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Original Article

Role of fixed parenchyma cells in blastema formation of the planarian *Dugesia japonica*

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ABSTRACT Observations of fixed parenchyma cells using electron microscopy were carried out in an attempt to understand the morphogenetic process of blastema formation in regenerating planarians. Fixed parenchyma cells could be found throughout one-day blastemata. In the mid-blastema region where migrating regenerative cells build up a compact cell aggregate, long and slender cytoplasmic processes of the fixed parenchyma cells were seen occupying spaces among regenerative cells. A characteristic feature of such processes was orderly arranged microtubules. Ruthenium red staining revealed thickened portions of cell coats on these processes and occasional formation of gap junctions between the cytoplasmic process of the fixed parenchyma cell and the regenerative cell undergoing migration. Colchicine treatment (M/1,000) caused detachment of the cytoplasmic processes from the regenerative cells. Microtubules within such processes became depolymerized. As a result, directional migration of regenerative cells was inhibited by colchicine treatment. To determine the extracellular site of fibronectin, immunoelectron microscopy was performed in one-day blastema. Immunogold labeling was detected at the surface area of fixed parenchyma cells and regenerative cells. In particular the reactivity was conspicuous at the cytoplasmic process of the fixed parenchyma cells. These observations suggest that the cytoplasmic processes of fixed parenchyma cell are related to directional movement of regenerative cells by providing a contact guidance system. The biological implications of this system are discussed in relation to the extracellular matrix components.

KEY WORDS: *fixed parenchyma cell, blastema, ruthenium red, fibronectin, planarian*

Introduction

When dissociated cells from planarian tissues are cultured, fixed parenchyma cells contribute to the accumulation of neoblasts (Betchaku, 1967, 1970). In spite of this interesting finding, most *in situ* studies of planarian regeneration have concentrated on regenerative cells or neoblasts. The phagocytotic activity of fixed parenchyma cells is well known (Pedersen, 1961; Bowen *et al.*, 1982; Morita and Best, 1984) but behavior of these cells during blastema development has rarely been studied. If fixed parenchyma cells cooperate with regenerative cells after wounding, it should be relevant to know how these cells are associated with blastema morphogenesis. One of the most remarkable phenomena occurring in the early stages is directional cell movement from the old stump region to the wound surface (Saló and Baguñà, 1989). From their polarized cell structure, the migration of regenerative cells seems most active in the mid-blastema region (Hori, unpublished data). It is generally accepted that the extracellular matrix influences a number of normal cellular and developmental activities such as cell migration, differentiation and proliferation (Thesleff *et al.*, 1979; Löfberg *et al.*, 1980; Hay, 1981). Therefore if we examine extracellular matrix components of the planarian blastema by various methods, it should be possible to gain more precise knowledge of the mechanism of blastema formation. The cationic dye, ruthenium red (RR), has been used as a stain for extracellular localization of proteoglycans. Since proteoglycans are found not only in cell coats but also in the extracellular ground substance (Luft, 1976; Alberts *et al.*, 1989), the use of such an electron-opaque tracer will provide a good opportunity for examining in greater detail the complex relationships between fixed parenchyma cells and regenerative cells. Fibronectin is also known as a multifunctional glycoprotein in the extracellular ground substance (Yamada *et al.*, 1985). Immunogold labeling of this molecule is expected to give additional information about its possible role in planarian regeneration.

The purpose of this study is to use electron microscopy to widen our understanding of the role of fixed parenchyma cells in blastema formation.

Abbreviations used in this paper: RR, ruthenium red.

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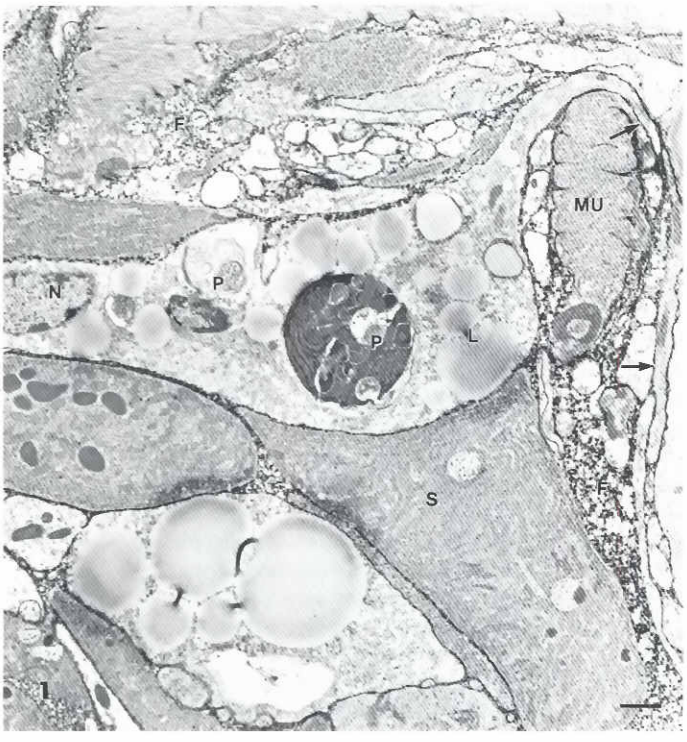


