells in the gut epithelium (grey arrows), but many cells in the epidermis (white arrows). Clusters of cells in the gonads (white arrowheads). Elongated nuclei of longitudinal muscle cells or differentiating neoblasts (grey arrowheads). Bar 100 μm.

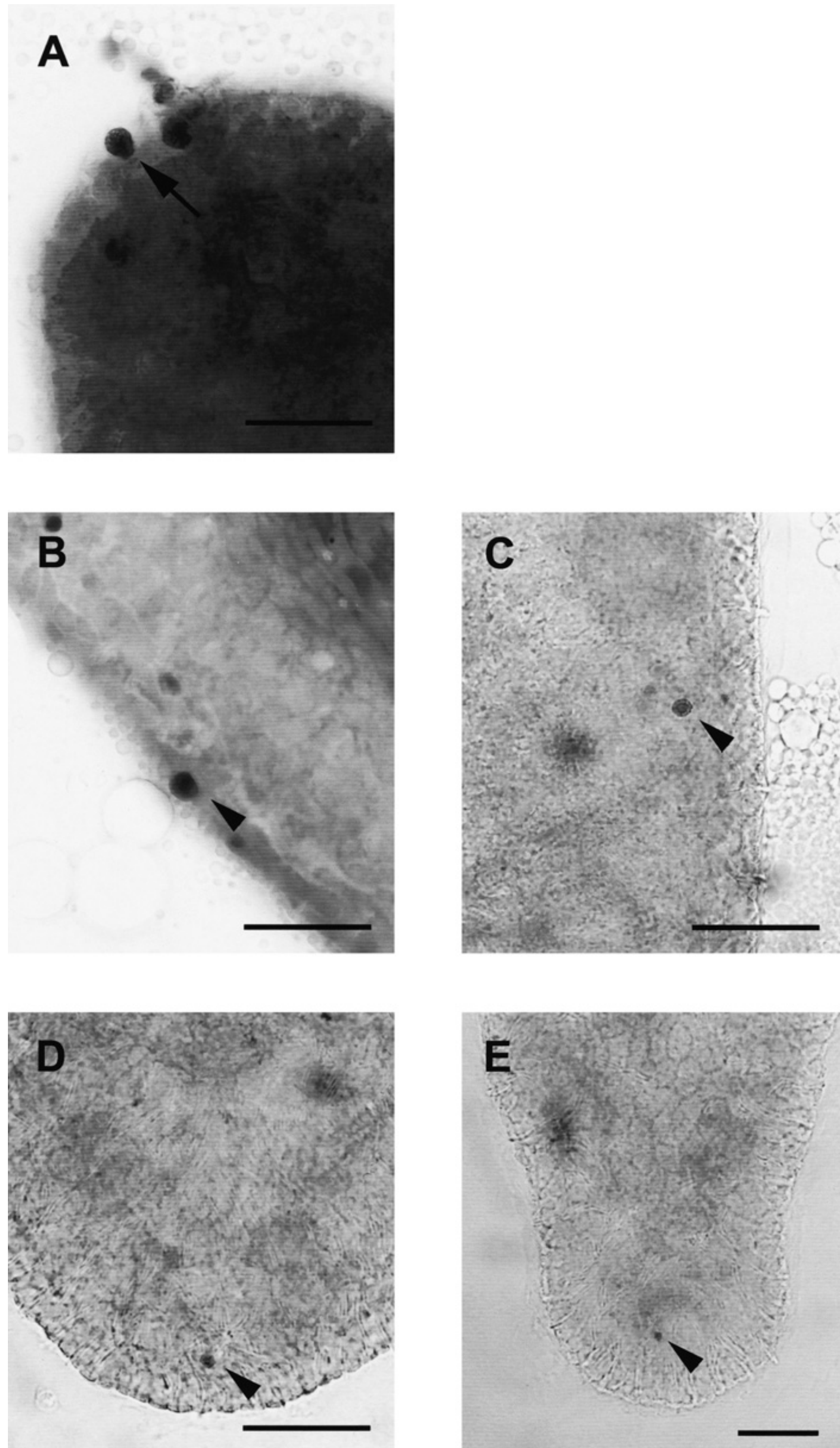

Fig. 2. Light micrographs of *Macrostomum* sp. Whole mount labelling with TUNEL-biotin-Streptavidin. (A) Apoptotic cell in the epidermis to be sloughed off (arrow). (B) Apoptotic cell in the epidermal layer (arrowhead). (C) Apoptotic cell (arrowhead) at the lateral side of one animal, dorsal view. (D) Dorsal view, posterior end of another animal showing one apoptotic cell below the epidermis (arrowhead). (E) Posterior end of another animal showing one apoptotic cell (arrowhead), dorsal view. Bars 50 μm.

