In Vitro Cell. Dev. Biol.—Animal 34:609–612, September 1998

1071-2690/98 $05.00+0.00

Letter to the Editor

STRAIN-INDUCED DUAL ALIGNMENT OF L6 RAT SKELETAL MUSCLE CELLS

Dear Editor:

Cells in the body are constantly subjected to mechanical perturbations including such forces as cyclic strain, shear stress, and ambient pressure (8). Studies from our laboratory and others have attempted to model these forces with a variety of *in vitro* devices to better comprehend the *in vivo* milieu (1,11). A common finding of cells subjected to cyclic strain has been the observation of perpendicular alignment in relation to the strain vector (8). This has been demonstrated in a broad array of cells including endothelial cells (14), vascular smooth muscle cells (7), keratinocytes (9), tendon cells (6), fibroblasts (3), osteoblast-like cells (4), and embryonic skeletal muscle cells (11).

In this study, we report strain-dependent, simultaneous, dual orientation of rat skeletal muscle cells (RSMCs) subjected to cyclic strain afforded by the heterogeneous gradient associated with a vacuum-generating strain apparatus. In particular, we report the novel finding of the parallel arrangement of RSMCs in response to intermediate strains of 1–12% in addition to the well-characterized perpendicular alignment of cells subjected to higher strains of 12–24% in the peripheral region.

L6 rat skeletal muscle cells (Batch #: F-12121, 1458-CRL, American Type Culture Collection) (13) were maintained in Dulbecco's modified Eagle's medium F-12 (DMEM/F-12), supplemented with 10% fetal calf serum, 100 U penicillin per ml, 100 μg streptomycin per ml, and 250 ng amphotericin B per ml. RSMC (fewer than 5 passages) were identified by their typical fusiform appearance, bundle-type arrangement of confluent cell populations, and ability to form myotubes when grown in serum-free medium (13).

RSMC were subjected to *in vitro* cyclic strain by the Flexercell strain unit (Flexcell. Corp., McKeesport, PA) (1,7,9). The strain unit consists of a manifold housed in a tissue incubator attached to a vacuum unit that is regulated by solenoid valves and a computer-controlled program to adjust frequency, magnitude, and duration of the cyclic strain. Cells are seeded onto type I collagen-coated, flexible membrane, six-well plates that can be deformed by vacuum. The strain on different regions of the membrane have been calculated by finite element analysis and verified by empiric measurement (6). Cells located in the center region experience much less strain than cells in the periphery with a 10% average strain. The membranes were subjected to vacuum deformation of 150 mm Hg (10% average strain), at a continuous frequency of 60 cycles/min (0.5 sec deformation alternating with 0.5 sec relaxation).

To measure RSMC alignment, RSMC were fixed with 3.7% formalin and stained with 2% crystal violet. To determine the adaptive cellular angle, the angle between the long axis of the cells in relation to the direction of the strain vector was calculated at the center, intermediate, and peripheral zones of the well. RSMC migration was performed according to the modified method of Bell et al. (2). Cells were plated on a flexible membrane with a "steel fence" to prevent spreading to one-half of the membrane. After the cells attained subconfluence, the fence was removed and the cells were subjected to cyclic strain. Migration was determined by phase-contrast microscopic analysis of RSMC movement (i.e., pattern and distance) to the unseeded side. Cellular morphological analysis of the pattern and distance was determined by our calculating the long axis of the cell to the short axis of 50 randomly selected RSMCs exposed to cyclic strain at the center, intermediate, and peripheral zones of the well and expressed as aspect ratios. The aspect ratio was compared to the ratio of static, control cells.

Under static conditions, RSMC are typically arranged in bundle-like perpendicular arrays of random orientation (Fig. 1 *A*). Exposure of RSMC to 3 d of 10% average strain (60 cycles/min) led to directed alignment according to the degree of strain (Fig. 1 *B*). In the peripheral zone subjected to high strain (12–24%), RSMC align perpendicular to the strain gradient. RSMC exposed to lesser strain (1–12%) in the intermediate zone aligned parallel to the strain gradient. These strain-dependent shifts in RSMC orientation were in marked contrast to the random orientation found in either the peripheral and intermediate regions of the static controls. The orientation of RSMC grown in the center of the well and subjected to minimal strain (0–1%) was not affected as compared to the static control.