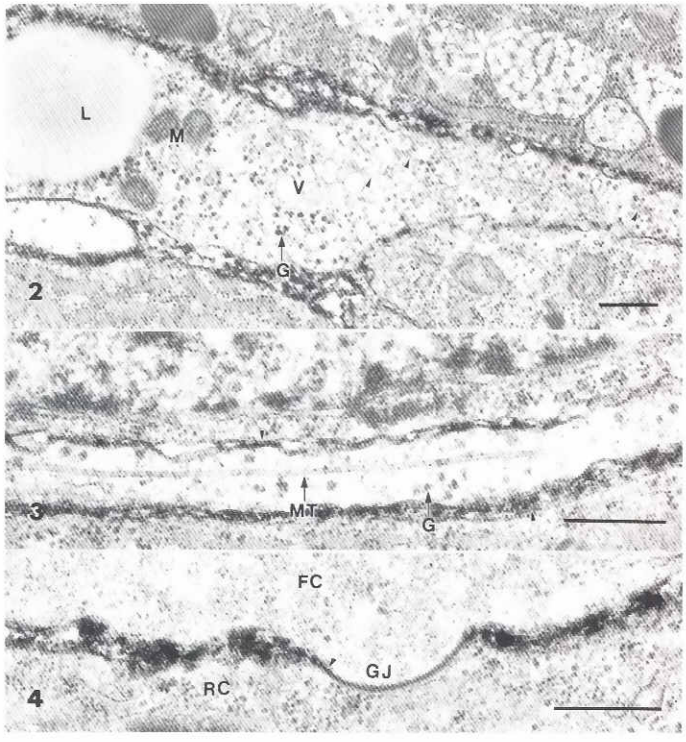


Fig. 1. Parenchymal region of the intact organism. *A typical fixed parenchyma cell is seen extending its long cytoplasmic processes (arrows). The proximal cytoplasmic region contains the nucleus (N), large phagosomes (P) and lipid droplets (L). Each cell is clearly delineated by RR-positive cell coats. F; reticular filaments, MU; muscle fiber, S; secretory cell. Bar= 1 μm.*

Fig. 2. Higher magnification view of the proximal region of the fixed parenchyma cell. *Intact organism. The cytoplasm is occupied by glycogen granules (G), mitochondria (M), lipid droplet (L) and vesicles (V). Microtubules are scattered along its long axis (arrowheads). Bar= 0.5 μm.*

Fig. 3. Adjacent section of the fixed parenchyma cell in Fig. 2. *The cytoplasmic process contains a microtubule (MT) running parallel to the axis. RR-positive surface coats are seen on the plasma membranes (arrowheads). G; glycogen granules. Bar= 0.5 μm.*

Fig. 4. A contact zone between the fixed parenchyma cell (FC) and the regenerative cell (RC) in the intact parenchyma. *Gap junction (GJ) is noted. RR-positive material penetrates the gap area (arrowhead). Bar= 0.5 μm.*

