Migration Into an In Vitro *Experimental Wound*

A Comparison of Porcine Aortic Endothelial and Smooth Muscle Cells and the Effect of Culture Irradiation

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The purpose of this study was to compare the group-cell migration characteristics of endothelial cells (ECs) and smooth muscle cells (SMCs) derived from the same source, the porcine thoracic aorta, as they moved into an experimental *in vitro* wound. The authors characterized migration by measuring two aspects of the migrating cells: the number of free cells in the wound and the distance of migration of the sheet of cells at the wound edge. The quantitative data showed that ECs migrated into the wound as a sheet of cells, while SMCs migrated as free single cells. In addition, since irradiated cells have been used to study cell migration and since the irradiated cells do undergo some shape changes, the distribution of the cytoskeletal microfilament fibres was compared in migrating irradiated and nonirradiated cells in order to see whether this feature of cell migration was different. Irradiated and nonirradiated migrating ECs showed a strikingly different pattern in the orientation of microfilament bundles when studied by immunofluorescence microscopy with antiserums to myosin and tropomyosin. (Am J Pathol 1981, 103:271–282)

CELL MIGRATION is an important function of both endothelial cells (ECs) and smooth muscle cells (SMCs) during the response of the arterial wall to injury.[1-7] Although SMC and EC migration has been studied *in vivo*, the way in which these cells move and the factors that influence this movement cannot be easily investigated. The development of techniques for isolating and maintaining medial SMC[8] and EC[9,10] in pure cultures has provided for an opportunity to study migration in more detail. In addition, the use of populations of irradiated cells[11] which do not proliferate but do keep their ability to migrate has allowed for the identification of specific factors that effect migration.[12,13] These studies were done with the assumption that the migration of irradiated cells and that of nonirradiated cells were similar.

The purpose of the first part of our study was to compare the patterns of group cell movement of SMC and EC. Since the movement of ECs and SMCs takes place in the same general environment of the injured vessel wall, it is important that one compare the characteristics of migration of these two populations of cells under similar conditions and using cells derived from the same source, in our case the porcine thoracic aorta. Using the experimental wound technique,[14] we have been able to observe and quantitate striking differences in the way in which populations of ECs and SMCs migrate.

The second purpose was to compare the migration of irradiated and nonirradiated cells. Both we and others have noted that the irradiated ECs are flatter than their nonirradiated counterparts,[12,13] and we were concerned that movement of irradiated cells did not truly reflect the migration of nonirradiated cells. Since the cytoskeleton is related to cell movement,[15-17] we studied the distribution of microfilaments in the irradiated and nonirradiated cells as they migrated into the experimental wound. Our data show that there is indeed a striking difference in the orientation of myosin and tropomyosin containing microfilaments in irradiated and nonirradiated migrating ECs.

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Materials and Methods

Cultures

Porcine thoracic aortas were obtained from a local slaughterhouse within 15 minutes of death. The aortic segments were brought to the laboratory in sterile Dulbecco's phosphate-buffered saline containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.25 μg/ml amphotericin.

EC Cultures

A modification of the method of Ryan et al[18] was used to harvest and culture ECs. The adventitial surface of the aortic segments was cleaned, and the lumen was washed several times with Puck's saline containing antibiotics. To harvest the ECs, we clamped a segment of the aorta at one end, using a hemostat, and filled the lumen with a 0.25% collagenase solution. The vessel was incubated at 37 C for 10 minutes under sterile conditions. The collagenase was removed and growth medium containing medium 199 in Earle's salts with 25 mM HEPES, 0.3 mg/l L-glutamine 20% fetal calf serum (FCS), and antibiotics was pipetted gently against the endothelial surface of the aorta. The growth medium was then removed and centrifuged at 900*g* for 5 minutes, and the pellet of ECs was resuspended in fresh growth medium and plated on 22-mm glass coverslips in 35-mm tissue culture dishes. The cells were incubated at 37 C in a humid atmosphere in 95% air and 5% CO_2. Initially there were clumps of ECs containing 10–20 cells, and by 8–10 days a confluent monolayer of polygonal shaped cells was present. Subcultures were established by treating the monolayer for 1–2 minutes with 0.05% trypsin and 0.02% ethylene diaminotetraacetic acid (EDTA) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline.

SMC Cultures

A method similar to that described by Ross was used.[8] The adventitia was dissected from the aortic segments, and the opened aorta was pinned down. A very thin strip of intima–media was removed and discarded, and a thin layer of media below the previously removed strip was peeled off and cut into 1 × 1-mm pieces. Six to ten pieces were placed in 35-mm tissue culture dishes and held in place by a glass coverslip. The explants were grown in Dulbecco's Modified Eagle's Medium (DME) supplemented with 0.03% L-glutamine, 10% FCS, and antibiotics, at 37 C in a humid atmosphere in 95% air and 5% CO_2. By 5–7 days cells grew out of the explant, and after 3 weeks there were enough cells to subculture using a 5-minute incubation with 0.05% trypsin and 0.02% EDTA. The SMCs were observed to contain bundles of myofilaments and dense bodies by transmission electron microscopy.

Wound

Both the SMCs and the ECs were seeded at 4×10^4 cells/ml in 60-mm dishes containing 22 × 40-mm sterile glass coverslips. Once a confluent monolayer was reached, we made a wound by completely removing the cells occupying about one third of the coverslip along the 40-mm edge with the flat edge of a wedge-shaped teflon spatula. The wound was examined microscopically to ensure that all ECs were removed, and three small linear scratches were made along the wound edge with the use of a sterile diamond pencil. The cells were then rinsed and incubated in the standard conditions used for culturing.

Measurement of Migration

Free Cells

At 44 hours the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4,[19] mounted in 50% glycerol-PBS, and then counted with phase optics on an inverted microscope with a 10× objective and a 10× ocular lens equipped with a 1 × 1-cm net micrometer. Free cells were considered to be cells in the wound that were not in contact with either the sheet of ECs or the network of SMCs extending into the wound edge. Twenty adjacent fields were counted along the edge of each wound, and the number of free cells was expressed as the mean number of cells per field. Three coverslips were counted for each experimental condition and a standard error of the mean was calculated.