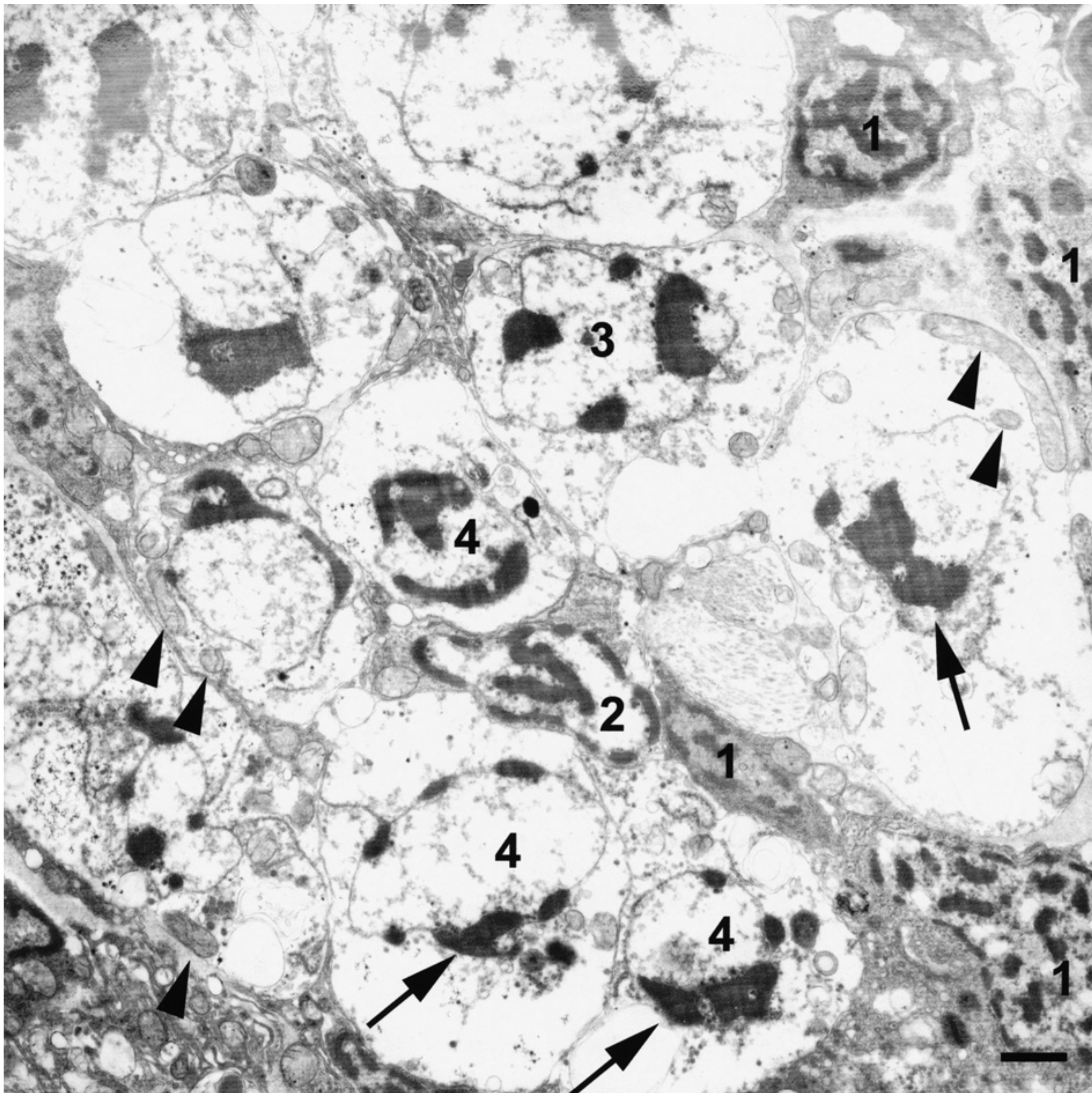

Fig. 3. Group of apoptotic cells in the brain region with stages of programmed cell death induced by colchicine. (1) Cells with intact nuclei, (2) the beginnings of apoptosis with nuclear condensation, (3) typical apoptotic cells with clumps of chromatin (arrows) at the nuclear margin, and (4) the beginnings of fragmentation of apoptotic cells with isolated nuclear material (asterisk) in the cytoplasm. All apoptotic cells show intact mitochondria (arrowheads). Bar 1 μm.

neoblast with cytoplasmic processes into the epidermal layer could be detected (Fig. 4b). Such neoblasts are thought to differentiate into young epidermal cells (Fig. 4c). In macerated cell suspensions of *Macrostomum* sp., individual TUNEL-labelled cells were detected (Fig. 5).

Apoptotic and necrotic cells were identified in macerated preparations using annexin V labelling. Annexin V positive cells were identified as apoptotic when the nuclei did not counterstain with a cell membrane impermeable dimeric Cyanin nucleic acid stain (YO YO, Molecular Probes) (Fig. 6a,c). In contrast, necrotic cells were doublestained with both annexin and YO YO (Fig. 6b,d).

Light-microscopical data showed cells in late stages of apoptosis. These cells were characterized by a TUNEL stained nucleus and a small, unstained cytoplasm. In one biotin-streptavidin TUNEL examination we identified a higher number of labelled epidermal cells. The