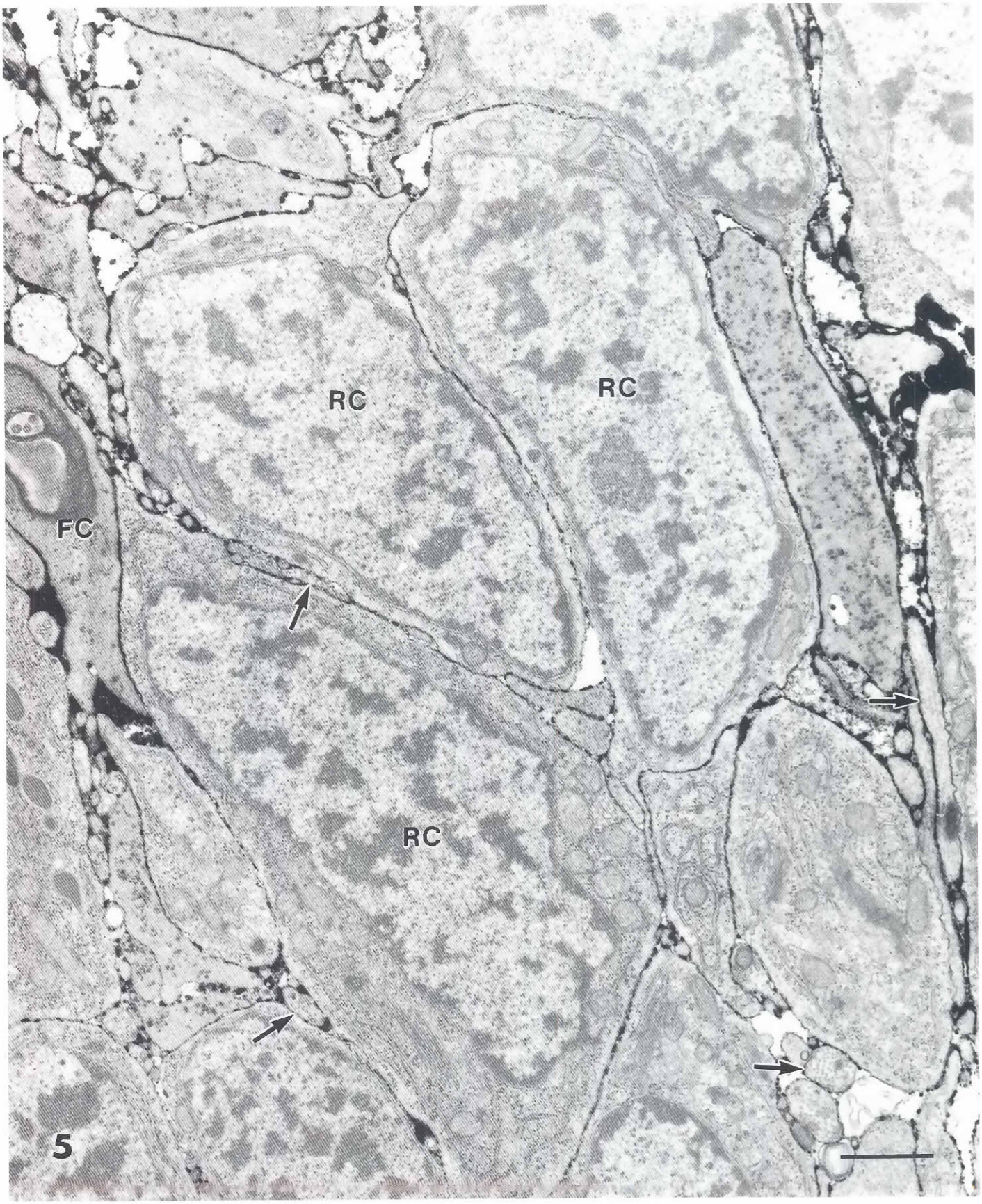
Results

Intact

The subepidermal region of planarians consisted mainly of muscle fibers whose intercellular spaces were filled by cytoplasmic portions of pigment cells and various gland cells. Deeper parenchyma regions were also cellular with a lesser part occupied by extracellular matrices. Large fixed parenchyma cells and small regenerative cells represented specific cell types in this region. The fixed parenchyma cells make full contact with surrounding cells by extending long cytoplasmic processes (Fig. 1). Thus they appeared to serve as connective tissue elements. Although their cytoplasm contained abundant lipid droplets and mitochondria with dense matrix, the cytoplasmic matrix was much less electron dense than that of other cells. The remaining cytoplasm contained a variable amount of glycogen granules, smooth-surfaced vesicles and microtubules except in preparations distorted by poor fixation (Fig. 2). One or more large phagosomes containing condensed cell debris or myelin figures were seen, suggesting their phagocytotic activity (Fig. 1). Microtubules were conspicuous in the proximal cytoplasmic portion (Fig. 2) while the slender distal processes measured 0.2-0.4 μm in width and contained only a few microtubules and glycogen granules (Fig. 3).

The fixed parenchyma cells were usually in contact with surrounding cells through RR-positive cell coats (Figs. 1-4). On occasions, typical gap junctions were observed between the fixed parenchyma cells and regenerative cells (Fig. 4). At these sites adjoining cells were separated by a constant space less than 7 nm wide, filled with RR-positive material. RR also revealed extracellular reticular filaments in the parenchyma. The filaments formed a network attached invariably to the cell coats (Fig. 1). Collagenous fibrils with periodic striation were never observed.