Sheets

The edge of the sheet of cells, or in the case of SMCs, the network of cells, was considered to be the point where the monolayer ended. The distance the sheet moved from the time the scratch was made was measured using a reticule with 10-mm divisions with a 10× ocular and 20× objective lens. The measurements were made at a fixed distance from each end of the three scratches, so that any alteration of the glass substratum by the scratch that might affect cell migration was excluded. Six measurements were thus made for each coverslip after wounding and at 44 hours. We calculated the distance of migration by subtracting the measurement at 1 hour from that at 44 hours. Three coverslips were measured for each experimental condition, and a standard error was calculated.

Irradiation

The monolayers to be wounded were irradiated at room temperature with 1500 rads of Cesium 137 for 16 minutes 1 hour before wounding. We measured the effect of 1500 rads of irradiation on the growth of low density SMCs and ECs on glass coverslips by doing growth curves,[20] using the standard growth conditions, using 10% FCS for SMCs and 20% FCS for ECs. We counted the number of mitotic cells per wound edge, ie, per 20 adjacent fields, in irradiated and nonirradiated wounds at 44 hours after wounding, using the same optics employed for counting free cells. Ten wounds were counted for SMCs and ECs, and standard errors were calculated.

Antiserums

The preparation of rabbit antiserums to rabbit anti-chicken breast muscle tropomyosin[21] has been previously described.

Pig uterine myosin was prepared from fresh slaughterhouse porcine uteri and purified essentially as described by Ash et al[22] with the use of the rapid method first described by Pollard et al.[23] The muscle was stripped clean of connective tissue and epithelium, ground up in a meat grinder, and stirred gently for 20 minutes with 3 ml of extraction buffer per gram of tissue. The extraction buffer used contained 0.3 M KCl, 50 mM PIPES (piperazine-N-N′-bis-[2-ethanesulfonic acid]) 2 mM adenosine triphosphate (ATP), 0.1 mM DTT (DL-dithiothreitol), 2 mM $MgCl_2$, pH 6.8, as modified from Ash.[22] The mixture was then filtered through cheesecloth, and the extract was centrifuged at 12,000*g* for 10 minutes. We further purified the supernatant by ammonium sulfate fractionation, taking the 35–55% saturated ammonium sulfate fraction, which was subsequently dialyzed overnight against 0.3 M KCl, 10 mM Tris-Cl, 0.1 mM DTT, pH 8.0. The dialysate was centrifuged at 12,000*g* for 20 minutes and the supernatant was dialyzed against a low ionic strength buffer which contained 50 mM KCl, 10 mM PIPES, 0.1 mM DTT, 5 mM $MgCl_2$, pH 6.8, in order to precipitate myosin. The pellet was recovered after centrifugation at 12,000*g* for 20 minutes and resuspended in an equal volume of KI buffer,[5] resulting in a concentration of 0.5 M KI, 10 mM Tris base, 2 mM ATP, 1 mM $MgCl_2$, and 5% sucrose, which was added to increase the density, pH 8.0. The mixture was centrifuged at 105,000*g* for 20 minutes, and the supernatant was applied immediately to a Biogel A 15m 200/400 mesh column (Bio-Rad Laboratories, Richmond, Calif) measuring 2.5 × 87 cm and equilibrated with 0.6 M KCl, 10 mM Tris base, 0.1 mM DTT, pH 8.0. Twenty milliliters of KI buffer was applied just ahead of the sample. The fractions were collected at 6 ml per 15 minutes. The fractions containing the purified myosin were located by their absorbance at 280 nm, and the purity of selected fractions was assessed by polyacrylamide slab gel electrophoresis.[24] The fractions to be assessed were selected on the basis of the Biogel A 15m gel filtration data of Pollard et al.[23] ATPase activity was not assessed. We pooled the fractions and dialyzed them against 50 mM KCl, 10 mM PIPES, 0.1 mM DTT, 5 mM $MgCl_2$, pH 6.8, for 24 hours to precipitate the myosin. The precipitate was collected by centrifugation at 12,000*g* for 20 minutes and redissolved in a 1:1 mixture of glycerol and 0.6 M KCl, 20 mM Tris-Cl, 1 mM DTT, pH 8.0. This solution was centrifuged at 150,000*g* for 1 hour, and the supernatant containing the purified myosin was stored at −20 C. All manipulations were performed on ice or in a cold room.

Sodium Dodecyl Polyacrylamide Slab Gel Electrophoresis

A slab gel system[24] was used with the discontinuous buffer system of Laemmli,[25] 6–15% linear gradient separating gels, and a 5% spacer gel. The gel and electrode buffer contained 0.2% sodium dodecyl sulfate (SDS), and the sample buffer contained 2% dodecyl sulfate and 2% 2-mercaptoethanol. Electrophoresis was performed at a constant current of 32 ma for 2 hours. The gels were fixed and stained overnight at 37 C with 25% isopropanol, 10% acetic acid in distilled water with 0.05% Coomassie brilliant blue R. Destaining was done with several changes of 10% methanol, 10% acetic acid in distilled water.

Preparation of Antiserums

Fractions free of actin and tropomyosin, as assayed by the SDS polyacrylamide gel electrophoresis, were pooled and used for immunization of the rabbits. Preimmune serum was collected. Lymph node injections in both popliteal fossae were carried out, and a total of 70 μg of purified myosin was injected in complete Freund's adjuvant. Subcutaneous booster injections of a total of 100 μg of myosin were injected in incomplete Freund's adjuvant in 8 separate areas on the back 3 weeks after the initial injection, and the animals were bled over a 2-week period 7 days after the booster injection. The antiserums were characterized by double immunodiffusion (Ouchterlony) plate reaction run in 0.3 M KCl, 10 mM Tris-Cl, pH 8.0

A monospecific antibody was made with the use of the affinity chromatographic technique described by

Ternynck et al[26] with the use of gluteraldehyde-activated Ultrogel (LKB, Industrie Biologie, France) and low pH elution.

