CYTOPLASMIC FILAMENTS AND GAP JUNCTIONS IN EPITHELIAL CELLS AND MYOFIBROBLASTS DURING WOUND HEALING

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ABSTRACT

During the healing of an experimental skin wound, epidermal cells and granulation tissue fibroblasts (myofibroblasts) develop an extensive cytoplasmic contractile apparatus. Concurrently, the proportion of epidermal cell surface occupied by gap junctions increases when compared to normal skin, and newly formed gap junctions appear between myofibroblasts; this suggests that epidermal cell migration and granulation tissue contraction are synchronized phenomena.

KEY WORDS epithelialization · microfilaments · gap junctions · anti-actin antibodies · cell movement · wound contraction

It is now well established that several nonmuscular cells contain contractile proteins (2, 15, 19, 24, 25, 33) which are mostly organized in the form of a cytoplasmic filamentous apparatus. Microfilaments appear *de novo* in epidermal cells during the healing of an open wound (14, 23) and in myofibroblasts of the underlying granulation tissue (12). The present paper reports observations on a concomitant neoformation of gap junctions in both these cell populations.

MATERIALS AND METHODS

15 male Wistar rats weighing ~150 g and two rabbits weighing ~2 kg were used for these studies. From the back of each rat and rabbit, a square of skin 4 cm^2 in area (with cutaneous muscle in rats and without it in rabbits) was removed on the first day of the experiment. By the 7th–8th days in rats (10th–15th days in rabbits) the wounds became markedly contracted and a rim of newly formed epithelium could be identified at their periphery. At this time, the animals were sacrificed and tissue samples were taken from the periphery of the wounds for fine structural and immunofluorescence studies (14). Samples of normal skin taken from the same animals at more than 3-cm distance from the wounds served as controls.

Electron Microscopy

Fixation was initiated by immersion of tissue samples in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 5 h at room temperature. The tissues were washed overnight at 4°C in cacodylate buffer and post-fixed for 90 min in 2% OsO_4 in S-collidine buffer (pH 7.4). They were then treated for 2 h at 4°C with 2% uranyl acetate (21) in 0.05 M sodium hydrogen maleate-NaOH buffer (pH 7.0), washed briefly in maleate buffer (pH 5.2), dehydrated in alcohol, and flat embedded in Epon 812. For light microscopy, 1-μm sections were stained with methylene blue-azur II. Thin sections were cut with a diamond knife on an LKB III ultrotome (LKB Instruments, Inc., Rockville, Md.) and examined after uranyl acetate and lead citrate staining on a Philips EM-300 microscope.

Morphometry

For morphometric evaluation, 70 × 70 mm pictures were taken randomly from normal rat epidermis (three animals, 15 blocks, 15 micrographs per block) and epidermis from the advancing edge of the wounds (three

animals, 7 blocks, 30 micrographs per block) at the constant direct magnification of 11,500. Positive films of these images were projected on a screen containing squares delimited by 12 equidistant vertical and 12 equidistant horizontal lines. We calculated the surface density (S_V, or total surface included in the unity test volume) of cells, desmosomes and gap junctions. This is represented by the general formula:

$$S_V = \frac{2N_i}{L_t},$$

where N_i = number of interactions between the test grid and the structure and L_t = total length of the lines of the grid divided by the magnification of the micrographs (in order to relate the results to the examined tissue) (38).

Freeze-Fracturing

Samples for freeze-fracturing were fixed in 3% glutaraldehyde, rinsed in cacodylate buffer, and infiltrated progressively with glycerol up to a 25% concentration. The tissues were frozen in Freon 22 and freeze-fractured at −115°C in a Balzers' 301 apparatus (Balzers AG, Balzers, Liechtenstein).