We quantified the precise changes in RSMC orientation after 1, 3, 5, and 7 d of cyclic strain (10% average, 60 cycles/min) by determination of the angle between the long axis of the cell and the strain vector in 50 cells per zone (i.e., center, intermediate, and peripheral) (Fig. 2). These data clearly show a strain-induced reduction of the angle in the intermediate zone as well an increase of the angle in the peripheral zone, reaching significant levels by Days 3 and 5, respectively. By Day 7, we observed a 100% angle reduction and hence a parallel arrangement in the intermediate zone. In contrast, the peripheral zone showed a greater than 200% angle increase and therefore a perpendicular arrangement.

Using the "fence technique" described in "Materials and Methods," we assessed RSMC migration in response to cyclic strain. As shown in Fig. 3, RSMC subjected to 5 d of cyclic strain migrated in a strain-dependent manner. RSMC in the peripheral, high strain zone (12–24%) migrated directly outwards (Fig. 3 *F*) within the peripheral region of high strain and in a more pronounced manner than static controls (Fig. 3 *E*). Because the proliferative response of RSMCs was unaltered by cyclic strain (*see* below), we conclude that the observed changes in migration were truly due to migration. However, we cannot rule out a minor component of strain-induced proliferation in mediating some of the migratory response into cell-free areas. RSMC in the intermediate strain zone (1–12%) turned 90 degrees and did not migrate to the unseeded area of the well (Fig. 3 *D*), unlike the static RSMC (Fig. 3 *C*). RSMC in the low-strain region (0–1%) in the center migrated directly outwards (Fig. 3 *B*) similar to the cells of static controls (Fig. 3 *A*) and to a lesser extent than stretched RSMC in the

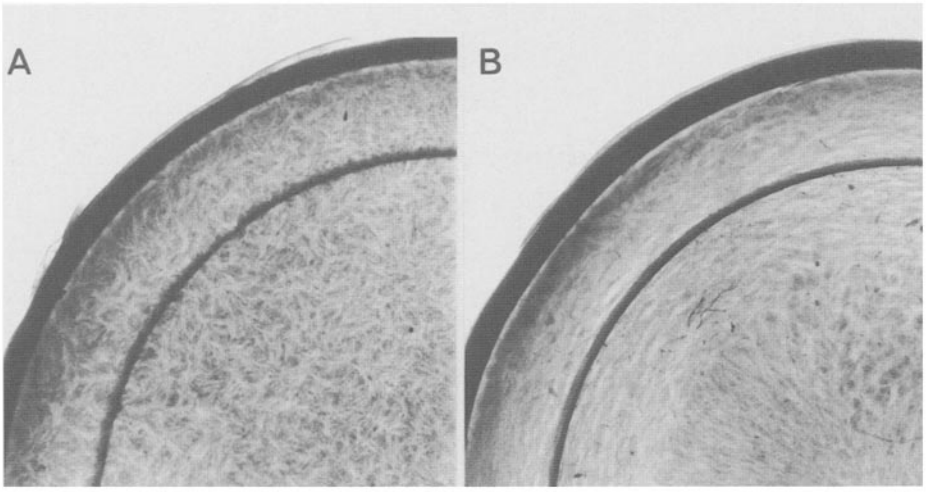

FIG. 1. Effect of cyclic strain on RSMC orientation. RSMC were exposed to 3 d of 10% average strain at 60 cycles/min under serum-fed conditions (*B*) as compared to static conditions (*A*). RSMC were washed, fixed, and stained as described in "Materials and Methods." Shown is a quarter section of the entire well from the center outward. Marked are the center, intermediate, and peripheral zones of the well. The *heavy line* represents the point of attachment of the flexible membrane. Data shown are from a representative experiment repeated three times.