Immunofluorescence

Methods previously described were followed.[19] Wounds were fixed at 44 hours in 3% paraformaldehyde for 20 minutes and then made permeable with 0.1% Triton for 4 minutes. The cells were then rinsed in PBS, pH 7.4, and treated with either antiserum to myosin diluted 1:10, or 25 μg/ml of affinity-purified antimyosin, for 20 minutes at room temperature. Tropomyosin antiserum was used at 1:20. The cells were then washed several times with PBS and incubated with a 1:10 dilution of fluorescein-labeled goat antirabbit IgG (Hyland, Costa Mesa, Calif) with a fluorescein/protein molar ratio of 3.7. After washing the coverslips in PBS and mounting them in 50% glycerol in PBS, we viewed the cells in a Zeiss photomicroscope II equipped with a mercury vapor lamp, epifluorescence optics, and interference filters. Photomicrographs were taken with Ilford FP-4 film and developed in Microphen. As controls, cells were stained with either pre-immune rabbit serum or pooled rabbit serum, or with the supernatant of an incubation of equivalent amounts of purified myosin or purified tropomyosin and the antiserum.

In order to quantitate the distribution of cytoskeletal fibres, we counted cells in the first row along the wound edge with a 40× objective and a 10× ocular lens with the photomicroscope. The orientation of the main fiber bundles stained with either antimyosin or antitropomyosin antibodies were observed relative to the position of the wound edge. Thus the bundles were either roughly parallel or perpendicular to the wound edge. The results were expressed as the percentage of cells with a given orientation in the total population of cells counted. About 200 cells were counted per coverslip. At least three coverslips were counted for each experimental condition, and standard errors were calculated.

Results

Cultures

The SMCs grew out of the explants in 5–7 days and were ready to be subcultured by 3 weeks. The cells showed the characteristic growth pattern especially in confluent cultures with focal areas of multilayering, the so-called hills-and-valleys pattern (Figure 1A). Ultrastructurally, the cells showed the typical features of porcine arterial smooth muscle cells,[20,27] with myofilaments, dense bodies, and occasionally an incomplete slightly electron-dense basement membrane.

The ECs used in these studies did not grow beyond confluency, and the confluent monolayer was characterized by flat polygonal shaped cells, giving a "cobblestone" appearance to the monolayer (Figure 1B).

Effect of Irradiation

The radiation dose of 1500 rads was sufficient to inhibit SMC and EC proliferation. Growth curves indicated that the irradiated cells were able to maintain a stable number over a 7-day period but did not proliferate (Figure 2). The number of mitotic cells counted along a wound edge at 44 hours was 31.3 ± 2.9 in non-irradiated wounds and 0.67 ± 0.66 in irradiated

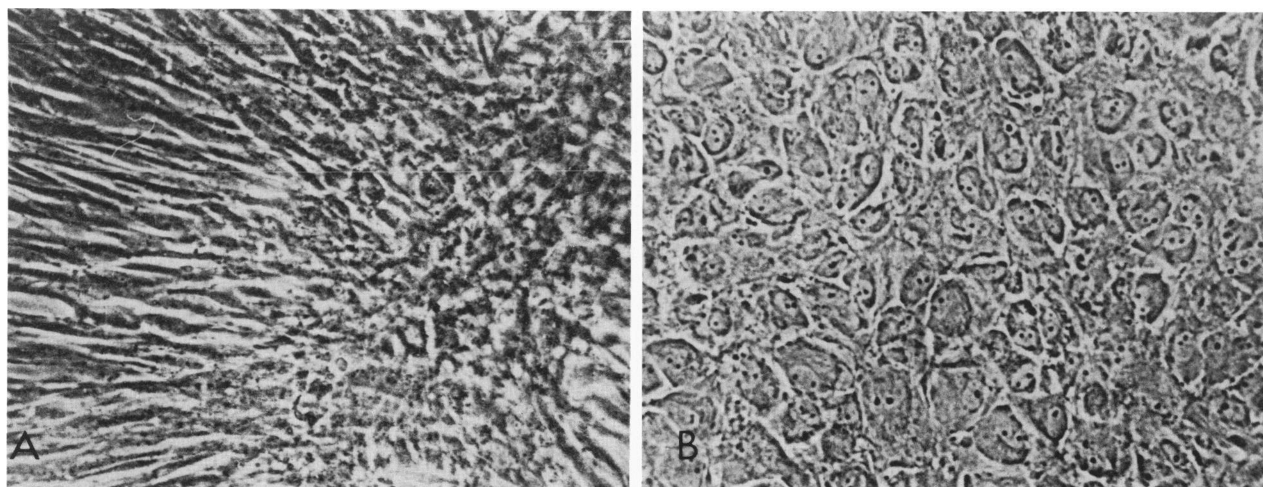


Figure 1—Confluent monolayer of cells 6 days after reaching confluency. **A**—Smooth muscle cells showing focal areas of multilayering the so-called hills-and-valleys pattern. (×125) **B**—Endothelial cells showing a contact-inhibited cobblestone monolayer. (×125)

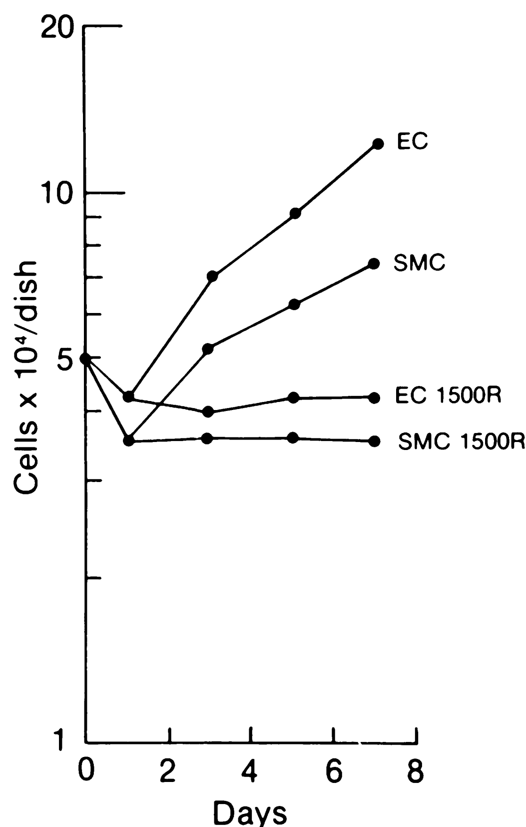


Figure 2—The growth of low-density irradiated EC and SMC cultures compared with nonirradiated cultures. ECs grown in 20% FCS and SMCs in 10% FCS. *R* = rads.