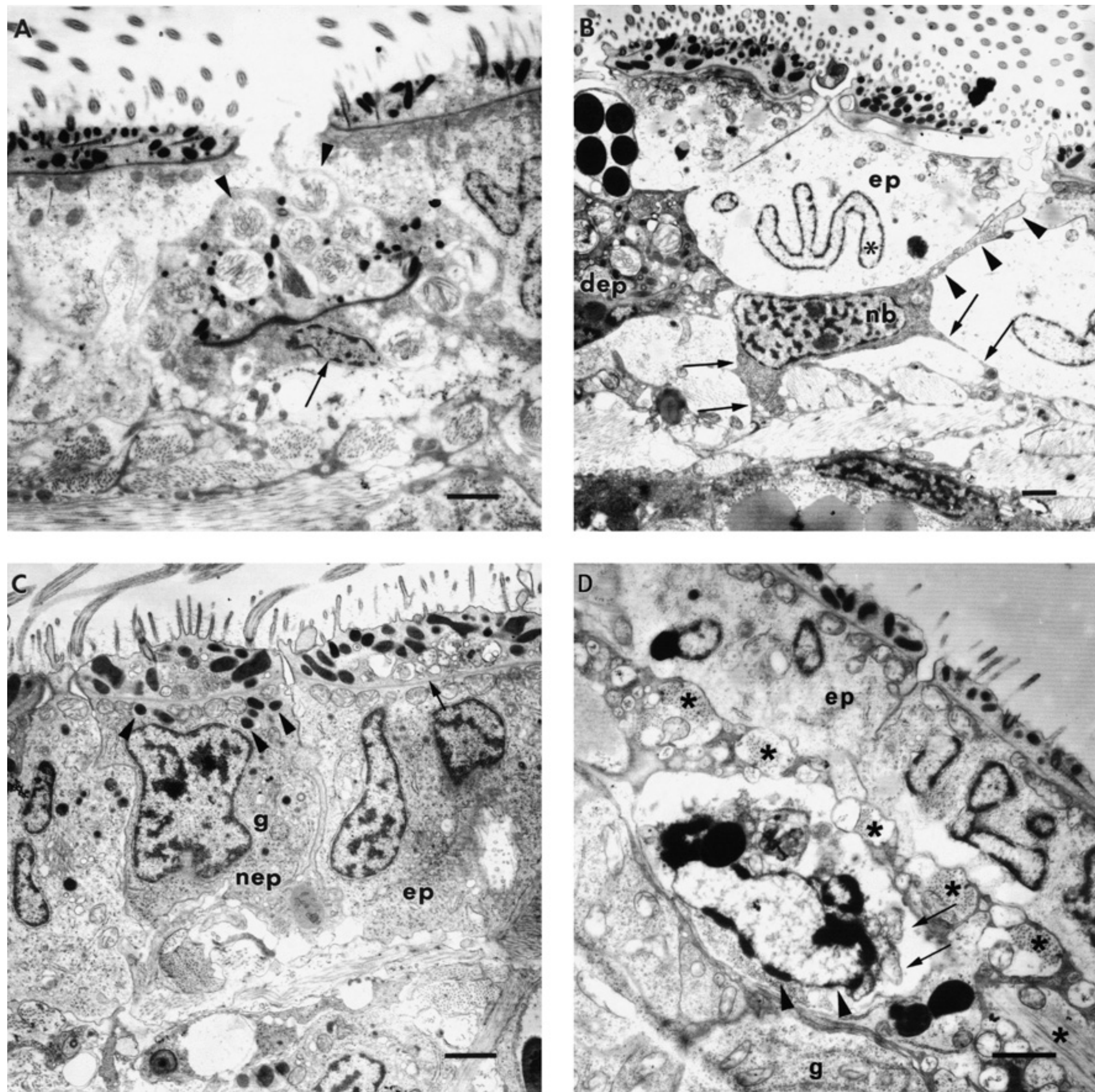

Fig. 4. (A) Degenerating epidermal cell, which is withdrawn from the epithelial layer. Cilia and microvilli are degenerating and can be found in vesicles (arrowheads). Note the intact nucleus (arrow). (B) Replacement of epithelial cell by a late stage neoblast. One cytoplasmic extrusion of the neoblast (nb) has already reached the apical part of the intact epithelium just beneath the junctional complex (arrowheads). Two other extrusions are still connected within the mesodermal muscle layer (arrows). On the left side of the picture a degenerating epidermal cell is visible (dep). Lobated nucleus (asterisk) of a fully differentiated cell (ep). (C) Differentiated and newly replaced epidermal cells are shown in this micrograph. A newly replaced epidermal cell (nep) which is completely integrated into the epidermal layer is shown. The nuclei of such cells are more compact and not extensively folded, as is characteristic of fully differentiated epidermal cells (ep). Ultrarhabdites (arrowheads) secreted by the Golgi apparatus (g) have not reached the apical cell region and are located beneath the terminal web (arrow). The cytoplasm appears denser due to the large amount of polyribosomes. (D) Apoptotic cell in the parenchyma with intact mitochondria (arrows) and the beginnings of fragmentation of the nucleus with condensed chromatin (arrowheads). Epidermis (ep), muscles (asterisks), gastrodermal cell (g). Bars 1 μm.

morphology of the nuclei of these cells was comparable to functional epidermal cells, with characteristic lobated nuclei (see Fig. 4b, Fig. 7).

Rhabdites and gland cell secretions showed background fluorescence (Fig. 8). This could, however, be distinguished from the fluorescence emitted after staining cells for apoptosis, i.e. the positive signal (see also discussion).