Immunofluorescence

Anti-actin antibodies (AAA) were obtained from the serum of two patients with chronic aggressive hepatitis (3, 15) having a titer of 1/1,280 and 1/640, respectively, when tested on rat intestinal smooth muscle. These sera were passed on columns of Sepharose covalently linked (4) with rabbit skeletal muscle actin (3), followed by elution of the antibody at pH 2.7. The specificity of these antibodies was tested by immunodiffusion (3, 10), immunoelectrophoresis, and immunofluorescence. With this test, AAA was fixed on striated (I bands) and smooth muscle, platelets, and cultivated fibroblasts. Anti-smooth muscle myosin antibodies (AMA) were raised in two rabbits after four subcutaneous injections of 1 mg of purified myosin from pregnant rat uterus (2). The titer of the antibody containing serum was 1/1,280 as judged by immunofluorescent staining of rat intestinal smooth muscle. The specificity of these antibodies was tested by immunofluorescence, immunoabsorption, and immunodiffusion, using the myosin utilized for immunization. Immunofluorescent staining was positive for smooth muscle, platelets, and cultivated fibroblasts (9) but not for striated and cardiac muscles. Incubation with purified myosin abolished the capacity of the antibody containing sera to stain smooth muscle, platelets, and fibroblasts. Immunodiffusions against crude and purified myosin gave a single precipitation line.

Cryostat sections, 4 μm thick, were fixed for 5 min in acetone at −20°C, then incubated for 15 min with AAA or AMA. After incubation, the sections were washed in phosphate-buffered saline (PBS) and then stained for 15 min either with fluorescein-conjugated IgG fraction of goat antiserum to human IgG (code 64.170, Miles Seravac, Lausanne, Switzerland) for AAA or with fluorescein-conjugated IgG fraction of sheep antiserum to rabbit IgG (Behring Werke AG, Marburg-Lahn, West Germany) for AMA. After rewashing in PBS and mounting in 90% glycerol in PBS, the level of fluorescence was compared with that found in control preparations treated with normal human serum instead of AAA or AMA sera.

RESULTS

The light microscope appearances of normal rat skin and of an 8-day-old healing wound are shown in Fig. 1*a* and *b*. Normal rat or rabbit skin consists of an epidermis composed of 2–3 layers of epithelial cells, and the underlying dermis consists mainly of collagen, fibroblasts, and small vessels. The base of the open wound consists of granulation tissue in which many large fibroblastic cells are seen closely connected with one another. At the periphery of the wound, the advancing edge of newly formed epithelium can be identified. When compared to their normal counterpart, these moving epithelial cells are paler and packed in several layers.

In agreement with previous observations (12, 14), immunofluorescence revealed that, contrary to their normal counterparts, myofibroblasts and newly growing epithelial cells fix AAA (Fig. 2*a* and *b*); moreover, they fix AMA (Fig. 3*a* and *b*).

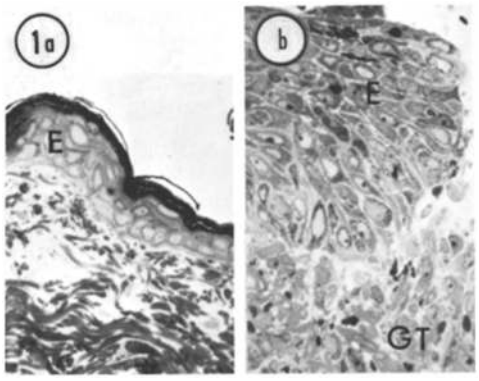


FIGURE 1 Methylene blue-azur II stained 1-μm thick sections of (*a*) control adult rat skin and (*b*) periphery of 8-day-old skin wound. In controls the epithelium (*E*) consists of 2–3 layers of cells. In the healing wound, the growing edge of epithelium (*E*) has paler, loosely packed cells as compared with control. The main cell components of the underlying granulation tissue (*GT*) are myofibroblasts. × 400.

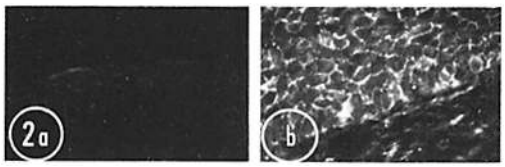


FIGURE 2 Cryostat sections incubated with AAA serum followed by fluorescein-conjugated antihuman IgG, from (*a*) control rabbit skin and (*b*) periphery of 15-day-old wound. Note bright immunofluorescent staining at the periphery of the newly growing epithelial cells. × 400.