wounds in 10 SMC wounds counted and 27.7 ± 0.88 and 0.33 ± 0.30, respectively, in 10 EC wounds. At 44 hours the irradiated ECs at the wound edge were flatter than nonirradiated ECs (Figure 3G). The irradiated SMCs appeared somewhat flatter, but the change was less pronounced than that of ECs (Figure 3H).

Migration

Immediately after wounding there was no morphologic evidence of migration (Figures 3A and B). Within 1 hour that part of the cell facing the wound showed a prominent leading edge with a ruffling membrane at the periphery. At 3 hours the lamellipodia were well developed (Figures 3C and D). There was no morphologic difference between irradiated and nonirradiated cells at this point. At 44 hours quantitative observations were carried out on the wound edge (Figure 4A and B).

Free Cell Migration

At 44 hours there was a significantly greater number of nonirradiated free SMCs than ECs in their respective wounds (Table 1). The difference was significant for both nonirradiated and irradiated cells, although the absolute numbers were less in the latter. There was no difference between SMCs grown in 10% and those grown in 20% FCS. While most of those SMCs showing the characteristic leading edge and elongated tail of a migrating cell were oriented with the leading edge away from the wound, some were oriented with the leading edge toward the wound. Preliminary studies with time-lapse cinemicrophotography confirmed that while most of the SMCs move away some do move toward the wound edge.

Phase contrast observations of EC wounds at several time points from 0 to 44 hours did not show any evidence that free cells had appeared in significant numbers and then reformed a cohesive sheet at 44 hours.

Sheet Migration

The nonirradiated ECs migrated into the wound as a uniform cohesive sheet of cells (Figure 3E). The cells in the first few rows made contact with adjacent cells at numerous points along the lateral margins. The nature of these contacts was not further studied at this time. The free SMCs that had migrated into the wound formed a network of cells behind the more distal free cells. This network of cells, connected to the monolayer, consisted of loosely arranged elongated cells whose long axes was randomly oriented with respect to the wound edge with some cells overlapping one another (Figure 3F). Thus the SMC network was not a cohesive migrating sheet of cells; instead it was formed by the contact and overlapping of free SMCs as they moved individually into the wound.

The nonirradiated EC sheet extended 0.65 ± 0.05 mm into the wound, while the SMC network extended a distance of 0.23 ± 0.01 mm (Table 1) over a 44-hour period. Irradiated cells showed a similar difference.

Myosin Purification

Fractions 27 to 31 (Figure 5A) of the KI-high-salt Biogel column contained highly purified porcine uterine myosin that was free of actin and tropomyosin (Figure 5B). The SDS polyacrylamide gel electrophoresis showed that the purified myosin consisted of a slowly migrating heavy chain and two light chains. The high-molecular-weight bands below the myosin heavy chain may be proteolytic fragments of the myosin heavy chain. Their proportion on overloaded gels increased as the stored myosin preparation aged.[28] The yield of purified myosin was 1–2 mg/10 g minced uterine tissue.

