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Hemidesmosomes in the epithelial cell line 804G: their fate during wound closure, mitosis and drug induced reorganization of the cytoskeleton

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Summary

Recently, we identified a novel epithelial cell line, 804G, derived from rat bladder, which readily forms hemidesmosomes *in vitro*. One of the major structural components of the plaques of 804G cell hemidesmosomes is a 230 kDa antigen recognized by autoantibodies in the sera of patients with bullous pemphigoid (BP). An additional polypeptide of 180 kDa also localizes to the hemidesmosome plaque of 804G cells as determined by immunoelectron microscopy. Using confocal fluorescence/phase microscopy, we have employed both 230 kDa and 180 kDa antibody probes to monitor the fate of hemidesmosomes following closure of *in vitro* wounds, during mitosis, and following drug induced disruption of the cytoskeleton. The punctate cell-substratum associated staining generated by the hemidesmosomal antibodies in stationary unwounded 804G cell cultures is greatly diminished or even lost in cells which enter wound sites, presumably in response to enhanced cell motility. Few, if any, hemidesmosomes are observed at the ultrastructural level in cells which have migrated into the wound area. However, as closure of the wound becomes complete, staining along the substratum attached surface of cells returns. During mitosis, there is no obvious loss of hemidesmosomal antigens along the basal surface of 804G cells, and formed hemidesmosomes can be observed in mitotic cells at the ultrastructural level. In 804G cells treated with colchicine, the typical subnuclear pattern of distribution of hemidesmosomal antigens is unaffected. In contrast, following treatment of 804G cells with cytochalasin D, hemidesmosomal antigens become concentrated at the cell periphery and no longer appear in the subnuclear region. Furthermore, formed hemidesmosomes are observed at the cell periphery of cytochalasin D-treated cells by electron microscopy. We suggest that hemidesmosomal plaques are mobile within the plasma membrane. We speculate that hemidesmosomal interactions with extracellular ligands are dynamic and we discuss a possible mechanism by which cytochalasin D induces reorganization of hemidesmosomes along the basal surface of 804G cells.

Key words: adhesion, cytoskeleton, anchorage device.

Introduction

Hemidesmosomes are cell surface anchorage sites for intermediate filaments (Kelly, 1966; Staehelin, 1974; Ellison and Garrod, 1984). They are located along the basal surface of an epithelial cell where it is in contact with the underlying substratum. The hemidesmosome is presumed to function as an epithelial cell-substratum attachment device (Staehelin, 1974). Indeed, in a variety of blistering diseases where there is a disruption of the hemidesmosomal complex, there is a concomitant perturbation in epithelium-connective tissue adherence (Schaumberg-Lever et al., 1972; Chapman et al., 1990).

The hemidesmosome is a complex morphological and biochemical entity. Its ultrastructural characteristics have been detailed by a number of workers (Weiss and Ferris, 1954; Kelly, 1966; Sheinvold and Kelly, 1976; Buck, 1983; Ellison and Garrod, 1984; Klatte et al., 1989). More recently, information concerning its molecular composition has been presented (Klatte et al., 1989; Schwarz et al., 1990; Owaribe et al., 1991; Tanaka et al., 1991). For example, regions of three polypeptides of 230, 200, and 180 kDa reside in the hemidesmosomal plaque to which keratin bundles attach (Klatte et al., 1989; Kurpakus and Jones, 1991). The 180 kDa hemidesmosomal polypeptide, originally characterized using human autoantibodies, has been shown to be a type II membrane protein (Klatte et al., 1989; Hopkinson et al., 1992).

The transmembrane integrin complex $\alpha_6\beta_4$ is also found in the hemidesmosome and the cytoplasmic domains of either the α_6 or β_4 subunits may play a role in cytoskeleton-hemidesmosomal interaction (Stepp et al., 1990; Jones et al., 1991; Quaranta and Jones, 1991; Sonnenberg et al., 1991). Although the extracellular ligand for the $\alpha_6\beta_4$ complex has not been defined biochemically, several groups have identified candidate molecules for this ligand (Kurpakus et al., 1991; Jones et al., 1991; Carter et al., 1991; Rousselle et al., 1991). The latter are apparently associated with the so-called anchoring filaments which underlie the hemidesmosome and connect it to the lamina densa region of the basement membrane (Staehelin, 1974).

We have recently begun to study hemidesmosome

dynamics using a novel cell line, termed 804G, which possesses the remarkable ability to form hemidesmosomes *in vitro* (Riddelle et al., 1991). Here we detail our studies on the fate of two hemidesmosomal plaque components of 230 and 180 kDa in 804G cells during healing of wounds prepared in confluent monolayer cultures *in vitro*, during cell shape changes associated with the mitotic phase of the cell cycle, and following drug-induced disruption of the cytoskeleton.

Materials and methods

Cell culture

The 804G cell line, derived from a rat bladder, was generously provided by Ryoichi Oyasu (Department of Pathology, Northwestern University Medical School, Chicago, IL), and is described by Izumi et al. (1981). These cells possess numerous hemidesmosomes when maintained in culture (Riddelle et al., 1991). The cells are maintained at 37°C in Minimum Essential Medium with Earle's Salts supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, and 10% fetal calf serum (Gibco Laboratories, Grand Island, NY).

For cytoskeletal disruption studies, 804G cells were maintained on glass coverslips for 48 h. The culture medium was removed, and medium containing either 1 μg/ml colchicine (Sigma, St. Louis, MO), dissolved in distilled water, or 5 μg/ml cytochalasin D (Sigma), dissolved in dimethyl sulfoxide (DMSO) (Sigma), was added to the cells. For controls, media containing equivalent amounts of either distilled water or DMSO was added to the cells.

Human sera and antibody preparations

Human autoantibodies directed against a 230 kDa plaque component of hemidesmosomes in a bullous pemphigoid (BP) serum sample have been described previously (Klatte et al., 1989; Kurpakus and Jones, 1991).

A monoclonal IgM antibody was generated against a fusion protein encoded by a 450 bp portion of the 180 kDa hemidesmosomal component gene (Hopkinson et al., 1992). This antibody is called mAb180 kDa. The region of the protein recognized by this antibody is located towards the amino terminus of the 180 kDa polypeptide (Hopkinson et al., 1992). The sequence of this domain and details of the preparation of the antibody are described elsewhere (Hopkinson et al., 1992).

A polyclonal antibody against keratin was provided by R.D. Goldman of the Department of CMS Biology, Northwestern University Medical School. A monoclonal antibody against alpha-tubulin was purchased from Amersham (Arlington Heights, IL). Rhodamine-conjugated phalloidin was obtained from Molecular Probes, Inc. (Junction City, OR).

Immunofluorescence

Cultured cells grown on glass coverslips were rinsed briefly in phosphate-buffered saline (PBS: 6 mM Na^+/K^+ phosphate, pH 7.4, 171 mM NaCl, 3 mM KCl) and then fixed in −20°C acetone for 2 min and air dried. In the case of cells processed for localization of filamentous actin, cells were prefixed for 10 min in 3.7% formaldehyde in PBS before extraction in −20°C acetone. Coverslips were incubated in primary antibody in a moist chamber overnight at room temperature. For double labels, coverslips were incubated overnight in a mixture of primary antibodies or a mixture of primary antibody and rhodamine-conjugated phalloidin as detailed by Jones and Goldman (1985). The coverslips were then extensively washed in PBS. In order to minimize nonspecific binding of secondary antibodies, cells were incubated for 30 min at 37°C in normal goat serum (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:20 in PBS. Coverslips were then incubated in an appropriate fluorescein-conjugated secondary antibody (Jackson ImmunoResearch) for single labels or double labels involving rhodamine-conjugated phalloidin for an additional 45 min at 37°C. In the case of double label immunofluorescence, a mixture of the appropriate rhodamine- and fluorescein-conjugated secondary antibodies was used. For double labelling experiments involving mAb180 kDa and the mouse monoclonal anti-tubulin antibody, fluorescein-conjugated goat anti-mouse IgM was mixed with rhodamine-conjugated goat anti-mouse IgG secondary antibodies. After extensive washing, coverslip preparations were mounted on a slide in gelvatol (Monsanto Corp., St. Louis, MO). Immunofluorescence controls included incubation of cells on coverslips in secondary conjugated antibodies alone and the use of antibodies that do not recognize epithelial cells. Stained cells were viewed using a Confocal Laser Scanning Microscope 10 (Carl Zeiss, Germany). Fluorescence and phase contrast micrographs were taken using Kodak Plus-X-Pan film (Eastman Kodak Co., Rochester, NY). Films were developed in Diafine two-stage developer (Acufine, Inc., Chicago, IL).

Conventional and immunoelectron microscopy

804G cells grown on glass coverslips were fixed in 1% glutaraldehyde in sodium cacodylate buffer, pH 7.4, for 1 h at room temperature. The preparations were then postfixed with 1% OsO_4 in sodium cacodylate, pH 7.4, containing 0.8% potassium ferri-

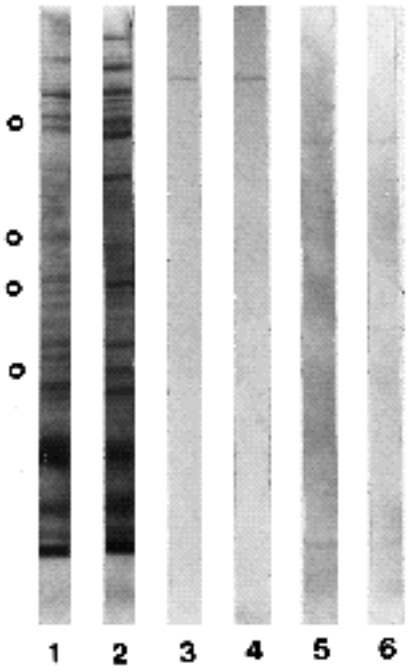


Fig. 1. A bovine tongue basal epithelial cell extract containing hemidesmosomal components (Klatte et al., 1989) (lanes 1,3 and 5) and a whole cell extract of 804G cells (lanes 2,4 and 6) were processed for SDS-PAGE and then transferred to nitrocellulose. Molecular mass standards are indicated by the open circles (from top to bottom, 200, 116, 97 and 66 kDa). Lanes 1 and 2 show amido black stains of transferred protein. Affinity-purified anti-230 kDa autoantibodies from bullous pemphigoid sera recognize 230 kDa polypeptides in both preparations (lanes 3 and 4). mAb180 kDa shows reaction with a 180 kDa polypeptide in both preparations (lanes 5 and 6).

cyanide ($K_3Fe(CN)_6$) for 4 h at 4°C. They were dehydrated and embedded in BEEM capsules in Epon/Araldite as previously described by Starger et al. (1978). The BEEM capsules containing cells were removed from the glass coverslip following immersion in liquid nitrogen. Sections were prepared both *en face* and perpendicular to the growth substratum. Thick sections (1 μm) were stained with toluidine blue for light microscopy. Thin sections (50 nm) were stained in uranyl acetate and lead citrate and viewed in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 60 kV.