The electron microscope appearance of normal rat and rabbit skin was in agreement with previous reports (1, 6). In particular, no microfilaments were visible in normal epidermal cells while many tonofilaments and junctions of the macula adherens "desmosome" type were observed (Figs. 4, 5, and 6). Small gap junctions were found only exceptionally in the stratum malpighii. In the underlying dermis, individual fibroblasts, segregated by large masses of collagen, were visible. Only occasionally, they contained few microfilaments. When compared with normal skin, the healing wounds revealed striking differences in both epithelial and fibroblastic cells. Epithelial cells showed a more irregular configuration with several microvillous cytoplasmic projections and contained well-defined cytoplasmic microfilaments predominantly located at the cell periphery (14) (Fig. 7). Tonofilaments were less abundant than in normal epithelium. The space between single epithelial cells was wide and irregular with few desmosomes when compared with controls. However, cytoplasmic projections of adjacent epithelial cells were interconnected by prominent gap junctions (Fig. 5). These gap junctions varied in size and number between different epithelial cells, but they could be easily identified in each electron microscope field of uranyl en bloc-treated preparations at a direct magnification of 11,500.

The following parameters were determined morphometrically in normal epidermal cells and in cells growing over the wound: (*a*) total surface density (S_{Vt}); (*b*) the proportion of cell surface occupied by gap junctions (S_{Vgj}/S_{Vt}) and (*c*) the proportion of cell surface occupied by desmosomes (S_{Vd}/S_{Vt}) (Table I). The evaluation of these results by means of Student's *t* test shows that the increase of S_{Vt} in epidermal cells over the wound when compared to normal cells is not statistically significant ($0.1 < P < 0.2$). On the other hand, the proportion of the cell surface occupied by gap junctions is significantly greater in epidermal cells growing over the wound than in normal cells ($2P < 0.05$). Moreover, the proportion of cell surface occupied by desmosomes is significantly lower in epidermal cells growing over the wound than in normal cells ($2P < 0.025$).

In the underlying granulation tissue, fibroblastic cells exhibited the characteristic cytoplasmic features of myofibroblasts (12). In particular, these cells were interconnected at several points by distinct gap junctions (Fig. 6); they contained a prominent microfilamentous cytoplasmic apparatus (Fig. 8) and had hemidesmosomelike specializations anchoring them to basement membranelike material situated beneath the plasma membrane (12).

Freeze-fracture replicas of normal rat skin disclosed epithelial cell membranes with sparse, randomly arranged intramembranous particles, as well as many heterogeneous clusters of 8- to 10-nm particles with the dimensions and distribution of desmosomes (6, 31). Only very few regularly packed plaquelike aggregates of small gap junctions were identified. Gap junctions were not seen on plasma membranes of occasional fibroblasts located among profiles of fractured dermal collagen. Freeze-fracture replicas prepared from the periphery of 7- to 8-day skin wounds revealed epithelial cell membranes with frequent gap junctions of various sizes. Some of the gap junctions occupied a considerable part of the exposed fracture face (Fig. 9). Desmosomes were also identified on epithelial cell membranes but less frequently than in normal rat epidermis. Fibroblasts were easily identified on replicas exposing granulation tissue in the proximity of the newly growing epithelium. Fracture faces of plasma membranes devoid of any particle aggregate were frequently seen. Some exposed fracture faces of fibroblast plasma membranes, however, showed zones of