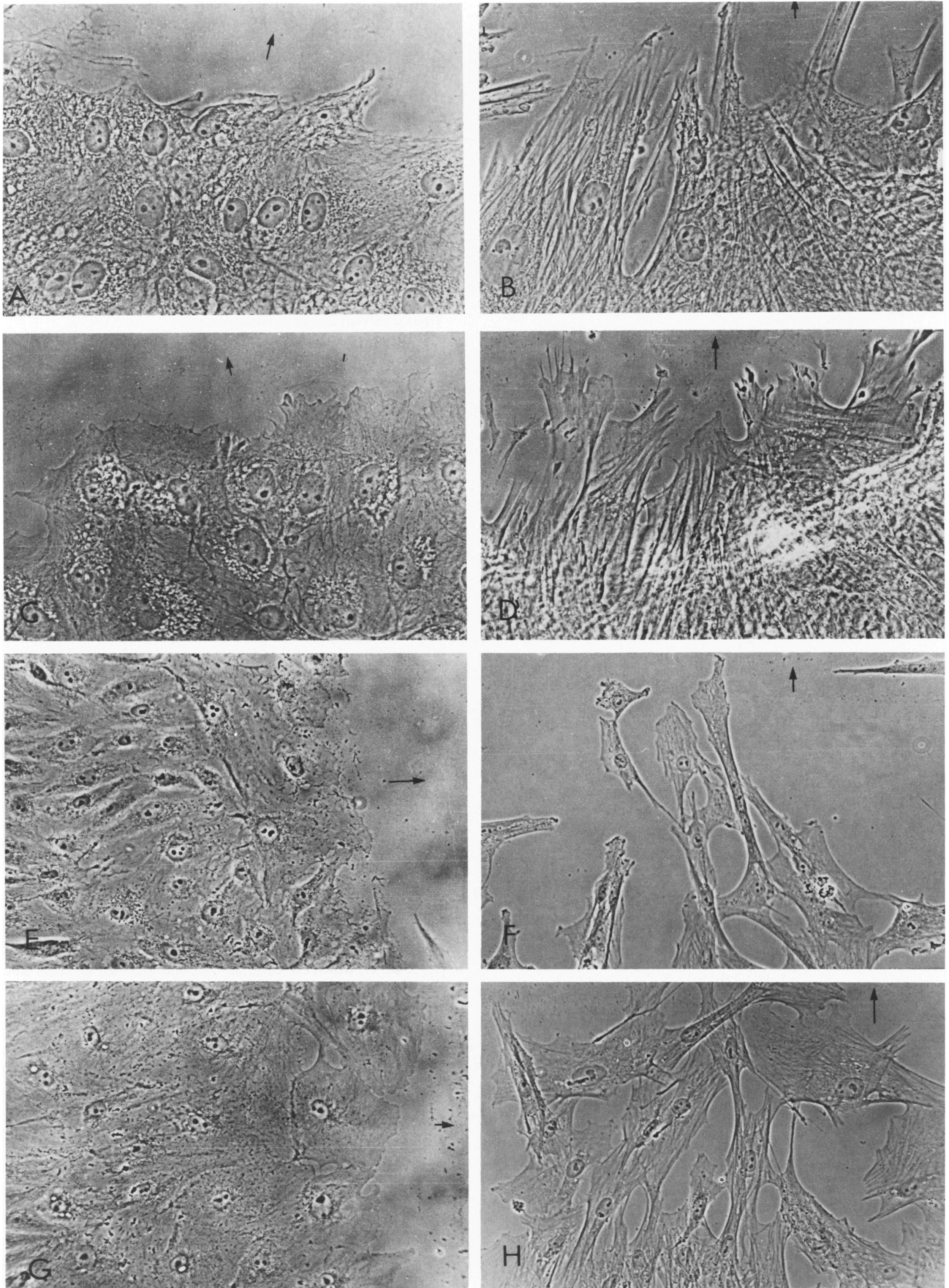


Figure 3—Cells migrating into an experimental wound—ECs (**A, C, E, G**) and SMCs (**B, D, F, H**). **A and B**—Immediately after wounding. (×340) **C and D**—Three hours after wounding. (×340) **E and F**—Forty-four hours after wounding. The ECs form a sheet of cells with contacts between adjacent cells. The SMCs shown are forming a network of cells. (×140; 100) **G and H**—Irradiated cells 44 hours after wounding, showing flattening. (×140) (*Arrow* perpendicular to wound edge, showing direction of movement.) (With a photographic reduction of 6%)

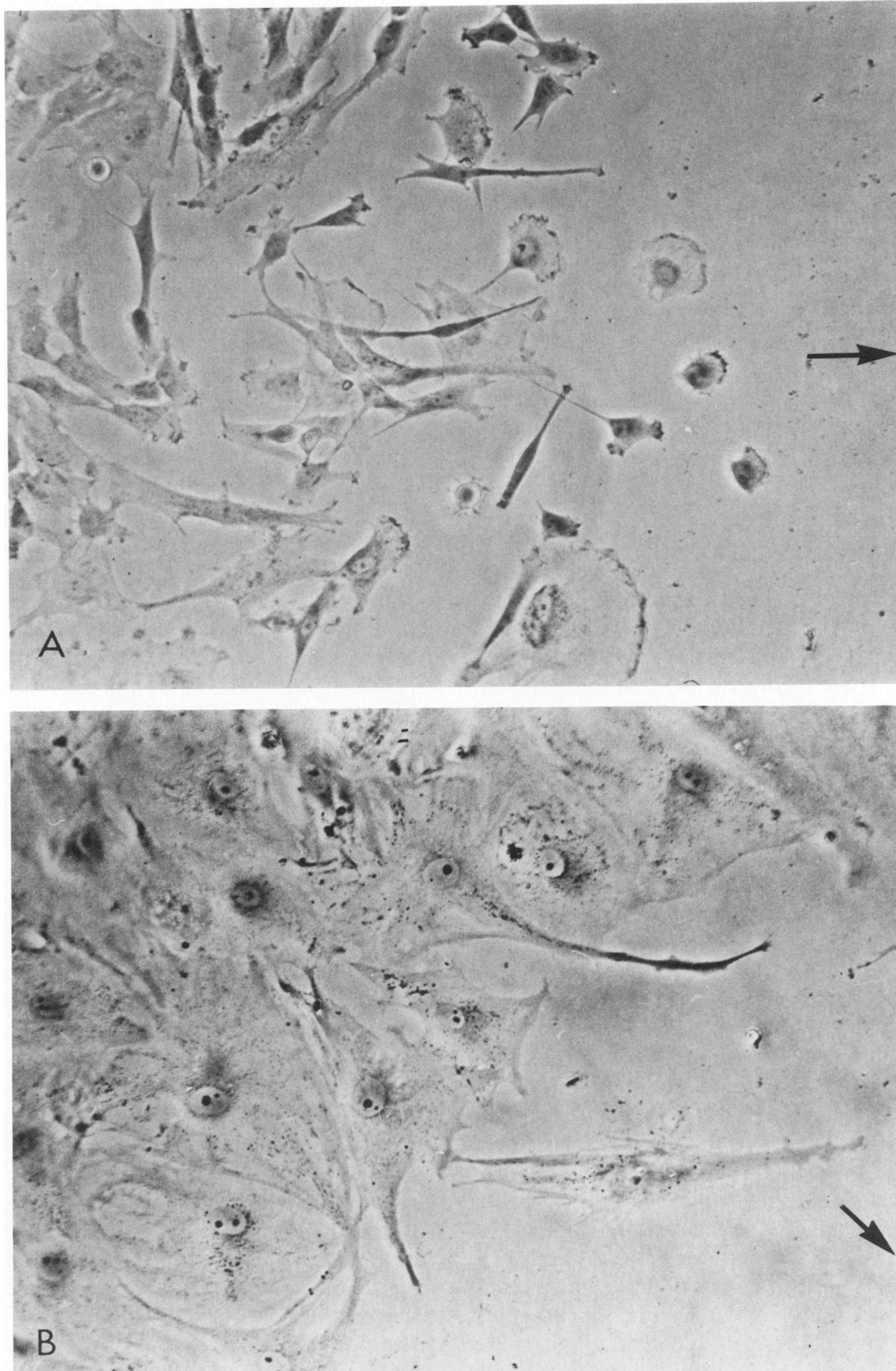


Figure 4—Micrograph of cells migrating into wound at 44 hours. **A**—SMCs show many free cells, overlapping cells, and a network of cells continuous with the monolayer. (×175) **B**—ECs show a migrating sheet with 1 free cell. (×225) (*Arrow* perpendicular to wound edge)