For immunogold localization, cells grown on glass coverslips were fixed in −20°C acetone for 2 min. The cells on coverslips were then incubated overnight in mAb180 kDa at room temperature. After thorough washing in PBS, the preparations were preabsorbed for 30 min in normal goat serum diluted 1:20. 5 nm gold-conjugated goat anti-mouse IgM secondary antibodies (Janssen Pharmaceutica, Beerse, Belgium) were applied to the cells, which were then incubated overnight at room temperature. The cells were washed extensively, fixed in 1% glutaraldehyde, postfixed in 1% OsO_4, dehydrated and embedded as described by Starger et al. (1978). Controls were similar to those detailed above for immunofluorescence with substitution of gold-conjugated secondary antibodies. Thin sections were prepared and viewed in the

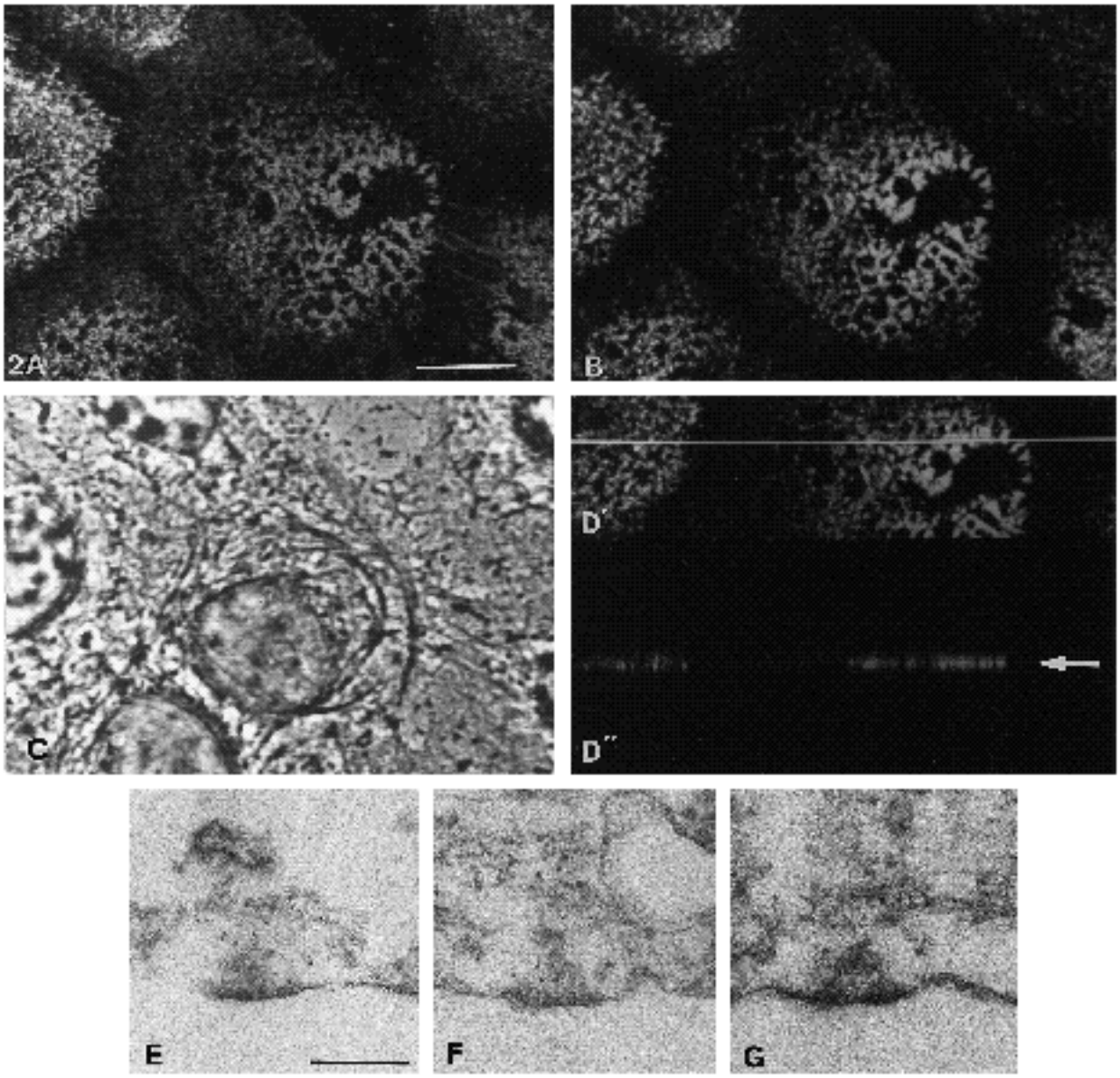


Fig. 2. Immunocytochemical localization of mAb180 kDa in 804G cells. 804G cells plated onto glass coverslips were processed for double label immunofluorescence confocal microscopy using 230 kDa autoantibodies present in a BP serum sample (A) and mAb180 kDa (B). (C) is a phase contrast micrograph of the same cells for orientation. Both the BP autoantibodies and mAb180 kDa generate identical cat paw-like staining patterns. The cell stained with mAb180 kDa is shown again in D′. The white line indicates a region through which a cross sectional profile (a z-section) of the cell has been made. This z-section is shown immediately beneath (D″) and reveals that the staining generated by mAb180 kDa is distributed towards the basal portion of the cell (arrow). Gold particles localize to the cytoplasmic aspect of the hemidesmosomal plaques of 804G cells processed for immunogold ultrastructural localization using mAb180 kDa (E,F and G). Bar in A, 10 μm; bar in E, 200 nm.

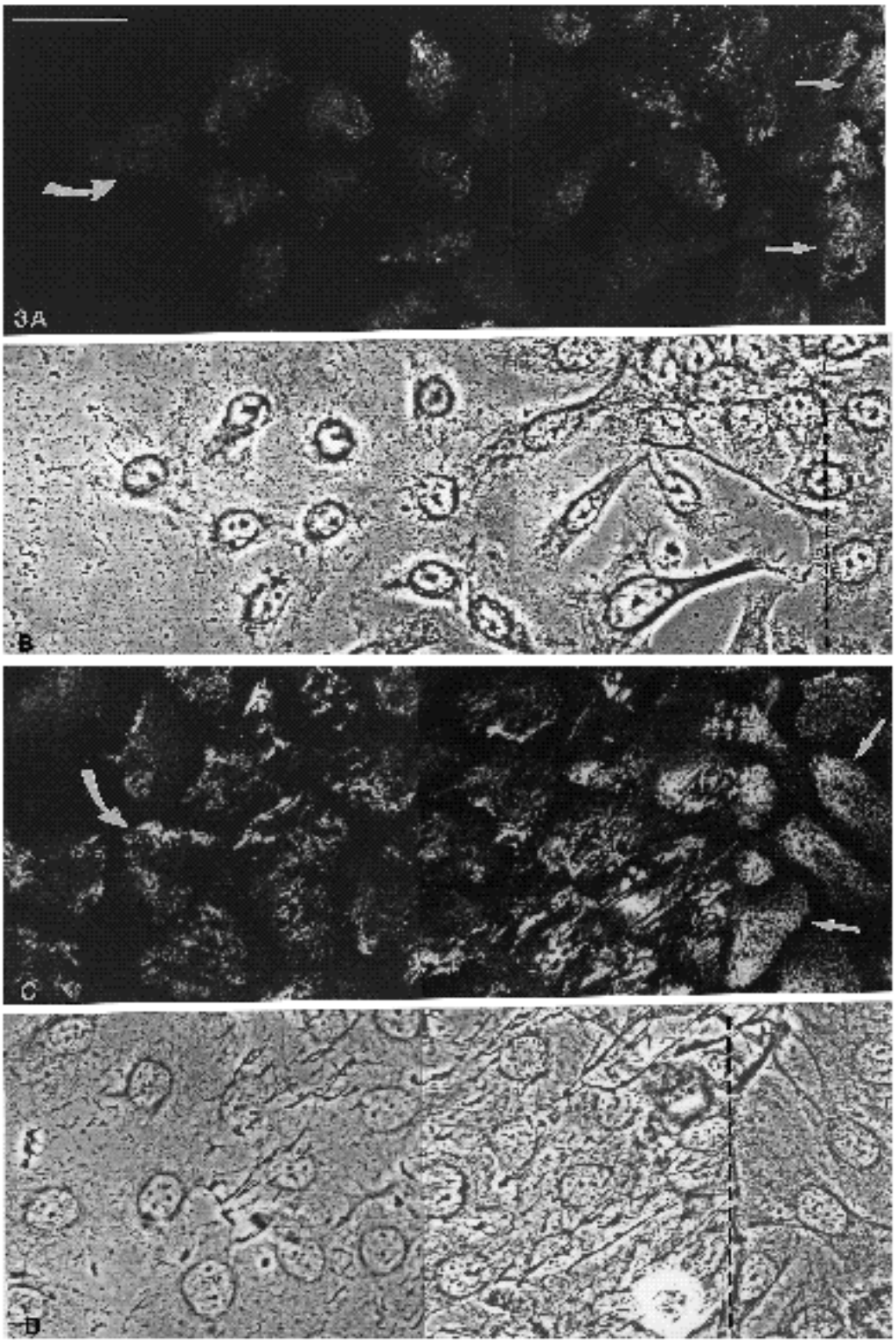


Fig. 3. Narrow scrape wounds were made in confluent monolayers of 804G cells. The cells were then allowed to cover the wound site and, at 12 h (A,B) and 24 h (C,D) post-wounding, preparations were processed for confocal immunofluorescence microscopy using mAb180 kDa. (B,D) are phase contrast images for orientation. The wound bed is to the left of the dashed lines in both B and D. mAb180 kDa generates the typical cat paw staining in cells outside the wound area (A,C, arrows). In A, cells within the wound site show minimal hemidesmosomal staining (curved arrow). At 24 h following wounding, most cells in the wound site are stained by mAb180 kDa. Cells that have migrated farthest into the wound (curved arrow in C) show staining that is concentrated at the cell periphery. This is quite different from the mainly subnuclear staining seen in cells that are close to the wound edge or cells that have not entered the wound site. Bar, 25 μm.