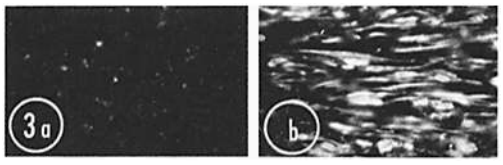
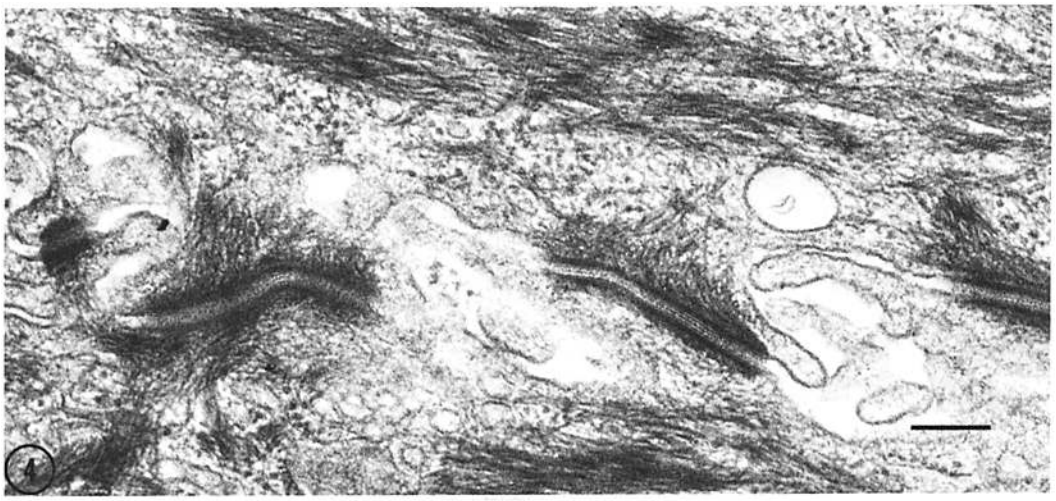
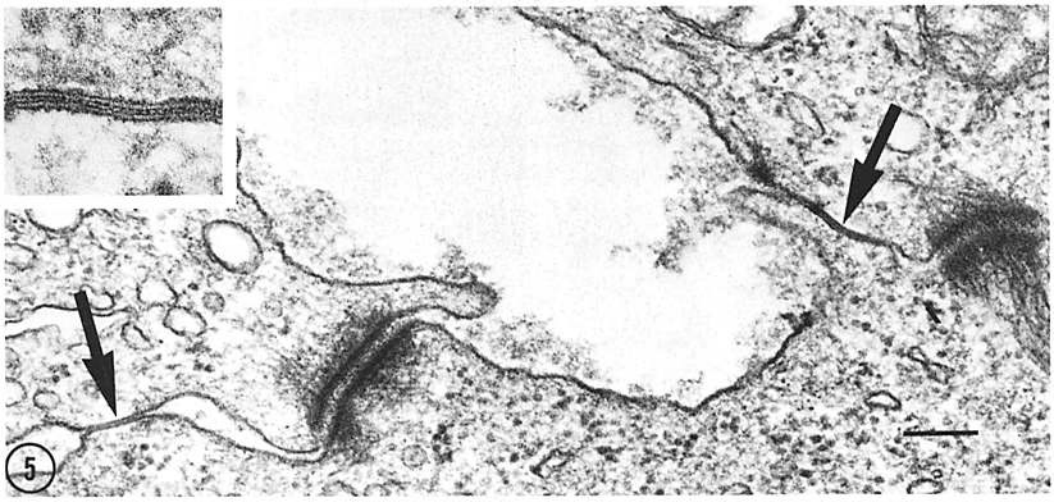


FIGURE 3 Cryostat sections of rabbit granulation tissue incubated with (*a*) normal rabbit serum and (*b*) AMA serum; both sera were followed by fluorescein conjugated antirabbit IgG. Note bright immunofluorescent staining of myofibroblasts. × 400.





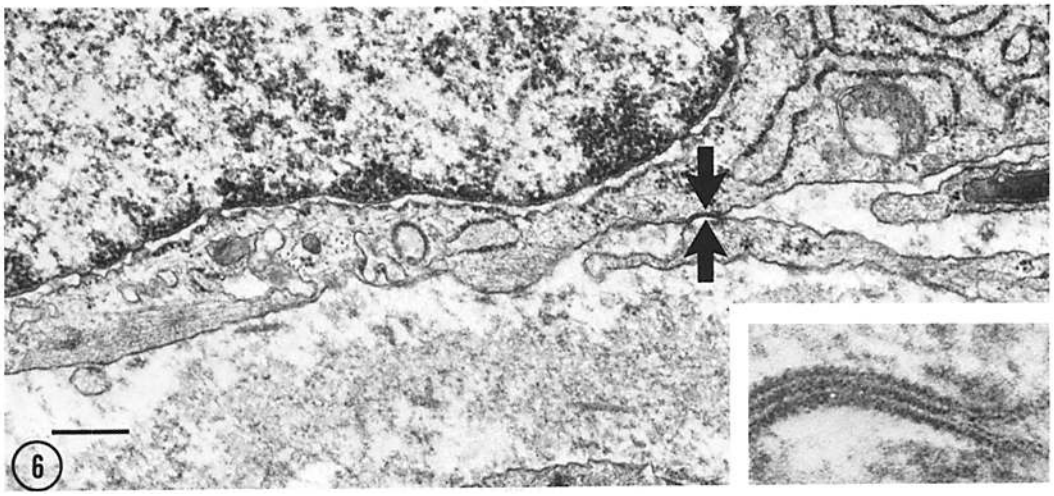


FIGURE 4 Electron micrograph of epithelial cells from the basal layer of epidermis of control rat skin. Prominent features are: abundance of tonofilaments in the cytoplasm and numerous desmosomes between adjacent epithelial cells. Bar, 0.2 μm. × 51,400.