Table 1—Endothelial Cell (EC) and Smooth Muscle Cell (SMC) Migration at 44 Hours

	Free cells			Sheet (EC) or Network (SMC)		
	X*	SE	P†	X‡	SE	P
Nonirradiated						
SMC						
10% FCS	18.8	4.5	< .01	0.218	.004	< .01
20% FCS	17.0	1.3	< .01	0.228	.01	< .01
EC						
20% FCS	3.6	0.7		0.646	0.048	
Irradiated						
SMC						
10% FCS	10.7	1.32	< .01	0.203	0.0158	< .01
20% FCS	10.3	0.97	< .01	0.284	0.079	< .01
EC						
20% FCS	2.0	0.06		0.646	0.007	

* Mean free cells/field (see text).
† SMC *vs* EC.
‡ mm/44 hours.

Myosin Antibody

The antiserums were characterized by double immunodiffusion (Ouchterlony) plate reaction and showed a single precipitin band against the purified myosin and a crude uterine extract that showed identity with each other (Figure 6). In addition, antiserums were precipitated with an equivalent amount of purified myosin and did not react with the crude extract.

The monospecific antibody stained the cells in a pattern similar to that of the antiserums; however, it lost its ability to precipitate in an Ouchterlony double immunodiffusion. The antibody stained smooth muscle cells,[29] endothelial cells, and adventitial fibroblasts.

Cytoskeleton Localization

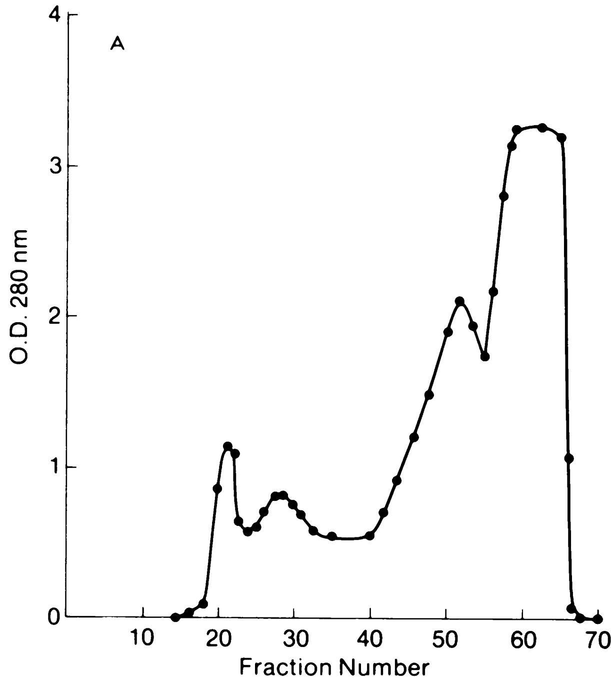
Immunofluorescent localization of intracellular myosin showed a difference in the orientation of the myosin-stained fibers in those ECs along the wound edge (Figures 7A and B). At 44 hours, the main fibers in 90 ± 1.3% of the irradiated cells were roughly parallel to the wound edge, while 10 ± 2.4% were roughly perpendicular to the wound edge (Table 2). In nonirradiated cells the figures were 55 ± 4.3 and 45 ± 4.9%, respectively. Localization of intracellular tropomyosin showed a similar difference in orientation of tropomyosin containing microfilaments (Figures 7C and D). At 44 hours 73 ± 7.2% of the irradiated cells showed the main fiber bundles roughly parallel to the wound, while 27 ± 7.2% were roughly perpendicular to the wound edge. In nonirradiated cells the figures were 45 ± 8.1% and 55 ± 8.1%, respectively. In SMCs there was no difference in the orientation of microfilaments stained with myosin and with tropomyosin when irradiated and nonirradiated cells at the edge of the network were compared (Table 2).

Discussion

We have been able to quantitate the differences characterizing the group-cell migration of SMCs and ECs derived from the same aortic source. SMCs migrate as unattached single cells with a much less cohesive network of cells forming behind the randomly arranged single cells, and ECs migrate into the wound as a cohesive sheet of cells with only a few free unattached cells present along the front edge of the wound. While this study was in progress, Thorgeirsson et al[11] described similar results when examining the effects of platelet factor on the migration of venous endothelial cells derived from umbilical veins and aortic medial smooth muscle cells obtained from infantile human aortas migrating on plastic under agarose gels. Although the conditions, the substratum, and the source of the cells were different than those in our experiment, their observations were similar to the quantitative data we have gathered.

Three types of cell migration have been described with respect to group cell movement. Fibroblasts have been shown to migrate as single cells.[31] Epithelial cells have been shown to migrate as contiguous cell sheets.[32] PtK_1 cells are described to form groups of cells, which move in unison.[33] The migration of smooth muscle cells is similar to that of fibroblasts. This single cell migration is the type of migration that would be most useful in migration from media into intima during the repair of vascular injury. On the other hand, the ability to migrate and still maintain a sheet is important for the role played by ECs in repair as the process of restoring the endothelial barrier is undertaken. The reason for the difference in migration is not apparent from our experiments. One possibility is that ECs remain as a sheet because of strong lateral adhesions between adjacent cells. We have observed that at 44 hours, there are areas along the lateral margins of the motile cells at the wound edge where the edges of adjacent cells either overlap slightly or just make contact. These sites may indeed be cell junctions. ECs have been shown to have junctional complexes between adjacent cells.[34]

Another consideration is our observation that the porcine ECs and SMCs behave quite differently in culture. The ECs are polygonal in shape and do not show any overlapping of cells, while the SMCs are elongated and characteristically show some overlapping even before a confluent monolayer is present. The



SMCs tend to remain as single cells in low-density culture, while the ECs grow as clumps of nonoverlapping cells and do not usually leave the expanding clump. Confluency of ECs occurs as each of the clumps expanding by cell proliferation come in contact with each other. Thus there appear to be inherent differences in these two cell types with respect to cell-to-cell interaction, and this appears to be reflected in their behaviour in the experimental wound.