Fig. 5. A middle region of the one-day blastema. *Regenerative cells (RC) build up a compact cell aggregation. Note an abundance of cytoplasmic processes of the fixed parenchyma cells which occupy narrow spaces among the regenerative cells (arrows). Each process contains bundles of neatly arranged microtubules. RR clearly delineates the boundary of blastema cells. FC; a proximal region of the fixed parenchyma cell. Bar= 1 μm.*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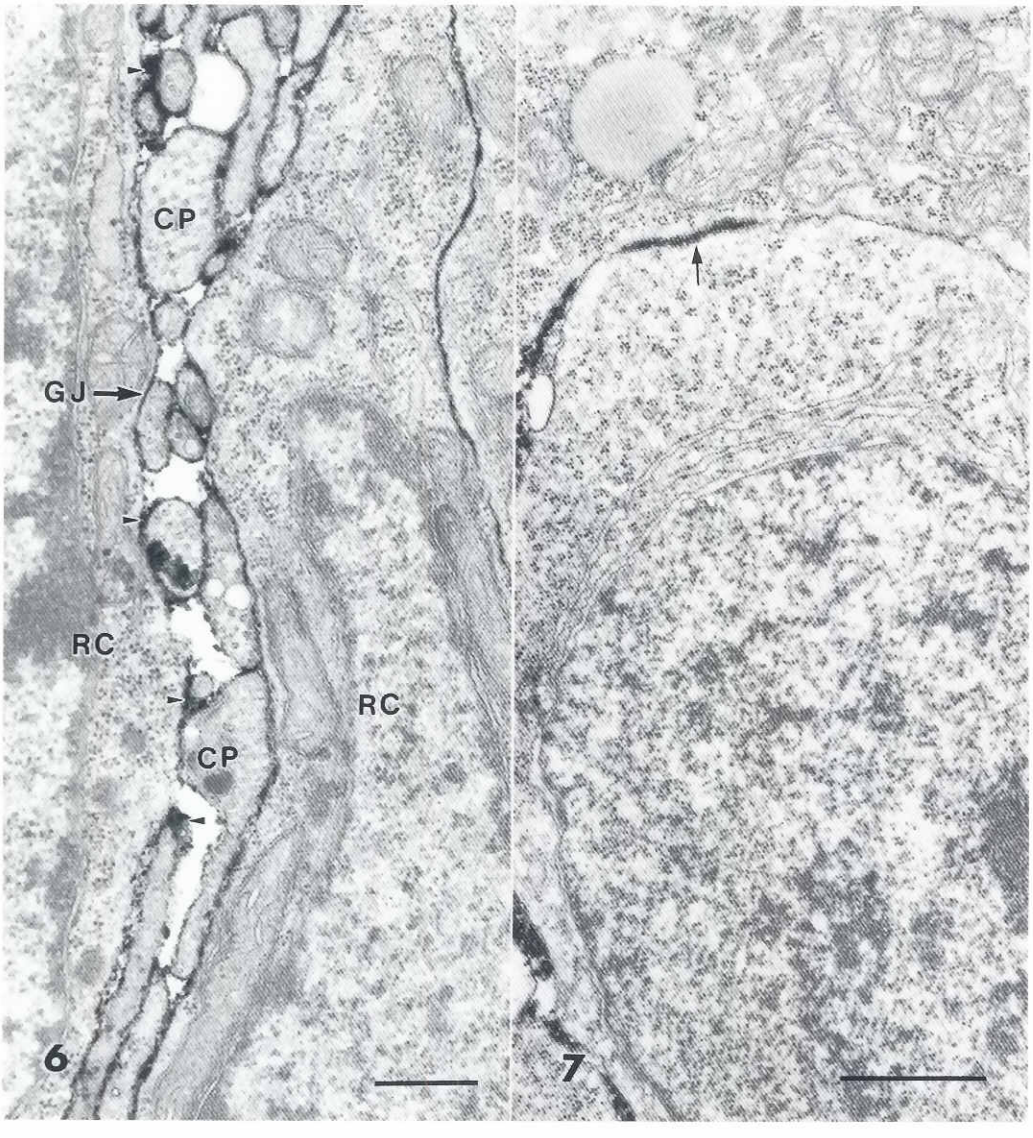