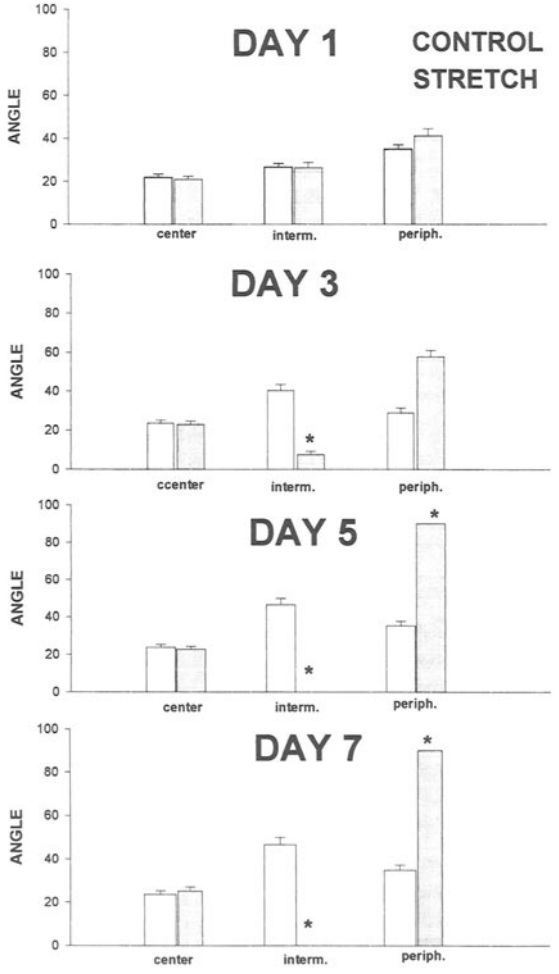

FIG. 2. Effect of cyclic strain on RSMC adaptive cellular angle. RSMC adaptive cellular angle was determined as the angle between the long axis of the cell and the strain vector. Angles were determined for 50 cells per field (i.e., center, intermediate, and periphery) under either control or stretch conditions (i.e., Day 1, Day 3, Day 5, Day 7) from one experiment and are representative of three separate experiments (* = $P < 0.05$).

periphery (Fig. 3 *F*). In contrast, static cells migrated evenly toward the unseeded area of the well (Fig. 3 *A,C,E*).

RSMC subjected to 10% cyclic strain at 60 cycles/min showed strain- and time-dependent alterations in morphology as measured by the ratio of long-axis- to short-axis aspect ratios. After 5 d of cyclic strain, RSMCs grown in the high strain, peripheral region were thinner and more elongated than the static control cells, as reflected by a significant (ANOVA with post hoc testing) ($P < 0.05$) increase in the aspect ratios (Table 1).

We next examined whether the strain-induced alterations in RSMC orientation and morphological and migratory features were related to changes in their proliferative rate. However, at a strain regimen that was found to be effective in altering these parameters (i.e., 10% average strain at 60 cycles/min) we failed to observe any significant change in the rate of RSMC proliferation. RSMC were not injured by this regimen as determined by lactate dehydrogenase release and absence of either vacuoles or desquamation (data not shown).

The data presented in this letter demonstrate strain-dependent dual alignment of L6 rat skeletal muscle cells. The overall distribution of cells in the culture well is striking in that cells in the periphery are oriented perpendicular to the strain gradient, cells in the intermediate zone are oriented parallel to the strain gradient, and cells in the center region are comparable to those observed in static controls.

In a recent study, Takemasa et al. (10) utilized a novel uniaxial device capable of generating a wide range of strain to study stress fiber orientation in response to acute cyclic strain in human umbilical vein endothelial cells (HUVECs). By studying regions of symmetrical coexisting confronting stress fibers, they were able to determine an accurate measure of orientation degree versus strain amplitude. They demonstrated a strong inverse correlation between the amplitude of strain and the degree displacement. HUVECs subjected to a lesser amplitude of strain were found to be aligned at a greater angular displacement from the line perpendicular to the direction of stretching and hence in a more parallel fashion to the strain vector and vice versa.

A few other reports have noted similar strain-dependent trends in orientation including a study by Dartsch et al. (5) in smooth muscle cells and Vandenburgh et al. (11) in skeletal muscle cells. Vandenburgh et al. (11) have demonstrated similar findings in primary avian