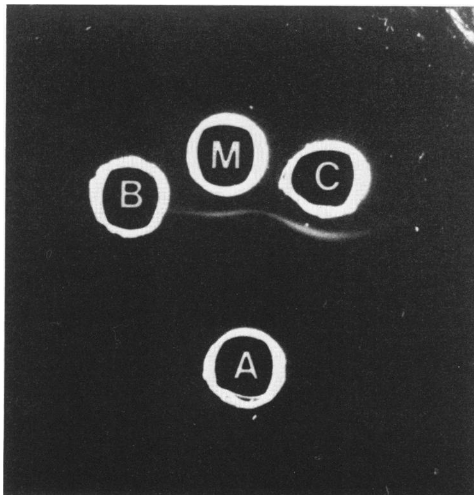


Figure 6—Double immunodiffusion reaction (Ouchterlony) of antiserums to porcine uterine myosin (*A*) against purified porcine uterine myosin (*M*), high-salt crude extract of porcine uterus (*C*), and high-salt buffer (*B*) run in 0.3 M KCl, 10mM Tris Cl, pH 8.0. Note single precipitin band and fusion of single precipitin bands between *A* and *M* and *A* and *C*.

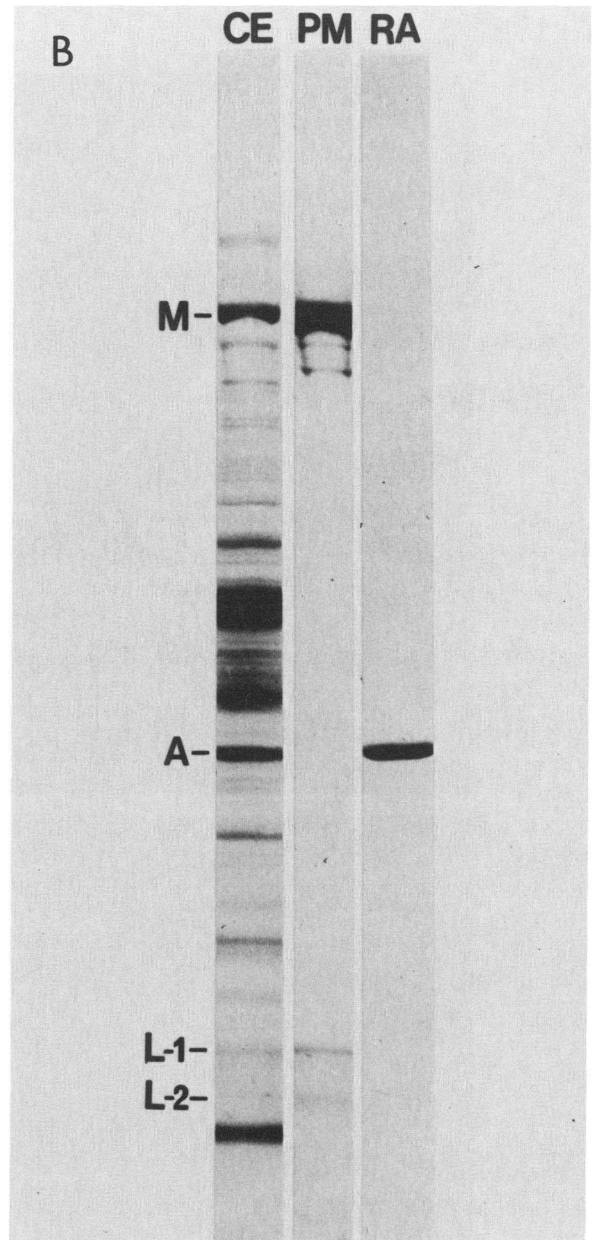


Figure 5—Purification of porcine uterine myosin. **A**—Gel filtration of an ammonium gel filtration of ammonium sulfate 35-55% fraction on Biogel A 15m column. **B**—Polyacrylamide gel electrophoresis of the pool of fractions 27-31, which was used to immunize the rabbits. *CE* = high-salt crude extract of porcine uterus. *PM* = purified porcine myosin used for immunization. *RA* = rabbit skeletal muscle actin.

Schwartz et al[6] have shown that there are indeed differences between EC regeneration *in vivo* and *in vitro*, especially with respect to the width and cell density of the regenerating zone. They showed that *in vivo* altered cell shape extended back from the edge for 60 to 100 rows of cells and the cell density within the regenerating zone was greater than that in the nonrespond-