FIGURE 5 Electron micrograph of adjacent epithelial cells from the growing edge of epithelium of an 8-day-old skin wound of rat. There are few desmosomes and adjacent epithelial cells are interconnected by gap junctions (arrows and *inset*). Bar, 0.2 μm. × 51,000; *inset* × 127,000.

FIGURE 6 Myofibroblasts from rabbit granulation tissue. Note microfilaments located mainly at the cell periphery and gap junction (arrows and *inset*) connecting two adjacent cells. Bar, 0.2 μm. × 24,000; *inset* × 163,130.

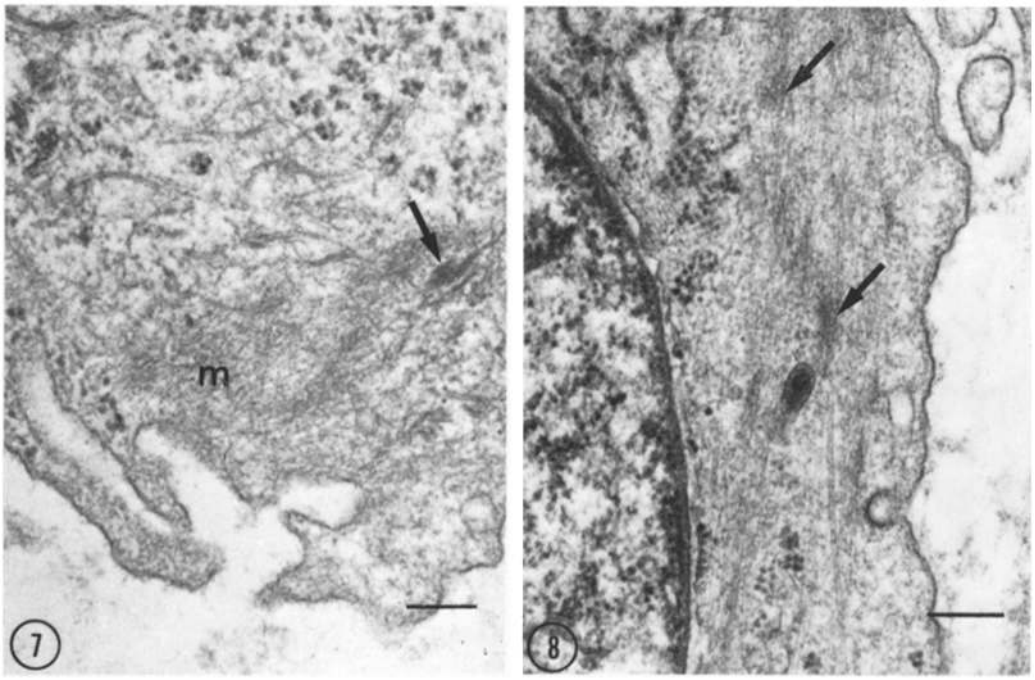


FIGURE 7 Rabbit epithelial cell regenerating over a 10-day-old wound. A microfilaments network (*m*) is visible at the cell periphery. Tonofilaments (arrow) are more centrally located. Bar, 0.2 μm. × 43,000.

FIGURE 8 Myofibroblasts from the granulation tissue underlying the wounds. Bundles of cytoplasmic microfilaments with electron-dense areas scattered in between (arrows) are visible. Bar, 0.2 μm. × 48,400.