Fig. 6. Higher magnification view of cytoplasmic processes of the fixed parenchyma cells. *One-day blastema. Microtubules are seen within every process (CP). A gap junction-like structure (GJ) is formed between a cytoplasmic process of the fixed parenchyma cell and the adjoining regenerative cell (RC). Partial thickening of RR-positive cell coats is evident at the processes (arrowheads). Bar= 0.5 μm.*

Fig. 7. Apical portion of the migrating regenerative cell showing polarity. *RR-positive cell coat is deposited irregularly because of ruffling of the leading edge (arrow). Bar= 1 μm.*

One-day blastema

A description of the general features of one-day blastemata, as seen in horizontal sections, has been given elsewhere (Hori, in press). During the first day of regeneration the blastema cell population increased by migration of cells forming cell aggregates beneath the wound epidermis. One-day blastema cells were not randomly oriented but usually pointed towards the wound surface. The directional cell movement was prominent particularly in the mid-blastema region where most regenerative cells showed cytoplasmic polarity; a leading edge at the apical region and a tapering posterior region were distinguished. It was noted that the one-day blastema consisted not only of such regenerative cells having a high karyoplasmic ratio but also of cytoplasmic processes of fixed parenchyma cells (Fig. 5). Fixed parenchyma cells could be found throughout the blastema varying greatly in size and shape depending on location. Both in the subepidermal and old stump regions, the fixed parenchyma cells appeared to devote themselves to phagocytizing degenerated cell debris since their cytoplasm was the only evidence of digestion in the vicinity of well-developed Golgi apparatus. In the mid-blastema region, however, these cells were characterized by extensive cytoplasmic processes which intervened between narrow spaces along lateral sides of migrating regenerative cells (Figs. 5, 6). Such processes never occurred around the leading edge of regenerative cells because the plasma membrane