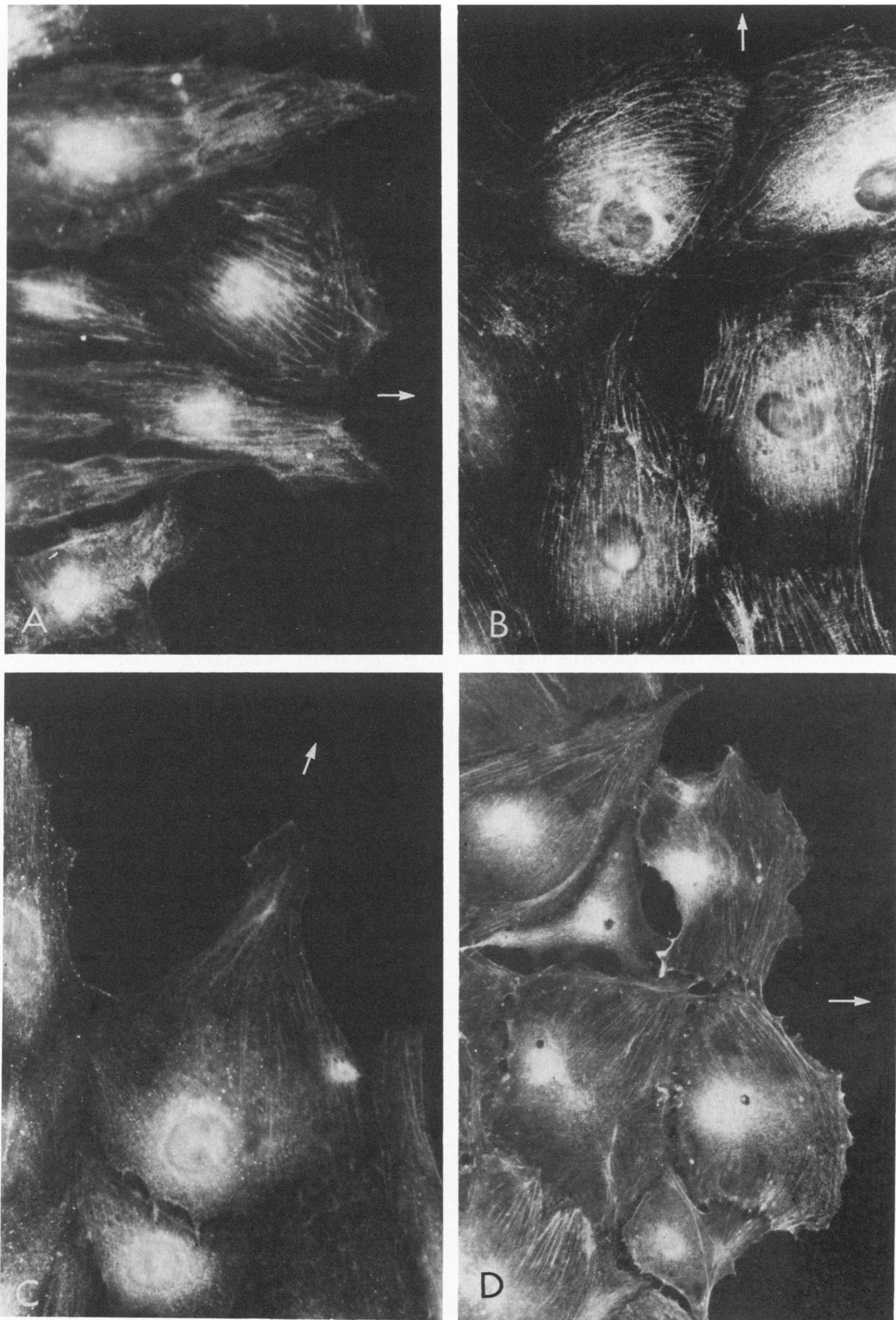


Figure 7—ECs stained with antiserum to myosin (**A, B**) and to tropomyosin (**C, D**) at 44 hours after wounding. The main fiber bundles in the first row of cells are both parallel and roughly perpendicular to the wound edge in nonirradiated cells (**A, C**), while in irradiated cells (**B, D**) they are more frequently parallel (*arrow* perpendicular to wound edge). (**A and D**, ×200; **B and C**, ×400)

Table 2—Orientation of Myosin and Tropomyosin Microfilaments Along Wound Edge at 44 Hours

	Myosin				Tropomyosin			
	Parallel		Perpendicular		Parallel		Perpendicular	
	$\bar{X}$*	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
EC								
Nonirradiated	55	± 4.3	45	± 4.9	45	± 8.1	55	± 8.1
Irradiated	90	± 1.3	10	± 2.4	73	± 7.2	27	± 7.2
SMC								
Nonirradiated	52	± 1.3	48	± 1.3	55	± 1.7	45	± 1.8
Irradiated	47	± 7.0	53	± 7.0	58	± 2.0	42	± 2.0

* Mean of three wounds.

ing endothelium, unlike the *in vitro* situation, where cell density remained the same. In addition, the flow of blood along the axis of the vessel appeared to influence regeneration, in that the regenerating ECs moved more rapidly in the axial direction than around the aortic circumference. Although the ECs along the wound edge *in vivo* appeared somewhat more elongated than our *in vitro* ECs, lamellipodia, which are important structures involved in cell movement, are described by Schwartz *in vivo* as extending from the cells at the wound edge onto the denuded surface similar to that which is seen in both of our EC and SMC wounds. Recognizing the differences between *in vitro* and *in vivo* findings, we think that the technique used in this study is useful in establishing some of the mechanisms of EC and SMC migration.

Scholley et al[11] showed that irradiation of an experimental wound could be utilized for the study of the migratory component in endothelial regeneration. Since agents may have a stimulatory effect on cell proliferation, it is important for one to have a system where the effect of a given agent on group cell migration can be assessed exclusively. ECs and SMCs exposed to the dose of radiation used in our and the other studies sited become flat as they spread forward to cover an area equal to that which they would have covered if cell proliferation remained intact. The migration of cells is characterized by a complex series of events and involves the formation and retraction of cell processes, the changing of the shape of the cell, and the displacement of the cell in a given direction. The mechanisms involved in these processes are presently unknown; however, studies suggest that the cytoskeleton plays an important role in cell migration.[15-17] Since the motile irradiated cells were flatter than the nonirradiated cells, it was likely that other elements involved in cell migration might show some differences. We carried out immunofluorescent studies to define the distribution and orientation of myosin and tropomyosin containing microfilaments in migrating irradiated and nonirradiated ECs and SMCs at the wound edge. The findings showed that the orientation of these microfilaments relative to the wound was different in ECs. This change in the orientation of the microfilament bundles appears to be associated with the flattening of the ECs. SMCs did not show a difference between irradiated and nonirradiated cells. This may reflect the general random orientation of the SMCs as they migrate at the wound edge in several directions, so that the orientation of the microfilament bundles is not a useful indicator of group cell migration of SMCs. The lack of change in orientation of the microfilaments should not be taken as evidence that the motility of SMCs is affected differently by irradiation from that of the ECs.

Immunofluorescent studies have shown that microfilament bundles contain actin,[35] myosin,[36] and tropomyosin[37] and that the direction of orientation of the main microfilament bundles in migrating cells is generally parallel to the direction of cell migration.[19,39] Thus although both irradiated and nonirradiated cells migrate forward into the wound, the change in the orientation of the microfilaments that is seen in irradiated ECs may indeed reflect a difference in the way in which the flat irradiated ECs migrate.

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