TABLE I

*Morphometric Evaluation of Cell and Junction Surfaces in Normal and Healing Epidermal Cells**

Structures evaluated	Normal cells	Healing cells
S_V gap junctions (S_{Vgj})	0.0005 ± 0.0004	0.0024 ± 0.0007
S_V desmosomes (S_{Vd})	0.1218 ± 0.0158	0.1014 ± 0.0135
S_V total cell surface (S_{Vt})	1.0441 ± 0.1289	1.3214 ± 0.0037
$\frac{S_{Vgj}}{S_{Vt}}$	0.0005 ± 0.0003	0.0018 ± 0.0005
$\frac{S_{Vd}}{S_{Vt}}$	0.1205 ± 0.0108	0.0765 ± 0.0102

* The values are expressed as mean ± standard error (μm^{-1}).

typical gap junction maculae. Within the maculae, the membrane particles were regular in both shape and dimensions, contrasting with the heterogeneity shown by the membrane particles of undifferentiated zones of the plasma membranes.

DISCUSSION

These immunofluorescence and electron microscope observations confirm previous findings on the development of actin filaments both in newly growing epithelium and in myofibroblasts of an open skin wound (12, 14). Antimyosin antibodies have revealed a parallel increase of myosin immunofluorescent staining in both cell populations, thus giving further support to the possibility that the presence of contractile proteins is related to contraction and/or locomotion of these cells. Furthermore, it appears that neoformation of contractile filaments is paralleled by the neoformation of gap junctions between myofibroblasts and by an

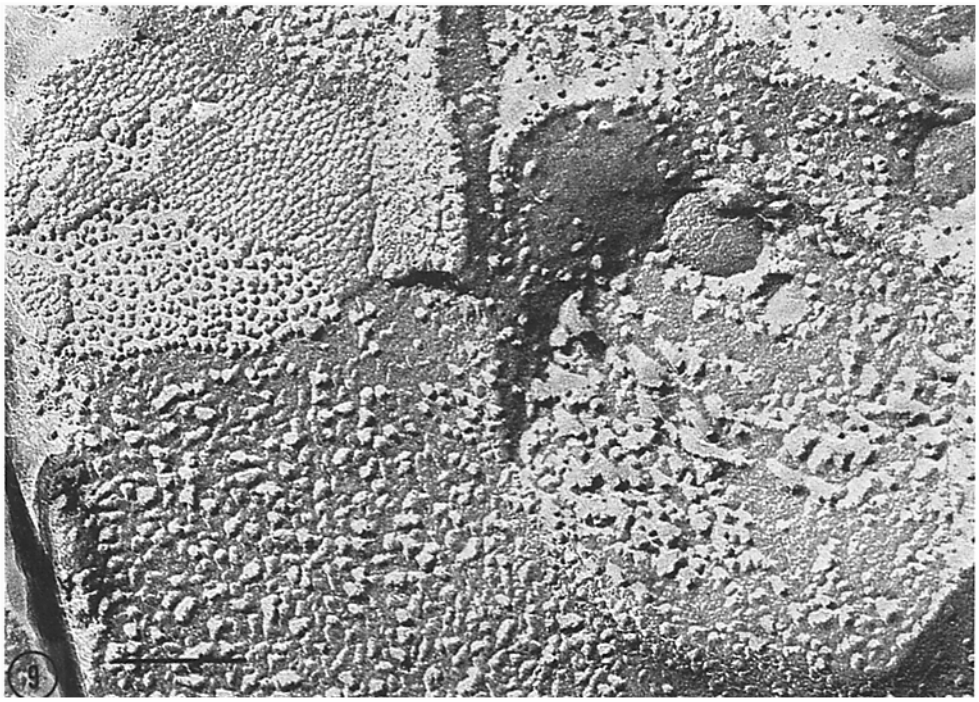


FIGURE 9 Freeze-fracture replica of newly growing epithelium from 8-day-old skin wound of rat. Note closely packed particles of a large gap junction on the P face of epithelial cell membrane and regular arrays of complementary depressions on the E-fracture face. Heterogeneous clusters of irregularly sized particles adjacent to the gap junction represent desmosomes on the P face. Bar, 0.1 μm. × 172,400.

increase of the surface occupied by gap junctions in regenerating epidermal cells when compared to normal cells. In normal adult animals, we have seen no gap junctions between dermal fibroblasts (10) and only occasional gap junctions between epidermal cells. These findings correlate with the observations reported in other animal species (6); however they are in contrast with the description of frequent gap junctions in the basal layer of human epidermis (18).