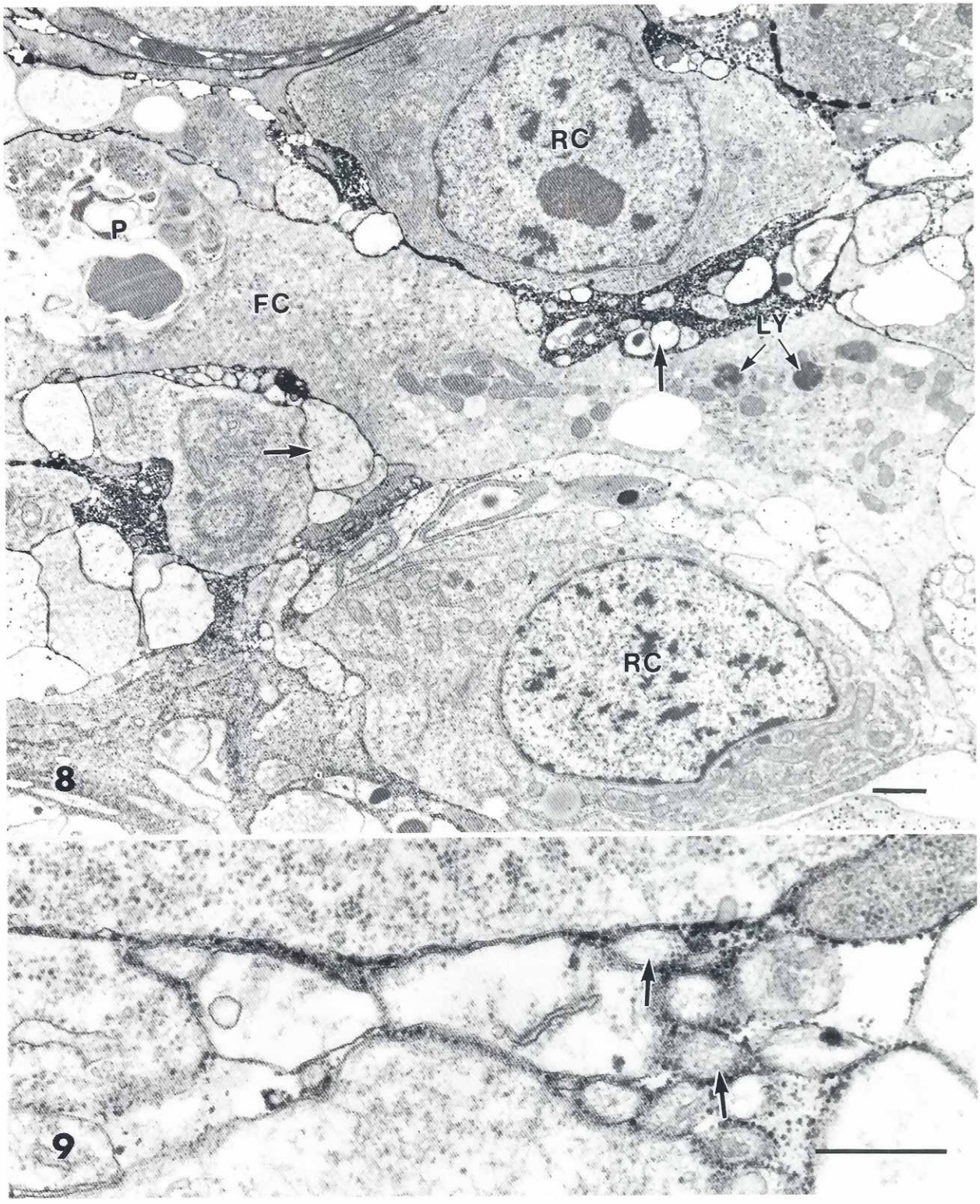

Fig. 8. One-day regenerate treated with colchicine. *Accumulation of regenerative cells is inhibited. The cytoplasm of a large fixed parenchyma cell (FC) contains phagosomes (P), RER, Golgi bodies and lysosome-like granules (LY). Note the absence of microtubules within the cytoplasmic processes (arrows). RC; regenerative cells with no polarization. Bar= 1 μm.*

Fig. 9. A higher magnification view of the cytoplasmic processes. *Colchicine treatment. Disintegrating microtubules are observed within the processes (arrows). Bar= 0.5 μm.*

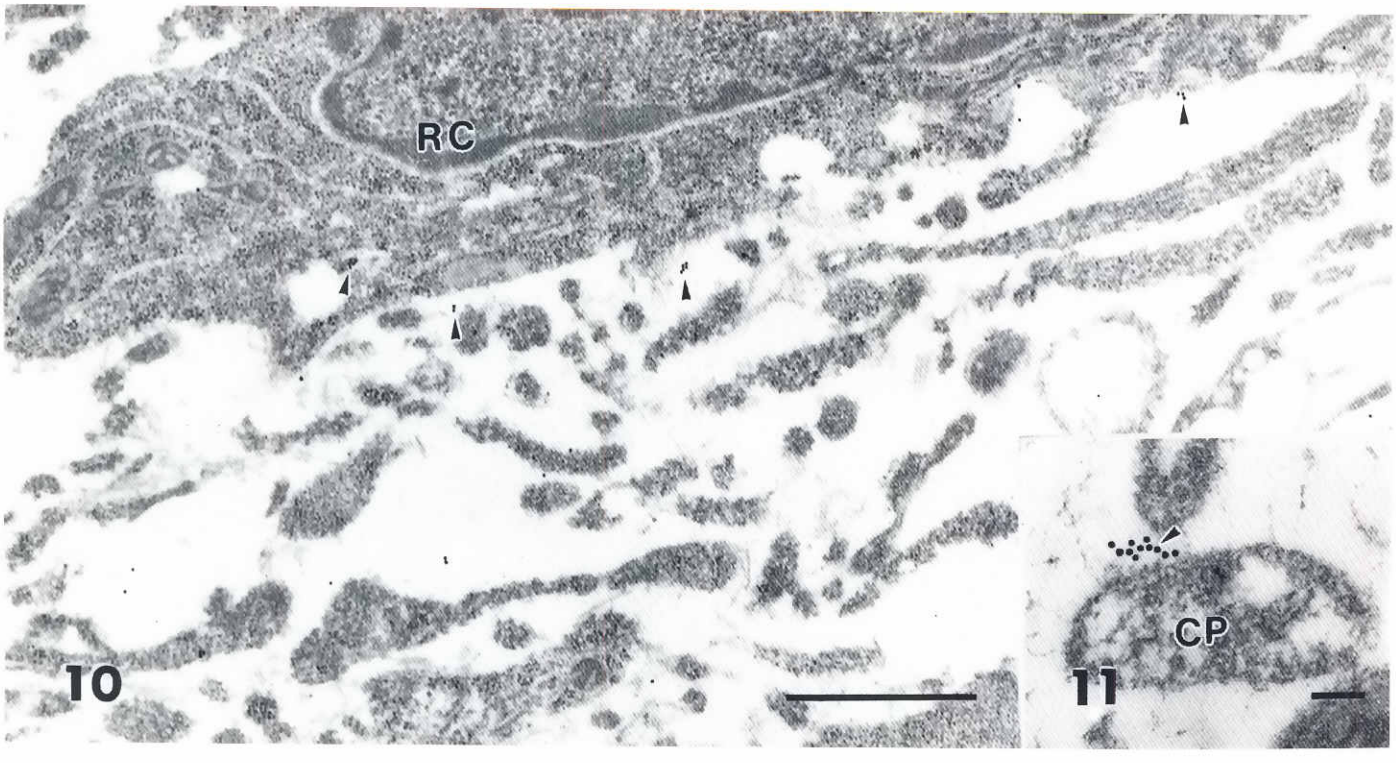

Fig. 10. Immunoelectron micrograph for fibronectin. *One-day blastema. Gold particles are located at the surface area of the regenerative cell (RC) as indicated by arrowheads. Bar= 1 μm.*