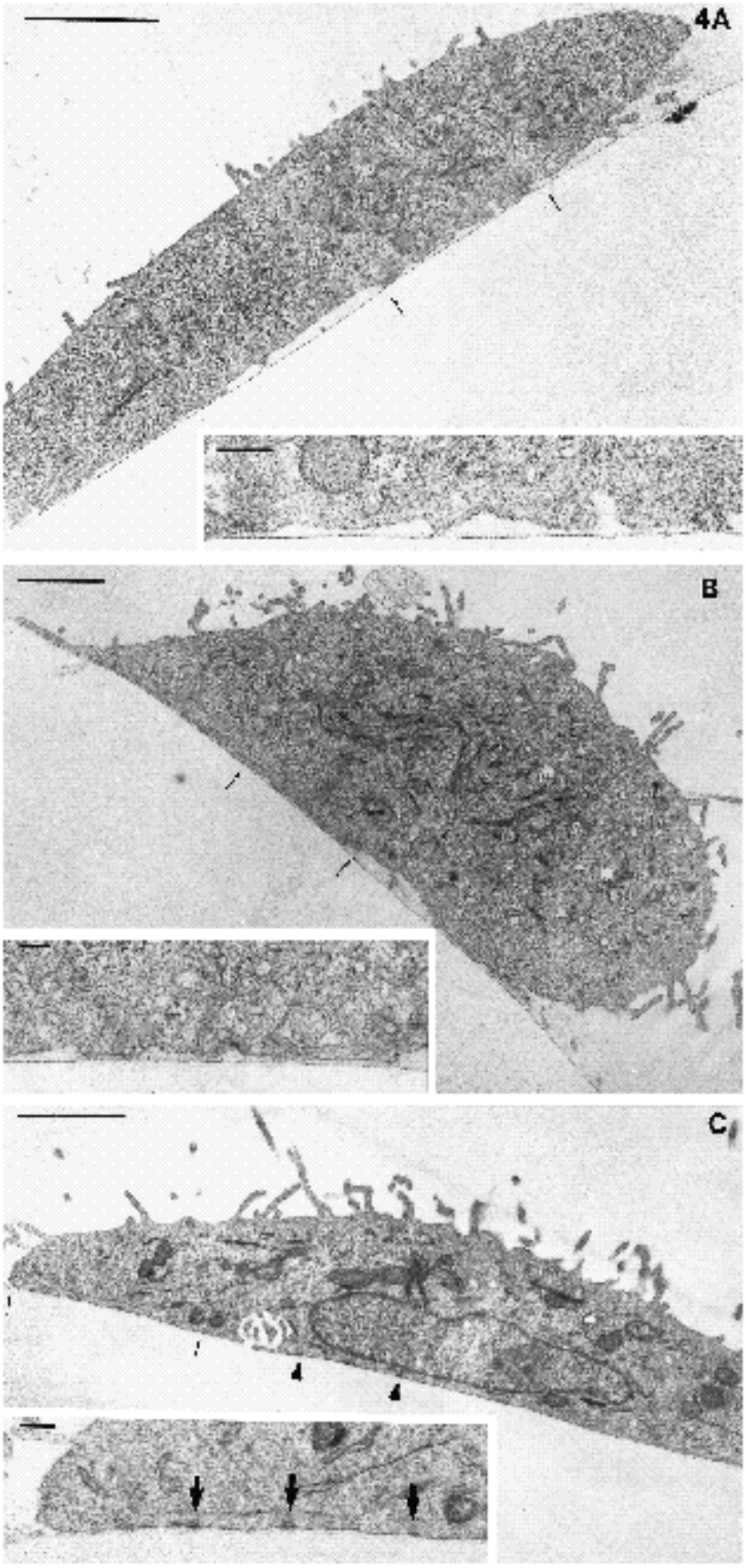


Fig. 4. 804G cells present in wound cultures were processed for conventional electron microscopy. Sections were prepared perpendicular to the substratum. Cells at 4 h (A) following wounding do not possess any obvious hemidesmosomes at the basal surface of the cells (contrast this with 804G cells shown in Fig. 4A of Riddelle et al., 1991 and Fig. 7a of Jones et al., 1991). The region between the arrows is enlarged in the inset. The cell in (B) is in a wound site at 12 h post-wounding. A portion of the basal surface indicated by the arrows is shown in the inset. This cell is also apparently devoid of formed hemidesmosomes. The cell shown in (C) is in the bed of a wound, 24 h after wounding. This cell possesses several normal-appearing hemidesmosomal structures (C and inset). Two hemidesmosomes underlying the nucleus are indicated by the arrow heads (C). The area between the arrows in C is shown at higher magnification in the inset. Several hemidesmosomes occur at the cell periphery (arrows, inset). Bar, 2 μm; bar in inset, 200 nm.

ondary antibodies. Thin sections were prepared and viewed in the electron microscope as described above.

SDS-PAGE and western blotting

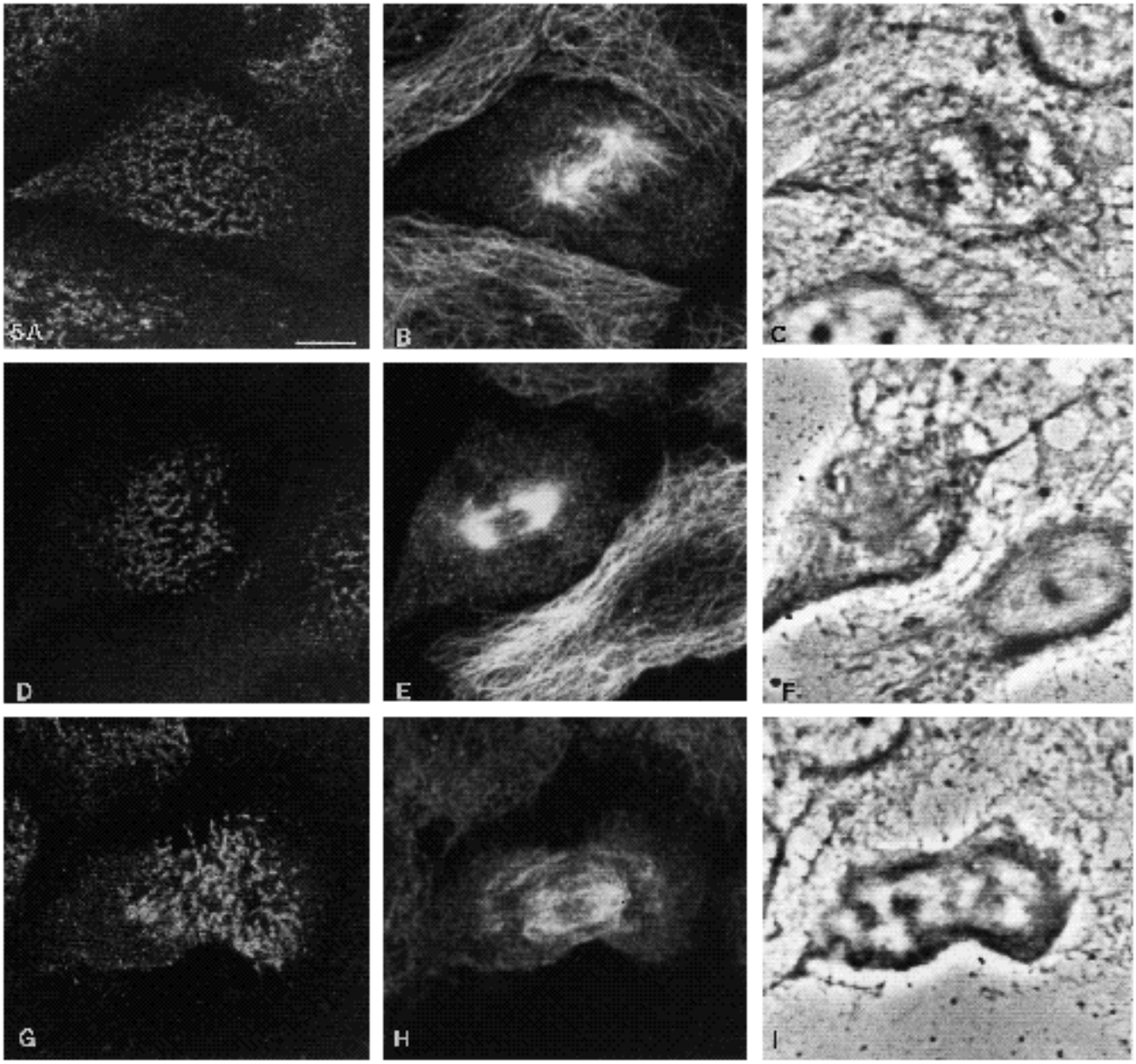
SDS-PAGE using 6.0% polyacrylamide gels with 3.0% stacking gels was performed on a basal cell extract of bovine tongue (Klatte et al., 1989) and 804G whole cell extracts according to Laemmli (1970). Approximately 20 μg of protein per gel lane was used. After SDS-PAGE, separated polypeptides were transferred to sheets of nitrocellulose (Towbin et al., 1979). Immunoblotting was carried out using the described antibodies as detailed by Zackroff et al. (1984).