Gap junctions are considered to be low resistance pathways for intercellular communication (8, 17, 31, 36). They are widely implicated in cell-to-cell transfer of ions (ionic or electrotonic coupling) (16, 31, 34) and in cell-to-cell transfer of metabolites (metabolic coupling) (16). The cells in most adult epithelia are interconnected by such communicating junctions (26, 27, 35). However, junctional interconnections are particularly widespread during early embryonic development (20, 21). The association of a contractile apparatus with gap junctions in epithelial cells growing over an open wound suggests a synchronized locomotion. It has been shown that the movement of epithelial sheets in culture results primarily from the locomotory activity of the marginal cells. However, no comments have been made in this study on the presence and function of gap junctions between moving epithelial cells (5).

The presence of gap junctions between myofibroblasts suggests that granulation tissue contraction is a synchronized phenomenon analogous with that in contractile tissues such as cardiac and smooth muscles, where association of contractile proteins with gap junctions is a characteristic feature (29, 30, 37).

Neoformation of cytoplasmic contractile filaments has been reported in several experimental or pathologic conditions (11, 12, 13, 14, 28). Similarly, membrane specializations such as tight or gap junctions were shown to increase after pharmacologic or hormonal stimulation (7, 8, 32). The observations presented here indicate that contractile proteins and gap junctions increase concurrently in such different tissues as epithelium and connective tissue after stressful changes in their microenvironment. In growing epithelium, individual cells gain the ability to

move, in order to rearrange themselves into an appropriate pattern; in granulation tissue, the contraction of a single myofibroblast is transmitted to other cells and to the stroma; in both cases, the response is probably synchronized rather than individual.

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REFERENCES

1. Berridge, M. J., and J. L. Oschman. 1972. Transporting Epithelia. Academic Press, Inc., New York.
2. Burridge, K., and D. Bray. 1975. Purification and structural analysis of myosin from brain and other non-muscle tissues. *J. Mol. Biol.* **99:**1-14.
3. Chaponnier, C., L. Kohler, and G. Gabbiani. 1977. Fixation of human anti-actin autoantibodies on skeletal muscle fibers. *Clin. Exp. Immunol.* **27:**278-284.
4. Cuatrecasas, P., M. Wolchek, and C. B. Anfinsen. 1968. Selective enzyme purification by affinity chromatography. *Proc. Natl. Acad. Sci. U. S. A.* **61:**636-643.
5. Dipasquale, A. 1975. Locomotory activity of epithelial cells in culture. *Exp. Cell Res.* **94:**191-215.
6. Elias, P. M., and D. S. Friend. 1975. The permeability barrier and pathways of transport in mammalian epidermis. *J. Cell Biol.* **65:**180-191.
7. Elias, P. M., and D. S. Friend. 1976. Vitamin-A-induced mucous metaplasia. An in vitro system for modulating tight and gap junction differentiation. *J. Cell Biol.* **68:**173-188.
8. Friend, D. S., and N. B. Gilula. 1972. Variations in tight and gap junctions in mammalian tissues. *J. Cell Biol.* **53:**758-776.
9. Fujiwara, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.* **71:**848-875.
10. Gabbiani, G., C. Chaponnier, A. Zumbe, and P. Vassalli. 1977. Actin and tubulin cocap with surface immunoglobulins in mouse B-lymphocytes. *Nature (Lond.).* **269:**697-698.
11. Gabbiani, G., J. Csank-Brassert, J. C. Schneeberger, Y. Kapanci, P. Trenchev, and E. J. Holborow. 1976. Contractile proteins in human cancer cells. Immunofluorescent and electron microscopic study. *Am. J. Pathol.* **83:**457-468.
12. Gabbiani, G., B. J. Hirschel, G. B. Ryan, P. R. Statkov, and G. Majno. 1972. Granulation tissue as a contractile organ: a study of structure and function. *J. Exp. Med.* **135:**719-734.
13. Gabbiani, G., R. Montesano, B. Tuchweber, M. Salas, and L. Orci. 1975. Phalloidin-induced hyperplasia of actin filaments in rat hepatocytes. *Lab. Invest.* **33:**562-569.
14. Gabbiani, G., and G. B. Ryan. 1974. Development of a contractile apparatus in epithelial cells during epidermal and liver regeneration. *J. Submicrosc. Cytol.* **6:**143-157.
15. Gabbiani, G., G. B. Ryan, J. P. Lamelin, P. Vassalli, G. Majno, C. A. Bouvier, A. Cruchaud, and E. F. Lüscher. 1973. Human smooth muscle autoantibody: its identification as anti-actin antibody and a study of its binding to "nonmuscular" cells. *Am. J. Pathol.* **72:**473-488.
16. Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (Lond.).* **235:**262-265.
17. Goodenough, D. A., and J. P. Revel. 1970. A fine structural analysis of intercellular junctions in the mouse liver. *J. Cell Biol.* **45:**272-290.
18. Hashimoto, K. 1971. Intercellular spaces of the human epidermis as demonstrated with lanthanum. *J. Invest. Dermatol.* **57:**17-31.
19. Ishikawa, H., R. Bischoff, and H. Holtzer. 1969. Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.* **43:**312-328.
20. Ito, S., and N. Hori. 1966. Electrical characteristics of Triturus egg cells during cleavage. *J. Gen. Physiol.* **49:**1019-1027.
21. Ito, S., and W. R. Loewenstein. 1969. Ionic communication between early embryonic cells. *Dev. Biol.* **19:**228-243.
22. Karnovsky, M. J. 1967. The ultrastructural basis of capillary-permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35:**213-236.
23. Krawczyk, W. S. 1971. A pattern of epidermal cell migration during wound healing. *J. Cell Biol.* **49:**247-263.
24. Lampert, I. A., P. Trenchev, and E. J. Holborow. 1974. Contractile protein changes in the regenerating rat liver. *Virchows Arch. Abt. B Zellpathol.* **15:**351-355.
25. Lazarides, E., and K. Weber. 1974. Actin antibody: the specific visualization of actin filaments in nonmuscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **71:**2268-2272.
26. Loewenstein, W. R. 1966. Permeability of membrane junctions. Conference on Biological Membranes: Recent Progress. *Ann. N. Y. Acad. Sci.* **137:**441-472.
27. Loewenstein, W. R., S. J. Socolar, S. Haga-