Programmed cell death could be induced with colchicine. Exposure for 24 h increased the number of apoptotic nuclei to 26.00 ± 10.62 (n=8) and incubation for 48 h increased it to 10.87 ± 5.89

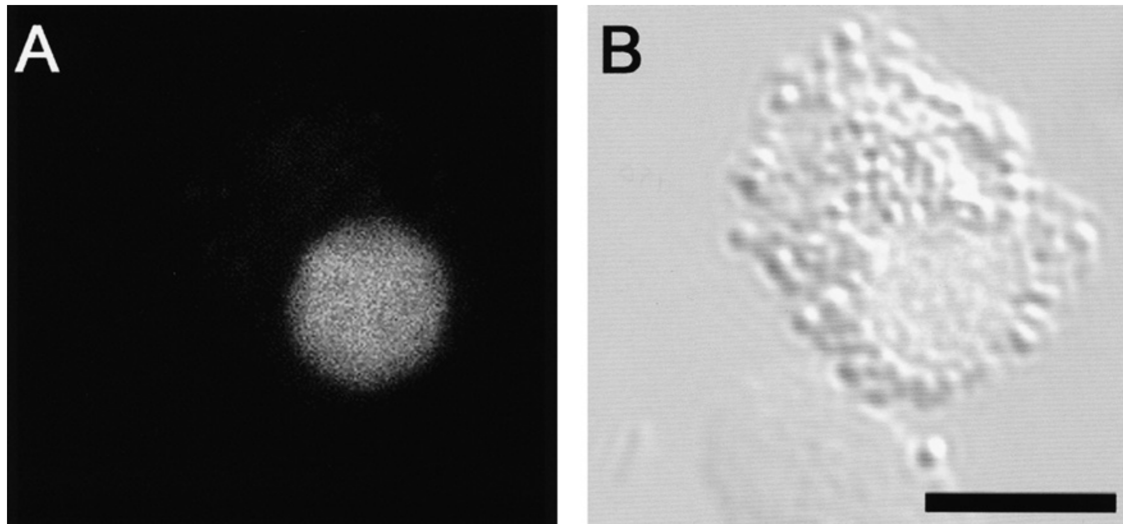

Fig. 5. Confocal projection of macerated cell of *Macrostomum* sp. (A) TUNEL labelled nucleus of apoptotic cell and (B) interference contrast micrographs of the same cells. Scale bar 5 µm.

(n=15). During incubation animals were not fed, so specimens were actually starved for 96 h in total. Exposure to actinomycin D (1 to 64 h) and starvation for up to 22 days had no effect on the number of apoptotic cells.

DISCUSSION

Occurrence of apoptosis in platyhelminths

In this study, we show the existence of programmed cell death and its importance for permanent renewal of tissues in the platyhelminth *Macrostomum* sp., an organism with an outstanding stem cell system. Platyhelminths have a key position at the transition from diploblastic to triploblastic organization (Rieger and Ladurner, 2001). The study of cell cycle characteristics in such a basal bilaterian is of special evolutionary and developmental biological interest.

Identification of S-phase cells in free-living flatworms has been performed using the incorporation of BrdU (Newmark and Sanchez Alvarado, 2000; Rieger and Ladurner, 2001; Gschwentner *et al.*, 2001). Our results confirm a high turnover of cells in *Macrostomum* sp. (Ladurner *et al.*, 2000), and indicate a high degree of conservation and the early appearance of apoptosis during the evolution of bilaterians (Vaux and Korsmeyer, 1999; Meier *et al.*, 2000). Apart from the well known occurrence of apoptosis in vertebrates (see for example Farber, 1994; Hale *et al.*, 1996), earlier studies have documented programmed cell death in several bilaterian model organisms, such as the nematode *Caenorhabditis elegans* (Metzstein *et al.*, 1998; Fraser, 1999; Liu and Hengartner, 1999; Meier *et al.*, 2000) and the fruitfly *Drosophila melanogaster* (Bergmann *et al.*, 1998; Vernooy *et al.*, 2000; Meier *et al.*, 2000; Bangs and White, 2000).

In this study, apoptotic cells were identified by the TUNEL technique, by specific binding of annexin and by electron microscopy. As mentioned above, programmed cell death seems to be a mechanism that has been conserved during evolution and can even be found in diploblasts such as *Hydra* (Jacobson *et al.*, 1997; Cikala *et al.*, 1999; Miller *et al.*, 2000).

Apoptosis and its detection methods

Several methods have been developed to identify apoptotic cells. The most widely used technique to detect programmed cell death is TUNEL. A very early event in apoptosis is the occurrence of DNA fragments (Willingham, 1999). Various fixatives can have dramatic effects on the efficiency of this method to detect apoptotic cells (Negoescu *et al.*, 1996; Willingham, 1999). Several authors have shown that modifications of the original method can improve sensitivity (Sanders and Wride, 1996; Negoescu *et al.*, 1996; Labat-Moleur *et al.*, 1998; Cuello-Carrion and Ciocca, 1999). In this way, 70–80% of apoptotic cells can be stained (Labat-Moleur *et al.*, 1998) with hardly any background (Sanders and Wride, 1996; Cuello-Carrion and Ciocca, 1999).

Rhabdites and gland cell secretions showed intrinsic background fluorescence (Fig. 8). This