Results

Immunochemical identification of 230 and 180 kDa hemidesmosomal polypeptides in 804G cells

To characterize the hemidesmosomal antibodies used in these studies, western immunoblotting analysis was performed on a bovine tongue basal epithelial cell preparation containing hemidesmosomal components and on 804G whole cell extracts. The blots were probed with mAb180 kDa and with human anti-230 kDa hemidesmosomal antibodies prepared as detailed in Klatte et al. (1989). The 804G cells possess immunologically distinct 180 and 230 kDa polypeptides recognized by these antibodies. Furthermore, these polypeptides comigrate with their bovine tongue equivalents (Fig. 1).

Double label confocal immunofluorescence microscopical analyses of 804G cells, using human anti-230 kDa autoantibodies and mAb180 kDa, show identical cell substratum associated staining, arranged in a “cat paw” type pattern (Fig. 2). A z-section, demonstrating a computer-generated cross-sectional profile of the same cell, reveals that the staining generated by mAb180 kDa is concentrated



along the substratum attached surface of the cell, as would be expected for localization to a cell-substratum attachment device such as the hemidesmosome (Fig. 2D″). Furthermore, immunoelectron microscopical analyses of 804G cells using mAb180 kDa, reveal that the mAb180 kDa antibodies localize to the cytoplasmic region of the plaque of the hemidesmosome (Fig. 2E-G).

In vitro wound healing

Using mAb180 kDa, we have monitored the fate of hemidesmosomes in 804G cells as the cells cover *in vitro* wounds. For these studies, 804G cells were grown to confluence on glass coverslips. The cell culture medium was removed and a narrow scrape wound, less than 1 mm wide, was made in the monolayer with a plastic pipette tip. The cells were briefly rinsed with PBS to remove cell debris. Fresh medium was added and the cells were returned to the tissue culture incubator. At various times after wounding, the coverslips were processed for either immunofluorescence or electron microscopy.

Within 4 h of wounding, cells begin to migrate into the wound site (result not shown). Between 4 and 12 h, cells that have migrated into the wound show minimal staining with mAb180 kDa. A few stained "spots" can be observed at the substratum attached surface of such cells (Fig. 3A). In contrast, cells in the unwounded region of the culture show a typical cat paw subnuclear staining pattern with this antibody (Fig. 3A).

In cells that have migrated farthest into the wound at 24 h, mAb180 kDa generates staining which appears concentrated at the cell-substratum surface both at the cell periphery and in the subnuclear region of the cell (Fig. 3C). This contrasts with the almost exclusive subnuclear staining generated by the same antibody in cells in the unwounded region of the culture and in cells, in the wound, which are closest to the wound edge (Fig. 3C). Indeed, the subnuclear distribution of hemidesmosomal plaque proteins only becomes the predominant pattern in cells, in the wound, upon complete cell coverage of the wound site (result not shown). This occurs between 36 and 48 h following wounding. It should be emphasized that at all time points in the above studies, the staining generated by mAb180 kDa and the 230 kDa antibodies colocalizes (not shown).

Ultrastructural analyses of 804G cells during wound healing

We have extended the above immunofluorescence results by examining the fate of hemidesmosomes at the ultrastructural level in the *in vitro* wound cultures of 804G cells. Wounded cell cultures were processed for conventional electron microscopy at 4, 12, and 24 h after wounding. Toluidine blue stained, thick sections (1 μm) of plastic embedded material allowed us to identify wound sites in

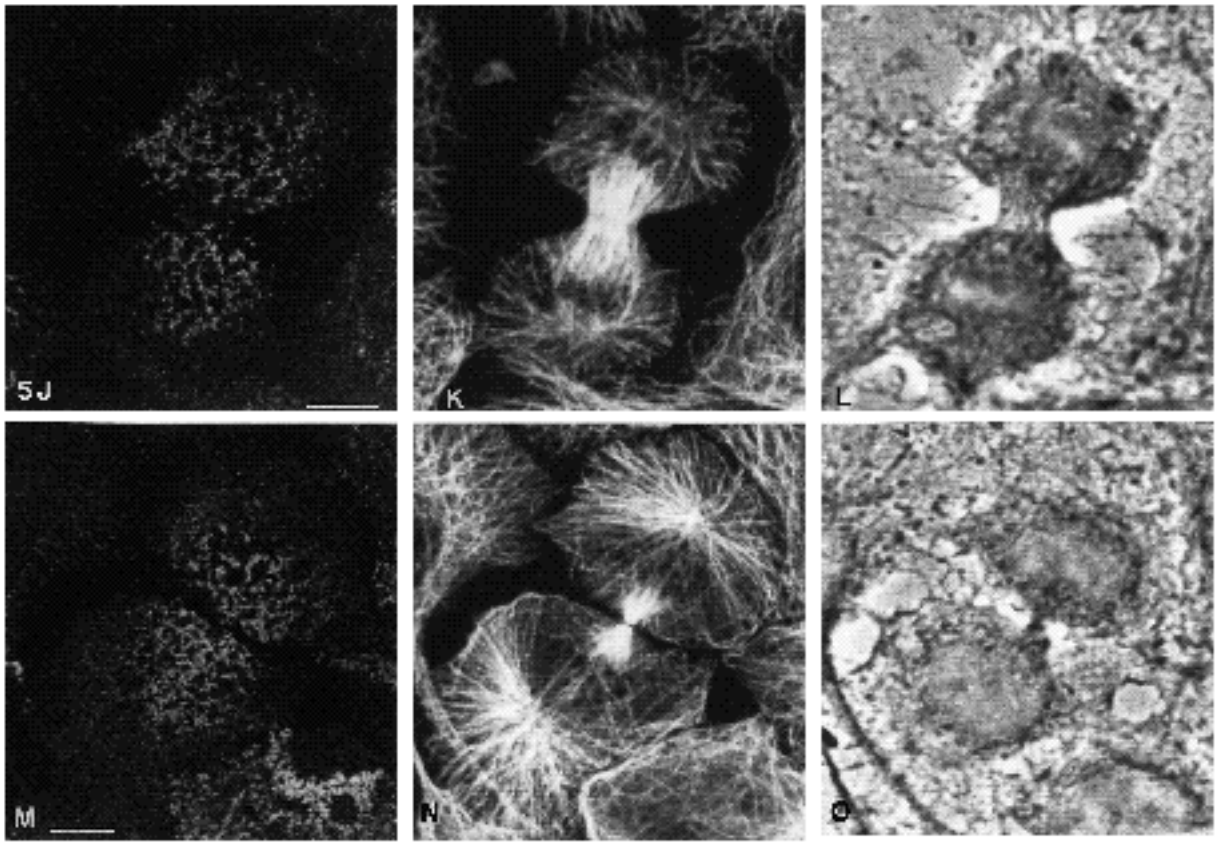


Fig. 5. Mitotic 804G cells were processed for double-label immunofluorescence with mAb180 kDa (A,D,G,J and M) and the monoclonal anti-alpha tubulin antibody (B,E,H,K and N). Phase contrast image are shown in (C,F,I,L and O). At every stage of mitosis, mAb180 kDa generates staining along the substratum attached surface of the cells. Bar, 5 μm.

the cultures (not shown) prior to thin sectioning. Cells away from the wound site possess numerous hemidesmosomes similar to those shown in Fig. 4 of Riddelle et al. (1991). Fig. 4A shows a cell present in the wound area 4 h after wounding. There are no obvious electron dense structures at the basal surface of this cell (inset). Likewise, at 12 h post-wounding, there is an absence of any hemidesmosome-like structures in cells located within the wound bed (Fig. 4B). In contrast, at 24 h after wounding, numerous hemidesmosome-like structures can be observed at the substratum-attached surface of the 804G cells that have migrated into the wound site (Fig. 4C). Each of these hemidesmosomes has a multilayered electron dense cytoplasmic plaque. In the cell shown in Fig. 4C, several hemidesmosomes appear towards the cell periphery as well as in the subnuclear region.

Fate of hemidesmosomes during mitosis

We wished to investigate the fate of hemidesmosomes in situations in which 804G cells undergo changes in shape. One such occasion is cellular rounding associated with mitosis when the 804G cells presumably become less adherent to their substratum (Fig. 5). For these studies, 804G cells were processed for double-label immunofluorescence microscopy using mAb180 kDa and a monoclonal anti-tubulin antibody, the latter being used as a marker for the stage of the cell cycle. At all stages of mitosis, mAb180 kDa produces immunofluorescence staining along the substratum attached surface of the cells in a pattern that looks essentially similar to that seen in interphase cells (Fig. 5). The same is true for the human 230 kDa autoantibodies (results not shown).

We have been unable to undertake a comprehensive analysis of mitotic 804G cells by electron microscopy because of the difficulty in identifying mitotic cells in thin sections of cross-sectional profiles of fixed populations of cells. Nevertheless, in the limited number of mitotic cells we have observed ultrastructurally, hemidesmosomes can be observed along the region of cell-substratum interaction (Fig. 6).

Cytoskeleton disruption and the fate of hemidesmosomes in 804G cells

We first investigated the effects of the microtubule-disrupting drug, colchicine, on hemidesmosome integrity in 804G cells. The cells were treated for 2 h in medium containing 1 μg/ml colchicine. In treated cells, colchicine does not perturb the localization of the 230 and 180 kDa hemidesmosomal components in 804G cells although it completely disrupts the microtubule network (result not shown). Furthermore, hemidesmosomes can be observed in the subnuclear region of colchicine treated cells (result not shown).