SHINO, Y. KANNO, and N. DAVIDSON. 1965. Intracellular communication: Renal urinary bladder, sensory, and salivary gland cells. *Science (Wash. D. C.)*. **149:**295-298.

28. MALECH, H. L., and T. L. LENTZ. 1974. Mirofilaments in epidermal cancer cells. *J. Cell Biol.* **60:**473-482.
29. MATTER, A. 1973. A morphometric study on the nexus of rat cardiac muscle. *J. Cell Biol.* **56:**690-696.
30. MCNUTT, N. S., and R. S. WEINSTEIN. 1970. The ultrastructure of the nexus. A correlated thin-section and freeze-cleave study. *J. Cell Biol.* **47:**666-688.
31. MCNUTT, N. S., and R. S. Weinstein. 1973. Membrane ultrastructure at mammalian intracellular junctions. *Progr. Biophys. Mol. Biol.* **26:**45-101.
32. MONTESANO, R., G. GABBIANI, A. PERRELET, and L. ORCI. 1976. In vivo induction of tight junction proliferation in rat liver. *J. Cell Biol.* **68:**793-798.
33. POLLARD, T. D., and R. R. WEIHING. 1974. Actin and myosin and cell movement. *CRC Crit. Rev. Biochem.* **2:**1-65.
34. REVEL, J. P., and M. J. KARNOVSKY. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33:**C7-C12.
35. REVEL, J. P., A. G. YEE, and A. J. HUDSPETH. 1971. Gap junctions between electrotonically coupled cells in tissue culture and in brown fat. *Proc. Natl. Acad. Sci. U. S. A.* **68:**2924-2927.
36. STAEHELIN, L. A. 1974. Structure and function of intercellular junctions. *Int. Rev. Cytol.* **39:**191-283.
37. UEHARA, Y., and G. BURNSTOCK. 1970. Demonstration of "gap junctions" between smooth muscle cells. *J. Cell Biol.* **44:**215-217.
38. WEIBEL, E. R., and H. ELIAS. 1967. Introduction to stereologic principles. *In* Quantitative Methods in Morphology. E. R. Weibel and H. Elias, editors. Springer-Verlag, Berlin. 89-98.