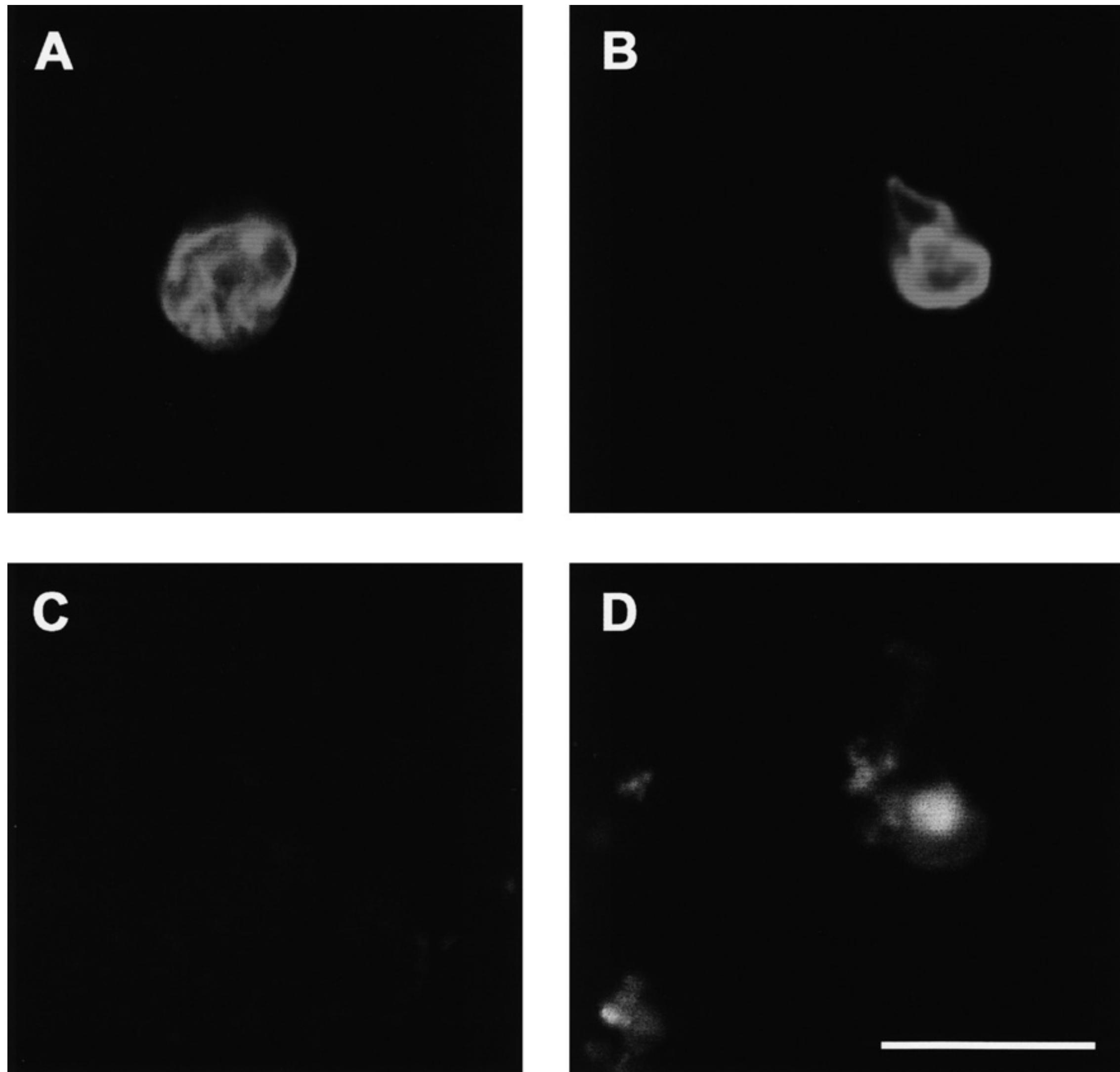

Fig. 6. Laser scanning projections of macerated cells of *Macrostomum* sp. Annexin–YO YO labelling of an apoptotic cell (A, C) and a necrotic cell (B, D). Apoptotic cells are labelled with annexin (A), nuclei do not show YO YO staining (C), necrotic cells are double stained with annexin (B) and YO YO (D). Bars 5 μm.

could, however, be distinguished from the positive signal and did not impair our results. DNA fragmentation is a hallmark of apoptosis, but it also appears during necrosis, although to a lesser degree (Goping *et al.*, 1999). Thus, TUNEL may stain necrotic cells, but preferentially labels apoptotic cells (Mundle and Raza, 1995). Although DNA fragmentation is one of the most characteristic processes of apoptosis (Gavrieli *et al.*, 1992), it is not necessary for cell death (Zweyer *et al.*, 1997; Nakamura *et al.*, 1997). Therefore, investigations on apoptosis should always be assessed by more than one technique (Cuello-Carrion and Ciocca, 1999).

Another characteristic of apoptotic cells, namely the translocation of the membrane lipid phosphatidyl-serine (Hale *et al.*, 1996) during early apoptosis (Willingham, 1999), was addressed. This alteration in the cell membrane is a distinguishing feature of apoptotic cells. Annexin V is a calcium-dependent protein that shows specific binding to phosphatidyl-serine (Hale *et al.*, 1996). Experimentation with living cells uses both the high affinity of annexin V for phosphatidyl-serine and the impermeability of the cell membrane to propidium iodide (PI) (Willingham, 1999). We double-stained living cells with annexin V and the dyes PI and YO YO in order to distinguish apoptotic from necrotic cells. Large dye molecules, such as PI and YO YO, cannot enter living cells with intact membranes, but can stain the nuclei of necrotic cells (Willingham, 1999). In macerated *Macrostomum*