Unlike colchicine, the microfilament disrupting drug cytochalasin D has considerable effects on hemidesmosomal protein localization in 804G cells. Within 2 h of incubating 804G cells in the presence of 5 μg/ml cytochalasin D, the microfilament system appears to collapse (Fig. 7). The cells change shape and arborize (Figs 7D and 8B). Each of the treated cells possesses several cell surface projections, which remain extending from the cell body as portions of the cell surface retract. Remarkably, cytochalasin D induces a redistribution of the 180 kDa polypeptide in 804G cells (Fig. 8A). Indeed, there is an apparent "inversion" of actin and hemidesmosomal protein staining in the cytochalasin D treated cells compared with their normal counterparts (compare Figs 7 and 8).

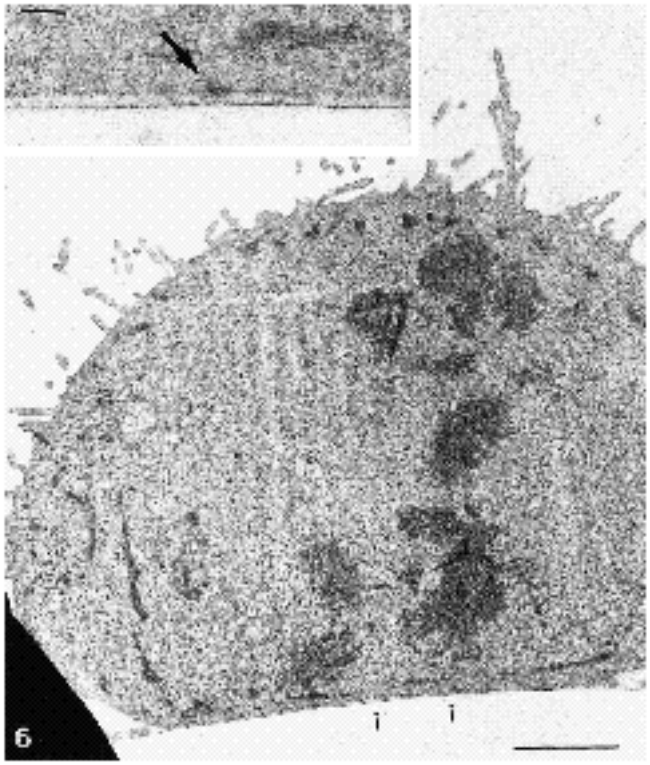


Fig. 6. An electron micrograph of an 804G cell prepared perpendicular to the substratum. This metaphase cell possesses several hemidesmosome-like structures along its substratum attached surface. The inset, a high magnification of the area between the arrows, shows one of these structures (arrow). Bar, 2 μm; bar in inset, 200 nm.

In cytochalasin D treated cells, accumulations of the 180 kDa hemidesmosomal plaque polypeptide appear to be arranged in rows in the cell projections mentioned above and are not observed underlying the nucleus (compare Figs 8 and 2). The same is the case for the 230 kDa polypeptide recognized by the human autoantibodies (Fig. 8C). Figure 8F″ is a z-section of a cytochalasin D treated 804G cell processed for immunofluorescence using mAb180 kDa. The staining produced by the monoclonal antibodies is mainly restricted to the substratum attached surface. Protein synthesis is not required for this change in staining, since cytochalasin D induces the same effect in cells pretreated with 10 μg/ml of cycloheximide, which we have already reported inhibits protein synthesis in 804G cells by greater than 93% (Riddelle et al., 1991).

At the ultrastructural level, hemidesmosomes are observed along the substratum-associated surface of cell projections of cytochalasin D treated cells (Fig. 9). The hemidesmosomes in such cells possess a normal-appearing plaque with which keratin bundles are associated. More-

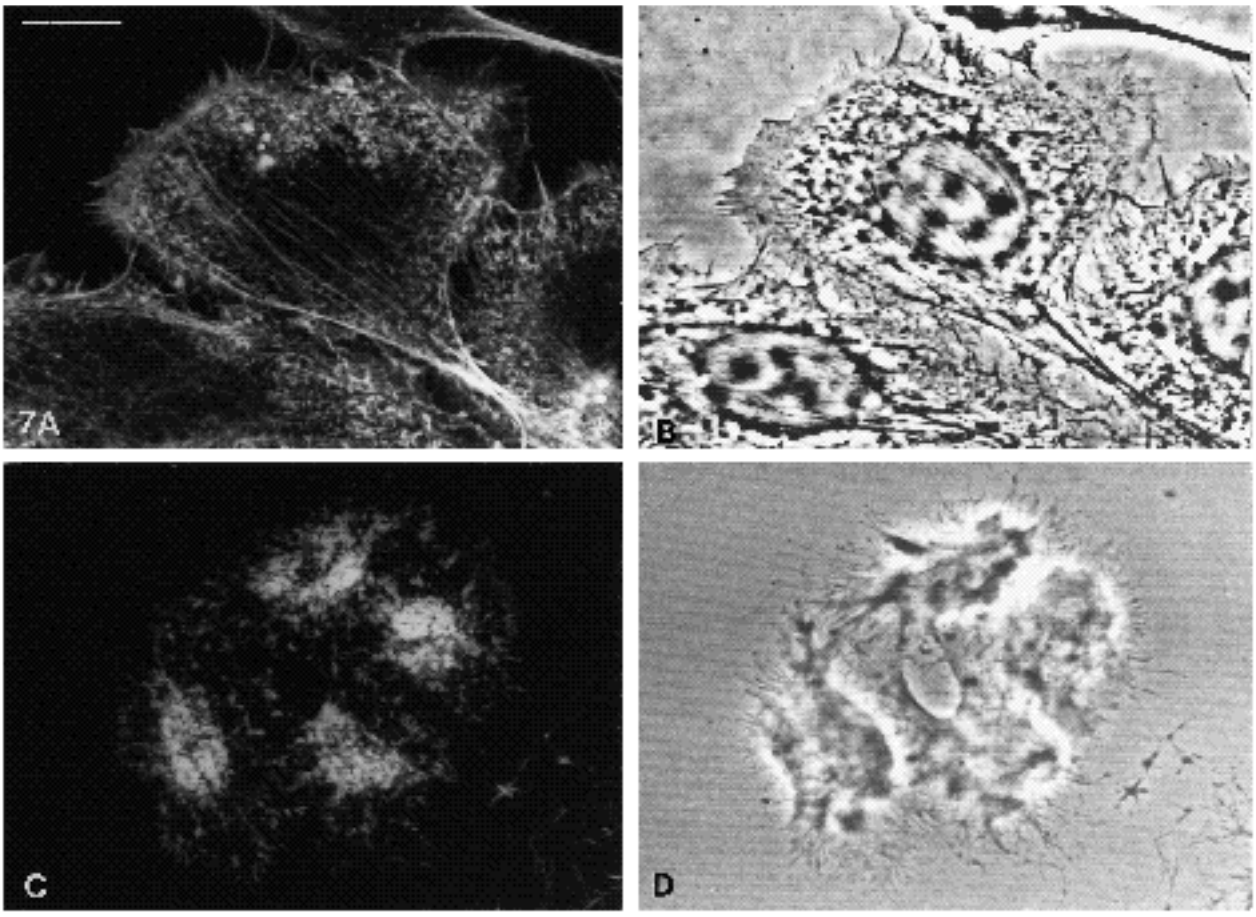


Fig. 7. Untreated 804G cells (A) and cells treated for 2 h in cytochalasin D (C) were processed for confocal fluorescence microscopy using rhodamine phalloidin. (B) and (D) are the corresponding phase contrast images. Rhodamine phalloidin stains prominent stress fibers in untreated cells (A). Cytochalasin D treatment of the cells induces cell arborization and a collapse of the actin cytoskeleton (C,D). Bar, 10 μm.

over, anchoring filament-like structures underlie each of the hemidesmosomes. *En face* sections close to the substratum attached surface of both untreated and cytochalasin D treated cells have also been prepared (Fig. 10). Electron dense structures, presumably representing hemidesmosomes, are clearly observed in the numerous cell projections of the drug treated cells (Fig. 10B). This is in contrast to untreated cells, where electron dense structures are primarily found towards the center of the cell (Fig. 10A).

The cytochalasin D treatment of 804G cells is completely reversible. Cytochalasin D treated 804G cells allowed to recover by removal of the drug and addition of fresh medium for 2 h, and subsequently processed for immunofluorescence using mAb180 kDa, show the typical cat paw staining pattern concentrated beneath the nucleus (result not shown). Furthermore, these cells possess numerous hemidesmosomes underlying the nucleus as determined ultrastructurally (result not shown).

Cytochalasin D induces reorganization of the keratin network in the 804G cells (Fig. 11). In untreated cells, keratin filament bundles appear as a wavy, coiled network, concentrated around the nucleus (Fig. 11B). In contrast, keratin filament bundles in cytochalasin D treated 804G cells extend radially in straight arrays from the nucleus into cell surface projections (Fig. 11E). Double-label immunofluorescence analyses of drug treated cells using the keratin antibody probe and mAb180 kDa reveal that the 180 kDa hemidesmosomal plaque protein is distributed along the keratin bundles in these cell projections (Fig. 11D and E).

Evidence of an interaction between intermediate filaments and microfilaments in 804G cells

The above results indicate that collapse of the microfilament network induces both a change in the distribution of hemidesmosomes and also reorganization of the keratin bundle network in 804G cells. We have no evidence that there is a direct association between hemidesmosomes and microfilaments. Rather fluorescence analyses of 804G cells using rhodamine-phalloidin reveal that microfilament bundles are concentrated at the cell periphery and not in the center of the cell where the majority of hemidesmosomes occur (Fig. 12). Furthermore, we have never observed an association between hemidesmosomes and microfilaments at the ultrastructural level. This is in contrast to certain keratin bundles which extend towards the cell periphery. These are often found in apparent contact with the microfilament network, as determined both by immunofluorescence and electron microscopy (Fig. 12).