Fig. 11. A higher magnification view of immunoelectron micrograph for fibronectin. *One-day blastema. Arrowhead indicates gold particles which are associated with the cytoplasmic process of the fixed parenchyma cell (CP). Bar= 0.1 μm.*

showed overlapping with anteriorly positioned cells (Fig. 7). Usually these cytoplasmic processes occurred in clumps. They were more numerous than the proximal regions. This suggests a highly branched form for the fixed parenchyma cells appearing in this region. The most conspicuous elements of the cytoplasmic processes were microtubules being compacted in bundles and oriented along the long axis (Figs. 5, 6). These microtubules were arranged at regular intervals which could not be observed in the proximal cytoplasm.

RR penetrates into cell contact zones measuring less than 8 nm in width; so individual blastema-forming cells are clearly delineated (Fig. 5). Cell coats or glycocalyx of the cytoplasmic processes displayed electron-dense thickening as revealed by RR staining (Fig. 6). Reticular filaments were absent between the cytoplasmic processes. Gap-type functional attachments were noted between the cytoplasmic process and the regenerative cells (Fig. 6). RR-positive cell coats were irregular at the leading edge of migrating regenerative cells because of ruffling or undulation of the plasma membrane (Fig. 7).

Effects of colchicine on one-day regenerates

The effect of colchicine (M/1,000) on the behavior of fixed parenchyma cells within one-day regenerates was studied. The drug inhibited the accumulation of blastema cells; the large fixed parenchyma in cells show increased phagocytic activity inthe wound region. Their cytoplasm contained large phagosomes and synthetic organelles. Lysosome-like granules were present in the vicinity of Golgi areas. Colchicine treatment also induced disintegration of microtubules within the cytoplasmic processes of the fixed parenchyma cells (Fig. 9). This resulted in detachment of cytoplasmic processes from regenerative cells (Fig. 8). Polarization of regenerative cells was lost.

Immunoelectron microscopy for fibronectin

The distribution of fibronectin was studied by immunoelectron microscopy using protein A-gold labeling of Epon-embedded sections. Fibronectin-immunoreactivity was mostly associated with the surface area of regenerative cells and fixed parenchyma cells forming the one-day blastema (Fig. 10). An abundance of immunogold particles were often seen around the cytoplasmic process of the fixed parenchyma cells (Fig. 11).

Discussion

Despite a wealth of information on the role and importance of regenerative cells or neoblasts in planarian regeneration, the presence of fixed parenchyma cells in the early regeneration blastema has passed largely unnoticed. The present results show numerous fixed parenchyma cells in the one-day blastemata and suggests a role different from that of the intact tissue.

Despite having been considered as «fixed» cells, fixed parenchyma cells are morphologically plastic cells that may somehow control the integrity of the developing blastema. It is reasonable to consider that accumulation of blastema cells after wounding is due mainly to the quick response of regenerative cells and fixed

parenchyma cells. Mobility of fixed parenchyma cells in the regenerating planarians has been previously suggested (Saló and Baguñà, 1985). When cultured *in vitro,* fixed parenchyma cells extend their filopodia of hyaline ectoderm and interdigitate among other cells (Betchaku, 1967). Thus, it is likely that such flexible cytoplasmic processes tend to associate with the lateral sides of migrating regenerative cells. Arranged at the lateral sides, the fixed parenchyma cells seem to maintain regular localization of regenerative cells. Glycogen granules, lipid droplets and mitochondria are concentrated in the proximal cytoplasm of each fixed parenchyma cell (Hay and Coward, 1975; Bowen *et al.*, 1982; Morita and Best, 1984). The distribution of these inclusions should be considered in relation to their function as energy storage. The slender cytoplasmic processes appearing in the mid-blastema region were characterized by an increase in neatly arranged microtubules. Immunocytochemistry for ß-tubulin has shown that microtubules are more abundant in the blastema than in the intact parenchyma (Hori, in press). This difference between regenerate and intact suggests high development of the fixed parenchyma cells giving rise to cytoplasmic processes.