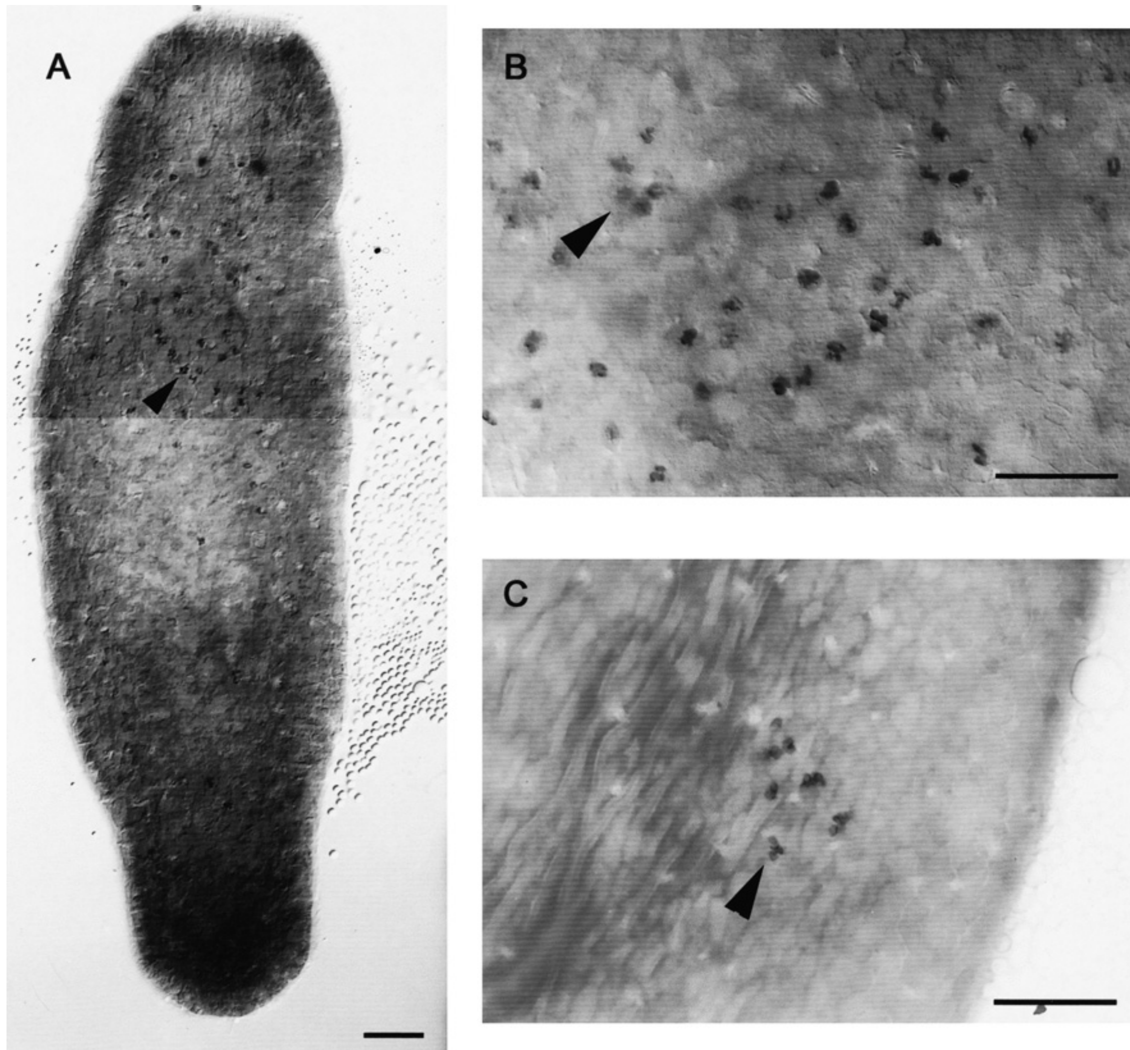

Fig. 7. Light microscopic images of *Macrostomum* sp. Whole mount labelling with TUNEL-biotin-Streptavidin. (A) Overview of a whole mount, dorsal view. TUNEL labelled epidermal cell (arrowhead). (B, C) Dorsal view of another animal. TUNEL labelled epidermal cells with lobated nuclei (arrowheads). Bars 50 µm.

sp. cell suspensions, apoptotic cells were labelled with annexin whilst the nuclei were not stained with YO YO, but necrotic cells were labelled with both.

Electron microscopy is a useful tool to distinguish between apoptosis (typical nuclear structure and intact mitochondria) and necrosis. In this study, we induced apoptosis in *Macrostomum* sp. by incubation with colchicine. An incubation of 24 h increased the number of apoptotic cells by 319.8%. Colchicine is known to increase the number of apoptotic cells in vertebrates (Duncan and Heddle, 1984; Giannakis *et al.*, 1991; Volbracht *et al.*, 1999; Ahlbom *et al.*, 1999) and *Hydra* (Cikala *et al.*, 1999). Induction of apoptosis by actinomycin D has been reported by several groups (Tiso *et al.*, 1995; Shiokawa *et al.*, 1997; Chou and Yung, 1997; Ljungman *et al.*, 1999). As Meijerman *et al.* (1999) found in the rat, actinomycin D cannot induce apoptosis in *Macrostomum* sp. It is known from planarians that starvation leads to a dramatic reduction in the size of animals and their cell number (Baguñà and Romero, 1981). We did not find an increased number of apoptotic cells in starving *Macrostomum* sp. This could indicate cell degradation based on mechanisms other than apoptosis.