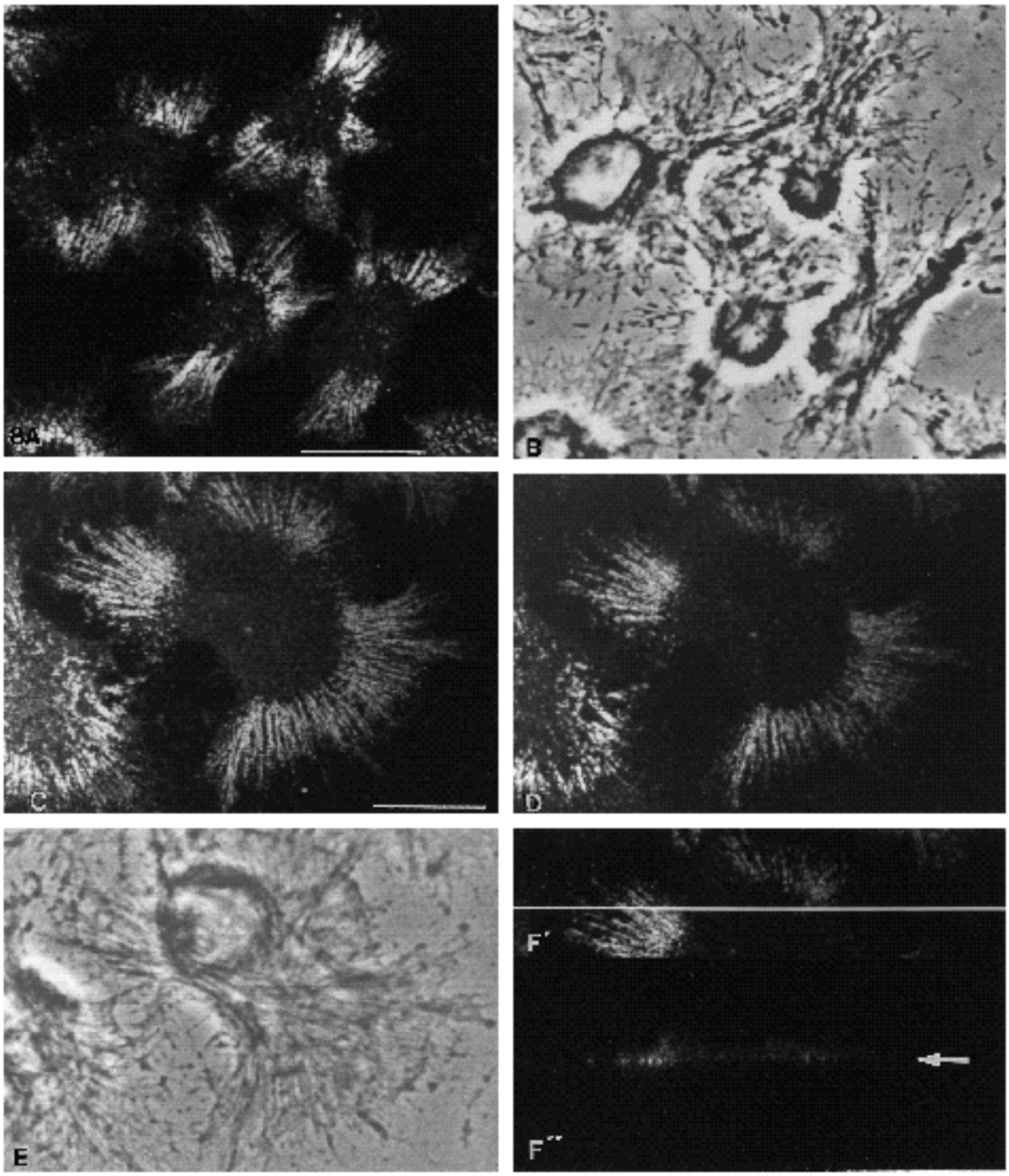


Fig. 8. (A) shows a group of 804G cells treated for 2 h with cytochalasin D processed for immunofluorescence microscopy using mAb180 kDa. A phase contrast image of the same cells is shown in (B). Note the dramatic change in shape of the treated cells (B) and the distribution of staining along cell projections (A). Double label immunofluorescence microscopy using 230 kDa autoantibodies (C) and mAb180 kDa (D) shows that both the 230 and 180 kDa antigens co-localize in cytochalasin D treated cells. (E) shows a phase contrast image of the cell shown in C, D. F′ is the same cell shown in D that was stained with mAb180 kDa. The white line in F′ indicates where a z-section profile was generated. This profile reveals that mAb180 kDa staining is concentrated along the substratum attached surface of the cell (arrow in F″). Bar, 10 μm.

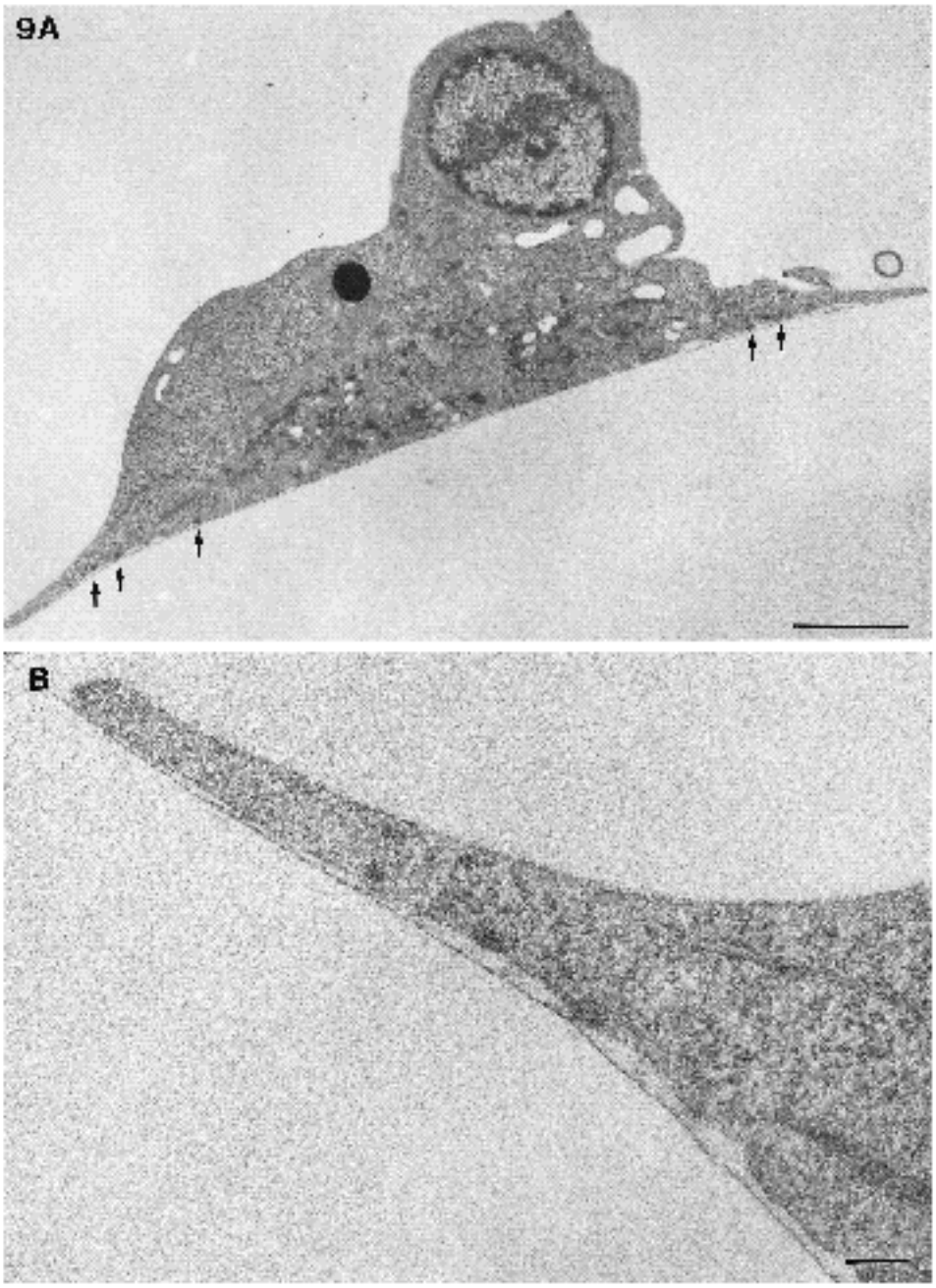


Fig. 9. An electron micrograph of an 804G cell treated for 2 h in cytochalasin D is shown in (A). The section of the cell was prepared perpendicular to the substratum. Several hemidesmosome-like structures can be seen along the bottom of the cell towards the cell periphery (A, arrows). The left hand edge of the cell is shown enlarged in (B). Bar in A, 2 μm; bar in B, 200 nm.

Discussion

Cells of the 804G line are unusual for their ability to form hemidesmosomes in culture (Riddelle et al., 1991). They therefore provide an ideal *in vitro* system in which to study hemidesmosomal dynamics. Indeed, Jones et al. (1991) have used these cells to show that the hemidesmosome-associated integrins $\alpha_6\beta_4$ play a role in hemidesmosome assembly. Here, we have analyzed the fate of hemidesmosomes in 804G cells during the cell cycle and under a number of experimental conditions, using a combination of electron and confocal laser scan microscopy.