Colchicine treatment caused the disintegration of microtubules in such processes of fixed parenchyma cells. The inhibition of directional locomotion of regenerative cells resulted in poorly formed regeneration blastema (Hori, unpublished data). Although the precise relationships between fixed parenchyma cells and regenerative cells in the early stage of blastema formation is poorly known, our results suggest a close cooperation between two types of cells, a fixed parenchyma cell and a regenerative cell. Gap junctions are very common in planarian epithelial cells. The lanthanum or ruthenium tracers are known to permeate freely the gap region of gap junctions as well as the extracellular spaces of the planarian parenchyma (Quick and Johnson, 1977; Hori, 1986). It is now generally accepted that the main function of the gap junction is to provide the basis for intimate communication between neighboring cells (Staehelin, 1974) and to serve synchronized cell populations for proliferative activity (Minkoff *et al.*, 1991). Therefore it is noteworthy that gap junctions were formed between the regenerative cell and fixed parenchyma cell during blastema formation. The existence of intercellular communication through the gap junctions may provide important pathways necessary for normal localization of each cell.

The extracellular matrix of multicellular organisms consists of various components influencing cell behavior and function (Hay, 1981; Yamada *et al.*, 1985; Gilbert, 1988). The planarian parenchyma is known to possess very primitive extracellular matrices since it contains no distinct fibrous elements (Pedersen, 1961; Hori, 1979). When tissue blocks were treated with RR, the bulk of RR-positive electron-opaque material was seen localized at the cell surface and reticular filaments. According to Jollie and Triche (1971), thickening of the cell coat indicates the area to which extracellular proteins attach. Thus, thickening of cell coats as revealed by RR staining is assumed to reflect activation of the cytoplasmic process of fixed parenchyma cells.

Fibronectin-like molecules have been detected in most invertebrates (Akiyama and Johnson, 1983; Yamada *et al.*, 1985) and in planarians (Lindroos and Wikgren, 1987; Lindroos and Still, 1988; Pascolini *et al.*, 1989). Many authors have suggested that such extracellular glycoproteins may control directional cell locomotion (Erickson *et al.*, 1980; Rovasio *et al.*, 1983; Gilbert, 1988). Furcht *et al.* (1978) have demonstrated that myoblasts have substantial amounts of cell surface fibronectin and that this molecule diminishes as the myoblasts differentiate. The presence of fibronectin-like molecules was evident on the surface area of fixed parenchyma cells and regenerative cells undergoing directional movement. This suggests a possible role of such molecules in providing a microenvironment in which cells migrate regularly and express their differentiation program.

Materials and Methods

Animals

Freshwater planarian *Dugesia japonica* was employed in this study. Worms were kept in tap water without food for one week, transected behind the auricles, and left regenerating in tap water at 18°C. Some of the worms were placed after transection in colchicine containing tap water (M/1,000) at 18°C. After 24 hours, regenerates from both groups were trimmed off carefully and prepared for electron microscopy.

Electron microscopy for ruthenium red staining

Specimens were prepared for ruthenium red (RR) staining as described in Luft (1966). Specimens were fixed for 60 min at 4°C in 1.2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) containing 0.1% RR (TAAB Lab.) and postfixed for 3 h at room temperature in 2% osmium tetroxide in the same buffer. Then, they were dehydrated with increasing concentrations of ethanol (50-100%) and embedded in Epon 812. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome using a diamond knife, and mounted on copper grids. After counterstaining with uranyl acetate and lead citrate, sections were examined in a Hitachi H-500 electron microscope.

Immunoelectron microscopy

Regenerates were fixed in PLP solution (McLean and Nakane, 1974) containing 2% paraformaldehyde and 0.1 M lysin in 0.05 M phosphate buffer (pH 7.4) overnight at 4°C . The specimens were washed in PBS, dehydrated in ethanol and embedded in Epon 812. Ultrathin sections mounted on nickel grids were stained with the PAG method (Roth *et al.*, 1978). Grids were washed in PBS containing 1% BSA and incubated overnight at 4°C with anti-human fibronectin antibody (Biomed. Tech. Inc.) diluted 1:500 in PBS-BSA. Control grids were incubated in PBS-BSA. After, they were treated with Protein A-Gold colloid diluted 1:10 in PBS-BSA for 45 min at room temperature. After washing in PBS and in distilled water they were stained with uranyl acetate and lead citrate.

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