The neoblast system and cell turnover

Baguñà and Romero (1981) have reported that the planarians *Dugesia tigrina* and *Dugesia mediterranea* are in a state of perpetual cell turnover. A 7 mm long *Dugesia tigrina* loses about 7000 cells per day while starving, and after feeding shows a

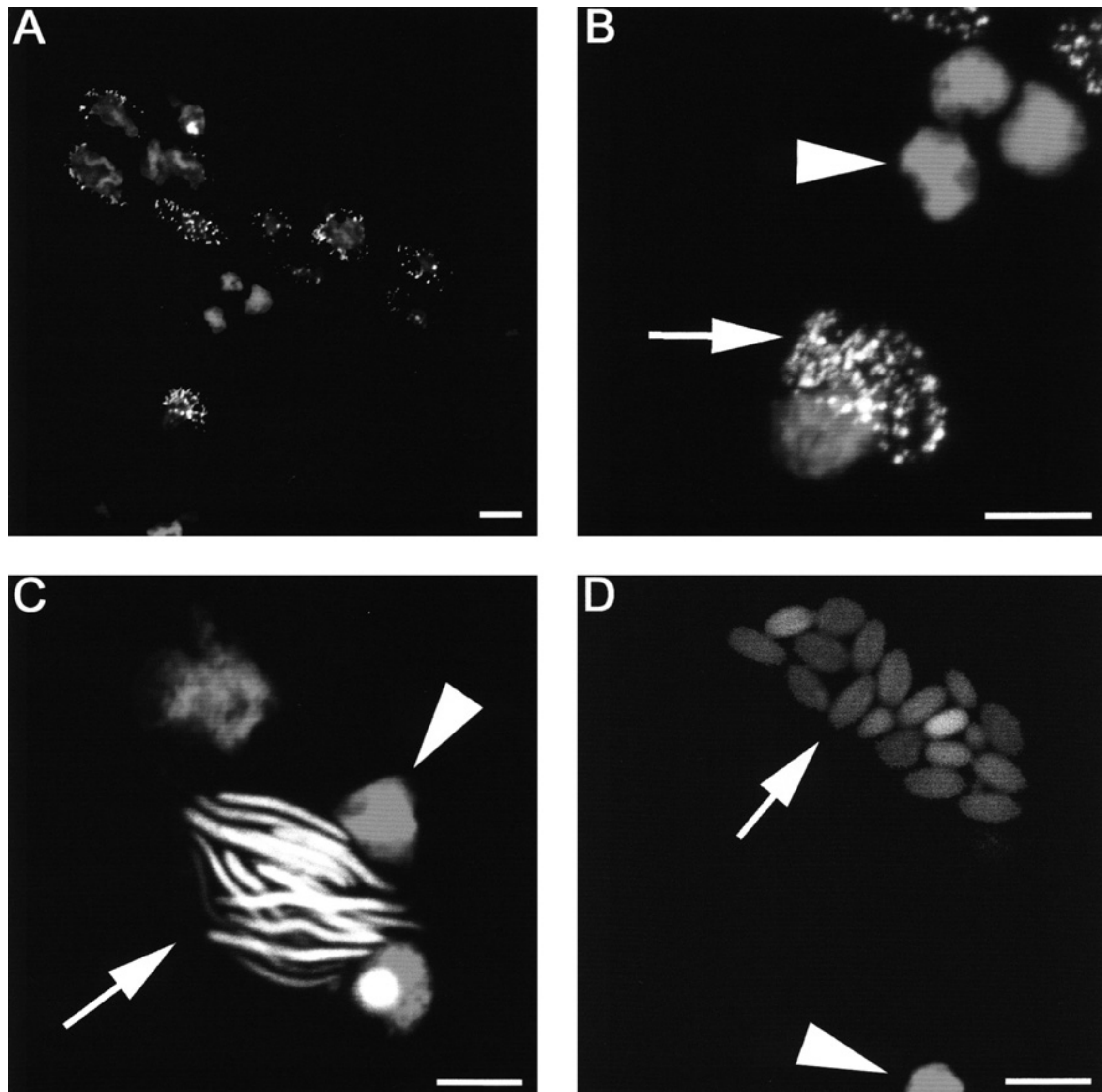

Fig. 8. Laser scanning projections of macerated cells of *Macrostomum* sp. demonstrating unspecific TUNEL labelling (arrows). Arrowheads indicate PI labelled cell nuclei. (A) Macerated cell preparation. All cells show PI labelled nuclei, while epidermal cells also have strong finely-scattered background staining (see magnification in B). (B) PI labelled roundish nuclei (arrowhead) and unspecific staining in an epidermal cell (arrow), probably due to ultrarhabdites in the apical cytoplasm of the epidermis. (C) PI labelled roundish nuclei (arrowhead). Bundle of rhabdites with strong unspecific labelling (arrow). (D) PI labelled roundish nuclei (arrowhead). Granules of shell glands (arrow). Bars 5 µm.

steady increase in cell number. In *Macrostomum* sp., Ladurner *et al.* (2000) estimated that 20% of all cells are neoblasts. More recent investigations suggest considerably lower values (8–9%, Bode unpubl.). 10% of neoblasts are in S-phase (Ladurner *et al.*, 2000). Continuous BrdU labelling for 14 days (Fig. 1) showed about 7000 labelled cells, indicating a high turnover rate of about 20.8 cells being replaced per hour. This implies a loss of about the same number of cells in adult animals under stable conditions.