In this study, we have used antibodies directed against two well characterized components of the hemidesmosome of 230 and 180 kDa, both of which were originally identified by Klatte et al. (1989), using human anti-hemidesmosomal autoantibodies. These workers showed that human autoantibodies directed against the 230 kDa polypeptide bind to the plaque of the hemidesmosome, and recent sequence analyses suggest that this polypeptide is entirely cytoplasmic (Tanaka et al., 1991). Similarly, affinity purified human autoantibodies directed against a 180 kDa polypeptide also localize to the cytoplasmic plaque of the hemidesmosome (Klatte et al., 1989). The 180 kDa fusion protein antibodies (mAb180 kDa) used in this report are highly species cross-reactive (Riddelle and Jones, unpublished observations). Unlike the affinity purified human anti-180 kDa autoantibodies used in a previous publication

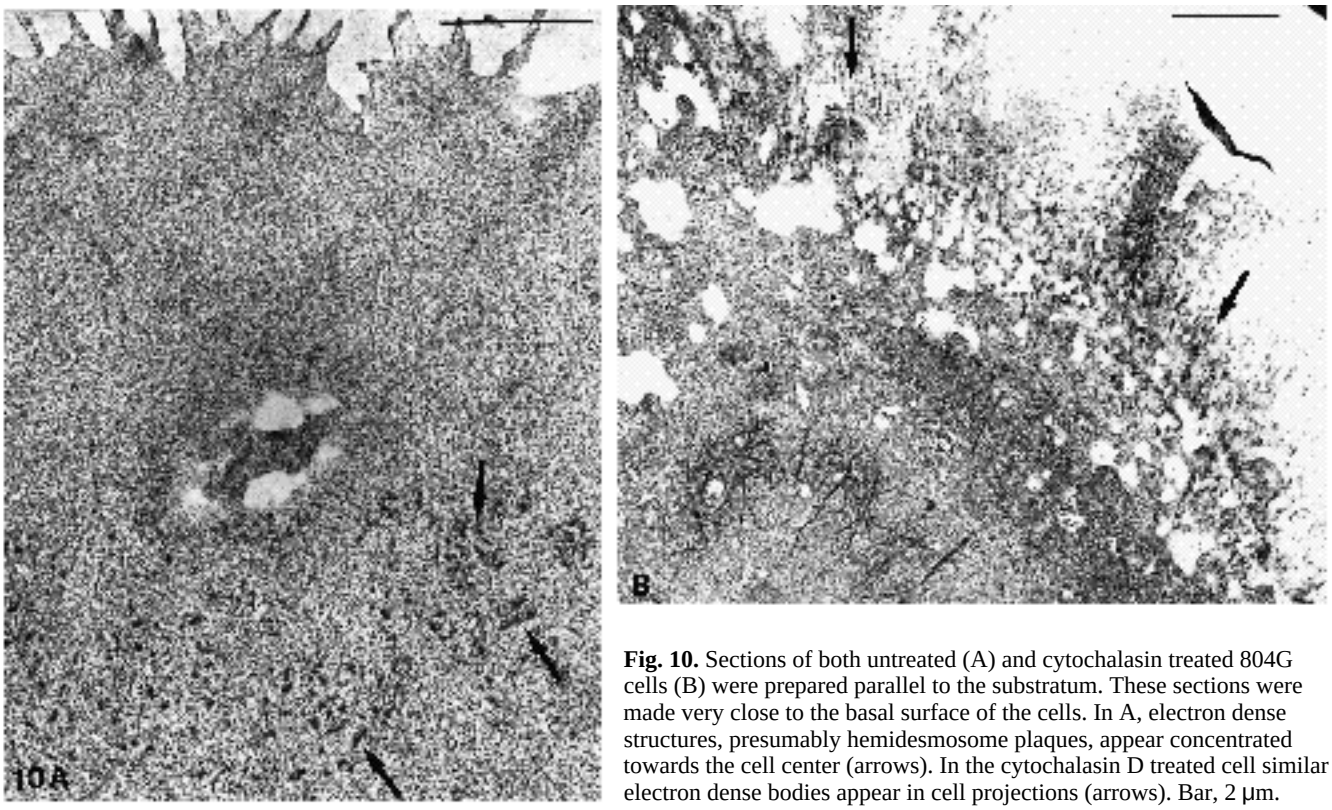


Fig. 10. Sections of both untreated (A) and cytochalasin treated 804G cells (B) were prepared parallel to the substratum. These sections were made very close to the basal surface of the cells. In A, electron dense structures, presumably hemidesmosome plaques, appear concentrated towards the cell center (arrows). In the cytochalasin D treated cell similar electron dense bodies appear in cell projections (arrows). Bar, 2 μm.

(Riddelle et al., 1991), mAb180 kDa recognizes the hemidesmosomes of 804G cells. Interestingly, molecular and biochemical characterization of the 180 kDa polypeptide indicate that it is a type II membrane protein and possesses collagen-like extracellular sequences (Giudice et al., 1991; Hopkinson et al., 1992). mAb180 kDa is directed against the amino terminus of the 180 kDa molecule (Hopkinson et al., 1992). Since the 180 kDa polypeptide is a type II membrane protein, the amino terminus would be located in the cytoplasm. This is consistent with the gold localization of mAb180 kDa that we present here.

During the healing of wounds, 804G cells which have migrated into the wound site do not possess formed hemidesmosomes. This also appears to be the case in epithelial cells populating wounds in tissue both *in vivo* and *in vitro* (Krawczyk and Wilgram, 1973; Hinter et al., 1980; Stanley et al., 1981; Fujikawa et al., 1984; Gipson et al., 1988; Gipson et al., 1989; Kurpakus et al., 1990). We presume that during the transition from a stationary to a migrating state, 804G cells may either disassemble or endocytose their hemidesmosomes (Takahashi et al., 1985), although we have no evidence that supports either phenomenon.

As the cells fill in the wounds and presumably become less motile, hemidesmosomal plaque components appear along the substratum attached surface of cells and the cells assemble hemidesmosomes, as determined by electron microscopy. In those cells which have migrated farthest into the wound, hemidesmosomal plaque elements commonly appear towards, but are not restricted to, the cell periphery. Consistent with this finding, electron microscopic examination of such cells reveals that hemidesmosome-like structures occur both at the cell periphery as well as in the subnuclear region. In contrast, hemidesmosomal plaque components are concentrated underneath the nucleus in cells which are close to the wound edge. Following complete coverage of the wound, hemidesmosome plaque components in the majority of cells appear underlying the nucleus. We speculate that this change in location of hemidesmosomal components is a result of their mobility in the plane of the membrane of 804G cells. We have already proposed such a phenomenon in a previous publication (Riddelle et al., 1991), based on a suggestion by Klymkowsky et al. (1983). We will return to this point later in the discussion.

We had expected that as 804G cells enter mitosis, their hemidesmosomes would disassemble as a result of the cell shape changes which occur during cell division. Although there may be subtle changes in the location of hemidesmosomal proteins along the substratum attached surface of mitotic cells compared with their interphase counterparts, we find no evidence of disassembly of the hemidesmosome plaque as 804G cells proceed through the mitotic cycle. This is particularly evident when comparing the fluorescence patterns generated by hemidesmosomal plaque antibodies in mitotic cells with those seen in 804G cells which have recently migrated into wound sites and which do not possess hemidesmosomes (contrast Figs 5 and 8). Thus, hemidesmosomes in 804G cells are maintained in an apparently assembled form despite the considerable shape change (i.e. rounding) which they undergo during mitosis. Of course, even though plaques of the hemidesmosomes remain apparently intact in mitotic cells, it is possible that there are either modifications in or even a loss of substra-