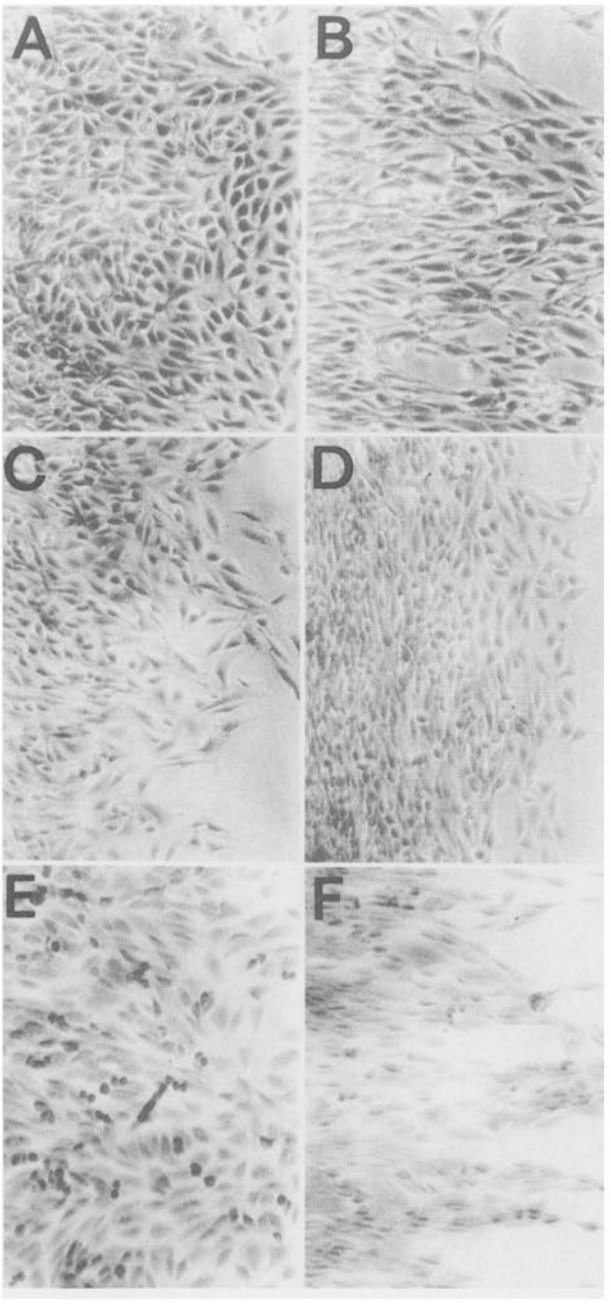

FIG. 3. Effect of cyclic strain on RSMC migration. RSMC migration was assessed by the "steel fence" technique to prevent spreading to one-half of the membrane. After the cells attained subconfluence, the fence was removed and the cells were subjected to 5 d of cyclic strain. RSMC migration was assessed by phase-contrast microscopic analysis of the pattern and distance of RSMC movement to the unseeded side. Shown are the results of one experiment repeated three times: (*A*) control, center; (*B*) stretch, center; (*C*) control, intermediate; (*D*) stretch, intermediate; (*E*) control, periphery; (*F*) stretch, periphery.

skeletal myoblasts using a different device that generates a more homogeneous strain and promotes the differentiation of myoblasts into myotubes. The extent of myotubule orientation is dependent on the magnitude of stretch. Thus, at an intermittent regimen with 17% stretch/relaxation for 48 h, myotubes orient perpendicular to the direction of strain throughout the well. Interestingly, in response to slow unidirectional continuous stretch, the direction of myotubule orientation is parallel to the force vector. Why the rat skeletal muscle cells grown in the intermediate zone in our system respond in a similar parallel manner to the strain vector is not understood but may reflect the sensing mechanism by which skeletal muscle cells sense and transduce mechanical perturbation.

TABLE 1

EFFECT OF CYCLIC STRAIN ON RSMC MORPHOLOGY[a]

Zone	Condition	Aspect Ratio
Center	Control	1.92 ± 0.09
	Stretch	2.12 ± 0.09
Intermediate	Control	1.86 ± 0.09
	Stretch	3.0 ± 0.11*
Periphery	Control	1.86 ± 0.08
	Stretch	4.34 ± 0.12*

[a]RSMC aspect ratios (long axis/short axis of 50 randomly selected cells) were measured from center, intermediate, and peripheral zones of the wells after cells were exposed to 5 days of cyclic strain (stretch) and compared with unstretched controls (control). Two additional experiments yielded similar results.

*Indicates values significantly different from controls at $P < 0.0$.

Despite the presence of a minor circumferential strain associated with the Flexercell apparatus, we believe that the predominance of the uniaxial, radial strain is more critical in dictating strain patterns. Moreover, as recently described by Wang et al. (12) albeit with a different model, substrate surface deformations may be quantified by two mutually perpendicular directions. Whether such local strain gradients exist and contribute to the observation of strain-induced dual alignment of RSMCs is beyond the scope of this letter but do merit attention. However, it should be noted that in the Wang study (12), melanocytes were found to respond solely with perpendicular alignment in response to a unidirectional cyclic stretching upon exceeding a strain threshold.

In summary, we report that cyclic strain stimulates a novel, dual alignment phenomenon not previously observed in a variety of other cell types including endothelial cells, smooth muscle cells, and keratinocytes. The dual alignment of RSMC constitutes a parallel arrangement in the intermediate region of the well of moderate strain (1–12%) and a perpendicular arrangement in the peripheral, high strain regions (12–24%). Strain-induced migration appeared to be directed in a manner consistent with the strain-induced changes in orientation. The strain-induced changes in orientation and migration were not related to changes in the proliferative rate of RSMC. The etiology and significance of the phenomenon remains unclear but application of cyclic strain may provide an interesting tool for investigators studying mechanotransduction processes in skeletal muscle.

ACKNOWLEDGMENTS

This research was supported by grants to B. E. S. [NIH R01 47345. VA Merit Review, and American Heart Association (National)] and to I. M. from NASA.

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