Apoptosis is a very fast process (Willingham, 1999). In mammals, TUNEL labels apoptotic cells for only a short period of 1–3 h (Gavrieli *et al.*, 1992). Tsubokawa and Wedeen (1999) have reported a period of less than 1 to 4 h in leech before apoptotic cells are phagocytosed. In our TUNEL experiments we found 8.13 ± 5.50 cells, i.e. only about one-third of the number of replaced cells (20.8 cells per hour). We assume that degradation mechanisms other than apoptosis play an additional role in the elimination of cells. An

alternative mechanism may be used to slough off old cells, particularly in epidermal tissue, but this was not detectable with the methods we used here. In one experiment the number of TUNEL positive cells was noticeably increased (19.8 ± 15.70, $n=19$). This could be due to individual differences, physiological effects or variations in the methods used.

For a more complete understanding of cell turnover, the rate of necrosis should be determined in addition to apoptosis. Also, the loss of BrdU label in neoblasts due to apoptosis and necrosis should be monitored in future experiments, in order to estimate the percentage of neoblasts that are eliminated without entering differentiation.

Functional implications

The interplay of cell division, differentiation and cell replacement is essential to multicellular organisms (Bergmann *et al.*, 1998). *C. elegans* has become established as a model organism for apoptosis, and investigation has concentrated on the mechanisms controlling programmed cell death (Hengartner, 1996; Hale *et al.*, 1996; Jacobson *et al.*, 1997; Metzstein *et al.*, 1998; Meier and Evan, 1998; Bergmann *et al.*, 1998; Fraser, 1999; Liu and Hengartner, 1999; Vernooy *et al.*, 2000; Meier *et al.*, 2000). In embryonic development in *C. elegans,* 1090 somatic cells are established, of which 131 undergo apoptosis (Hale *et al.*, 1996; Metzstein *et al.*, 1998; Meier and Evan, 1998; Meier *et al.*, 2000). Programmed cell death has been identified as an important factor in vertebrate development (Jacobson *et al.*, 1997; Meier and Evan, 1998; Meier *et al.*, 2000), as well as in invertebrates such as the annelid leech (Tsubokawa and Wedeen, 1999) and insects.

In *Drosophila,* programmed cell death plays a key role during embryonic development and metamorphosis (Bergmann *et al.*, 1998; Vernooy *et al.*, 2000; Bangs and White, 2000). Apoptosis also plays a crucial role in the homeostasis of tissues (Gavrieli *et al.*, 1992; Hengartner, 1996). It is a rare event in slowly renewing tissues (Gavrieli *et al.*, 1992). Programmed cell death is essential in the immune system (Hale *et al.*, 1996; Nagata, 1997), as a guarantee for quality control and repair mechanisms (Meier *et al.*, 2000), and is correlated to tumor suppressors such as p53 (Hale *et al.*, 1996; Schmale and Bamberger, 1997; Israels and Israels, 1999). So far we can definitely say that apoptosis plays a role in cell turnover in the epidermis of *Macrostomum* sp. In colchicine-treated animals, programmed cell death was also found in other tissues, such as brain. We have no information about the role of apoptosis during embryonic development in *Macrostomum* or other flatworm species, or the role of programmed cell death in tissue repair.

Evolutionary implications

Programmed cell death is a highly conserved mechanism within almost all eukaryotic organisms (Jacobson *et al.*, 1997; Nicholson and Thornberry, 1997; Bergmann *et al.*, 1998; Liu and Hengartner, 1999; Aravind *et al.*, 1999; Huang *et al.*, 2000; Frohlich and Madeo, 2001). Control mechanisms are necessary in multicellular organisms, even plants. In plants, a completely different mechanism would be expected, due to the cell wall making phagocytosis impossible (Jacobson *et al.*, 1997; Ranganath and Nagashree, 2001). DNA fragmentation as a hallmark of apoptosis was proven during fission in cnidarians (Mire and Venable, 1999). Investigations into apoptosis in *Hydra* revealed its importance in oogenesis (Miller *et al.*, 2000). Cikala *et al.* (1999) identified caspases in *Hydra vulgaris* that are involved in programmed cell death. Los *et al.* (2001) reported that caspases are more than killers involved in the control of T-cell proliferation and cell-cycle progression. No caspases have been identified in *Macrostomum* sp. to date.

Our investigations show programmed cell death in a member of the platyhelminths. We have visualized two important characteristics of apoptotic cells: (I) DNA fragmentation and (II) translocation of phosphatidyl-serine. A third hallmark of apoptosis is the complex system of caspases involved in cell degradation. In the model organisms *C. elegans* and *D. melanogaster* numerous proteases have been identified and homologues of them have been found in mammals. Further investigation, therefore, has to be focused on the detection of homologues of these caspases in platyhelminths. Apoptosis should also be inducible by light (Chen *et al.*, 1999), including UV (Godar, 1996; Ljungman *et al.*, 1999) and gamma rays (Duncan *et al.*, 1983; Ruifrok *et al.*, 1998; Ljungman *et al.*, 1999).

Conclusion

This study investigated two extremes of cell fate in platyhelminths—birth and death. Eukaryotic organisms have had to evolve a complex system to maintain tissue homeostasis. This implies a precise balance between cell proliferation and cell loss. The techniques we used here allowed us to visualize

both S-phase cells as markers of cell proliferation, and programmed cell death as one mechanism for the elimination of cells.

The proliferative activity of neoblasts resulting in a high turnover of tissues implies the existence of an effective replacement system for worn out cells. We have shown that DNA fragmentation and translocation of phosphatidyl-serine are important properties of apoptosis in a basal flatworm. The number of apoptotic cells was lower than expected from the proliferation rate, implying that alternative methods of cell replacement must be involved.

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