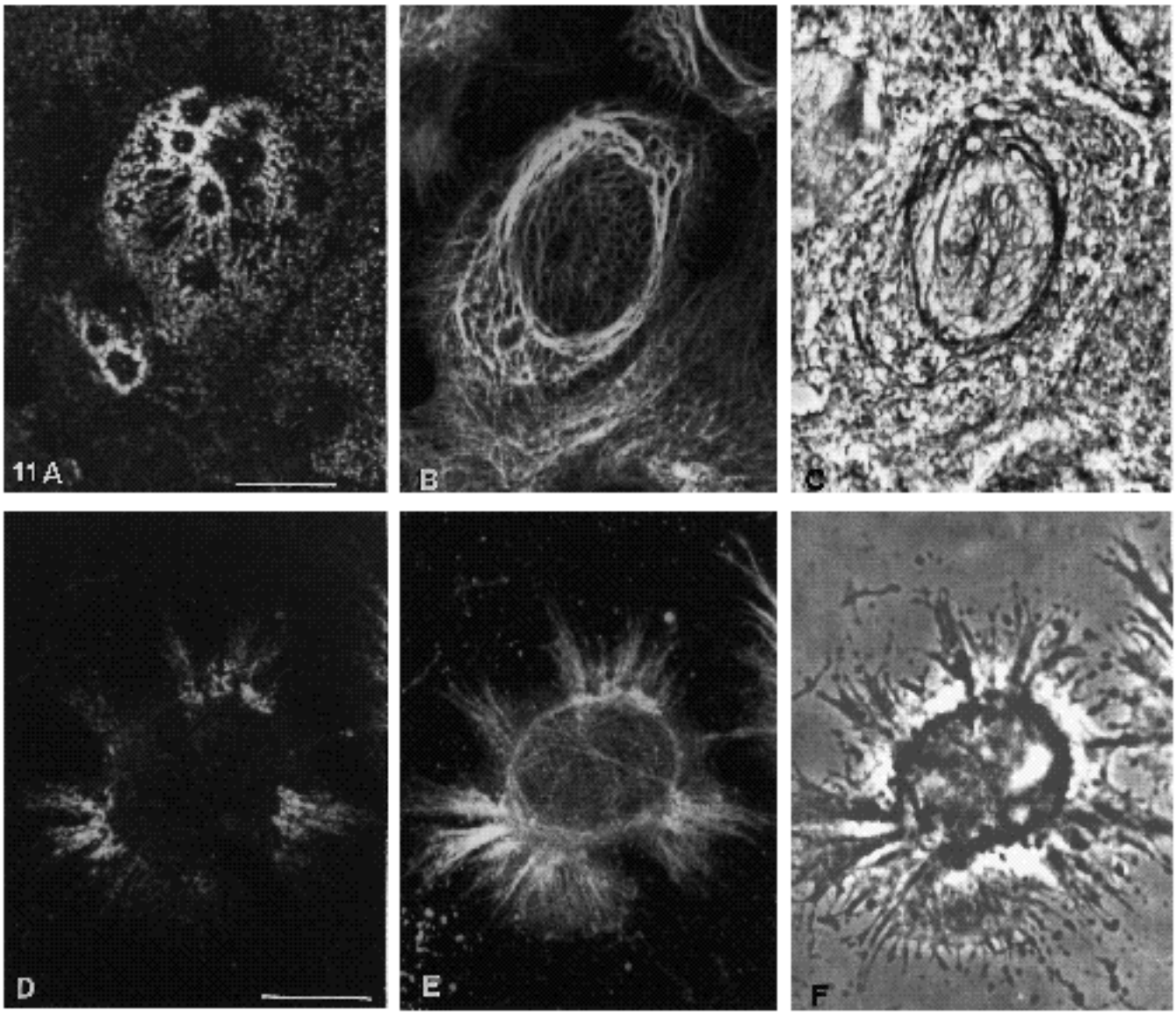


Fig. 11. Untreated cells (A-C) and cells treated for 2 h with cytochalasin D (D-F) were processed for double label immunofluorescence using mAb180 kDa (A,D) and a keratin antibody preparation (B,E). Phase contrast images of cells are shown in (C) and (F). In the control cells, highly coiled keratin bundles are concentrated around the nucleus (B). In contrast, in cytochalasin D treated cells the 180 kDa antigen is located along cell projections (D) which also contain linear arrays of keratin bundles (E). Bar, 10 μm.

tum interactions of such hemidesmosomes. The latter would be consistent with a previous finding that the plaque of the hemidesmosome remains on the cell surface of freshly trypsinized cells (Riddelle et al., 1991). We have already proposed that 804G cells may simply reuse these plaques during re-establishment of cell-substratum interactions as the cell spreads over a substratum following replating (Riddelle et al., 1991). The same phenomenon may occur in cells at the conclusion of mitosis. This obviously implies that the interaction of the hemidesmosome with the substratum may be dynamic, and our studies on the effects of cytoskeletal disrupting drugs on 804G cells support this contention.

In cytochalasin D treated 804G cells, there are dramatic changes in the location of hemidesmosomes. In untreated cells or in cells whose microtubule network has been depolymerized by colchicine, hemidesmosomes are concentrated underneath the nucleus. In contrast, in 804G cells incubated with cytochalasin D, hemidesmosomes become concentrated at the periphery of the 804G cells in cell projections. Protein synthesis is not required for this change in location of formed hemidesmosomes. In order to explain this phenomenon, one could suppose that hemidesmosomes in cytochalasin D treated cells are first disassembled. Hemidesmosomes would then be assembled in their new location either from stores of cytoplasmic protein or by reuse of components of the disassembled hemidesmosomes. However, we feel it unlikely that both the disassembly of hemidesmosomes and *de novo* assembly of hemidesmosomes could occur in the short time within which cytocha-

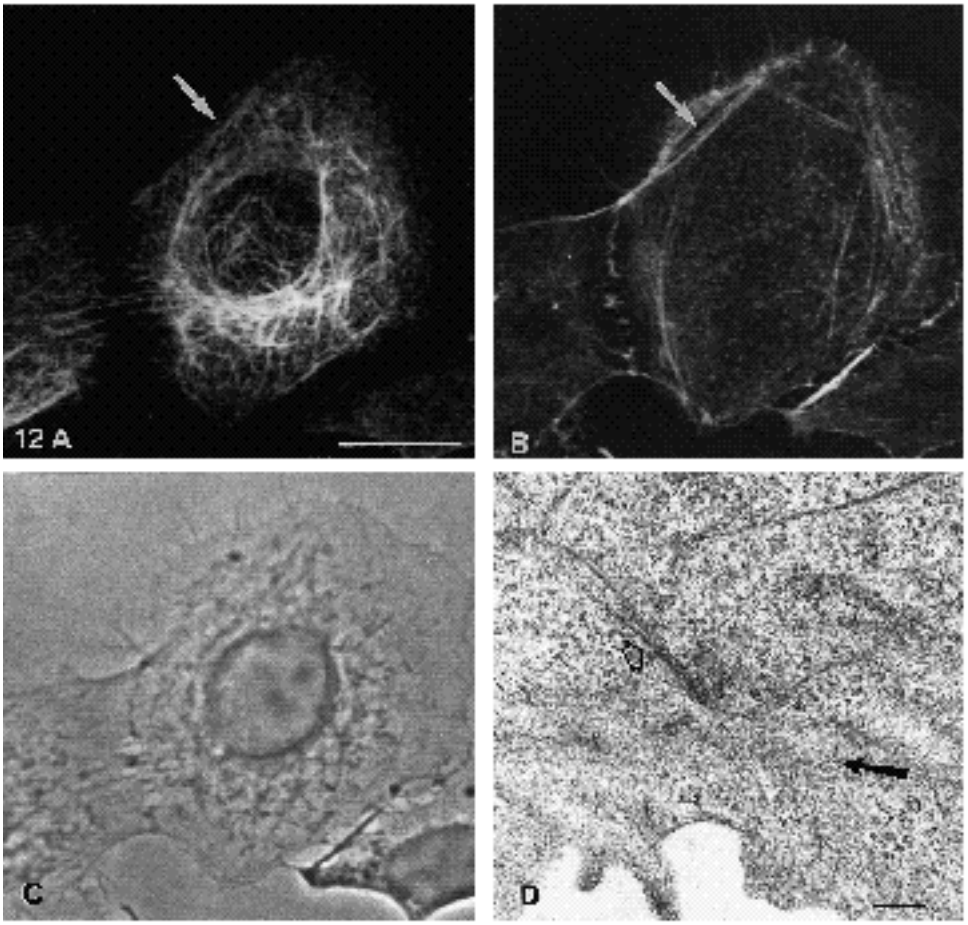


Fig. 12. An 804G cell was processed for double label fluorescence microscopy using a keratin antibody (A) and rhodamine-conjugated phalloidin (B). (C) shows the corresponding phase contrast image of the cell. Note that towards the cell periphery there is co-localization of a bundle of keratin with a bundle of actin filaments (A,B, arrow). An interaction of intermediate filaments (open arrow) and microfilaments (closed arrow) is also seen at the ultrastructural level (D). Bar in A, 5 μm; bar in D, 200 nm.

lasin D treatment induces both a cell shape change and the apparent change in location of hemidesmosomes. In this regard, we have observed redistribution of hemidesmosomes within as little as 30 min of addition of cytochalasin to cells. Rather, we favor the possibility that the plaques of hemidesmosomes, including both cytoplasmic components such as the 230 kDa polypeptide and transmembrane components such as the 180 kDa polypeptide, are induced to move as a complex in the plane of the basal surface membrane of 804G cells by cytochalasin D treatment.

Why should the disruption of the actin cytoskeleton network in 804G cells result in the relocation of the plaque of the hemidesmosome, as we are proposing? We speculate that the relocation of hemidesmosomes in cytochalasin D treated cells is a secondary consequence of the reorganization of the intermediate filament system in the cells. In untreated cells, the keratin network is concentrated in tight coiled bundles around the nucleus. These bundles are surrounded by microfilament arrays. Since the intermediate filament network in 804G cells appears to associate with the microfilament system as is the case in a number of cell types (Geiger and Singer, 1980; Schliwa and van Blerkom, 1981; Knapp et al., 1983a,b; Goldman et al., 1986; Wolf and Mullins, 1987), it is tempting to suggest that an intact microfilament system acts in some way to maintain the coiled form of the keratin bundles. Upon depolymerization of the microfilaments by cytochalasin D, the keratin bundles uncoil and can be found extending into cell projections along which hemidesmosomes are located. Thus the plaques of hemidesmosomes, to which the intermediate filament network is attached, may be "pulled" along the basal surface of the cell during this drug induced movement of the keratin bundles. Of course, this can only occur if the interaction between the cell and the substratum via the hemidesmosome were non-static.

In summary, in 804G cells, hemidesmosomes are disassembled as cells become motile and populate wounds. This is consistent with a variety of studies which have analyzed *in vivo* wound healing (reviewed by Kurpakus et al., 1990). In contrast, during cell shape changes which result during mitosis and following disruption of the cytoskeleton, hemidesmosomal plaques remain intact but there are apparent perturbations in hemidesmosome-substratum attachment. These results taken together strongly suggest that the hemidesmosome is a much more dynamic structure than previously considered. This may appear, at first, contradictory to the notion that hemidesmosomes provide a "stable" anchorage device for an epithelial cell in intact tissues. However, it should be borne in mind that anchoring fibrils underlie each hemidesmosome in a tissue. One could suppose that anchoring fibrils, through their interaction with anchoring filaments, may restrict the mobility of the hemidesmosome within the plane of the membrane *in vivo*. Since 804G cells do not possess anchoring fibrils, this restriction on their mobility would therefore be removed.

What could a dynamic nature of hemidesmosome-sub-

stratum interaction mean for cells *in vivo*? Hemidesmosomes are found in tissues, such as the skin, where there is a relatively rapid turnover of cells. Thus, retention of intact hemidesmosomal plaques through mitosis may allow daughter cells to quickly and efficiently re-establish stable connection with the substratum following cell division. In this scenario, the distribution of pre-existing anchoring fibrils in the connective tissue may determine the position of hemidesmosomes in the post-mitotic epithelial cells. This would have a precedent, since Gipson et al. (1983) have shown that when epithelial sheets are placed on connective tissue material, hemidesmosomes appear directly above preformed bundles of anchoring fibrils.

To further these studies, we are in the process of analyzing the molecular basis of hemidesmosome-substratum interactions. It is our goal to identify those matrix molecules which act as the linkers between the membrane molecules of the hemidesmosome and anchoring fibrils.

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