In Vitro Cell. Dev. Biol.—Animal 34:640–645, September 1998

1071-2690/98 $05.00+0.00

FIBROBLAST MOVEMENTS DURING CONTRACTION OF COLLAGEN LATTICES—A QUANTITATIVE STUDY USING A NEW THREE-DIMENSIONAL TIME-LAPSE TECHNIQUE WITH PHASE-CONTRAST LASER SCANNING MICROSCOPY

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(Received 12 January 1998; accepted 7 April 1998)

SUMMARY

In this study we assessed the behavior of fibroblasts during contraction of collagen lattices. We applied a new technique for three-dimensional time-lapse studies of movements of living cells using phase-contrast laser scanning microscopy. Five anchored and five floating collagen lattices were studied regarding the activity of cells during a 7-h period of active contraction. Three-dimensional reconstructions of the fibroblasts and their extensions were made from datasets of 16–26 "optical sections" 5 μm apart recorded hourly during the period of measurements. The distance between fibroblast nuclei in the floating lattices decreased by a mean of 6.8 μm, but remained constant in the anchored group. Only minor variations were found in the angle between a line connecting any two nuclei and the tangent of the lattice margin. The lengths of the cellular extensions continuously changed by shortening and extending, and an increasing number of intercellular contacts were established with time. The angle between the extensions and the periphery of the lattice varied continually, and no distinct pattern of arrangement of the extensions was seen. In conclusion, we have shown in living cells *in vitro* that fibroblasts do not appear to move around within lattices during contraction but rather send out and withdraw cellular extensions continuously. This speaks against cellular locomotion or movement as a main feature of contraction. Time-lapse scanning laser microscopy has also been shown to be a suitable method to study cellular behavior quantitatively in three dimensions during lattice contraction.

Key words: collagen gel contraction; laser scanning microscopy; cellular extension; cellular locomotion; fibroblast.

INTRODUCTION

Contraction of connective tissue occurs during normal tissue repair as well as in pathological conditions such as Dupuytren's contracture and contracture around breast implants (16,20). Contraction is performed by fibroblasts and can be studied *in vitro* in collagen lattices which are able to contract to 25% of their original size within 48 h. The mechanism by which the fibroblasts contract the lattices is unknown however, but has been ascribed to cellular movements or locomotion within the gel. These conclusions are mainly based on studies on fixed lattices with fluorescence microscopy (5) and electron microscopy (9). Another hypothesis states that cells show repeated protrusion and withdrawal of processes and thereby collect collagen fibrils into their vicinity (22). All these presumptions are based on morphological data and no actual studies on living and contracting cells appear to have been performed. To better understand the kinetics of fibroblast behavior during contraction, there is a need for methods to observe and register the behavior of living cells during active contraction.

In the present study we took advantage of the fact that microscopic phase-contrast mode gives a resolution in the focal plane which is similar to that obtained in confocal microscopes (10). We studied living cells in collagen lattices during active contraction using a laser scanning microscope in the phase-contrast mode with a motor-driven objective stage, and sequential images along the *z*-axis (5 μm apart) were recorded over time. The laser beam easily penetrated the collagen gel so that the transmitted light could be recorded with a photomultiplier tube. The series of optical sections obtained were stored in a computer and three-dimensional reconstructions were made of the cells and their extensions. Three-dimensional time-lapse recordings of living cells over time were thus performed. Using this technique, we estimated the behavior of fibroblasts during contraction of anchored and floating collagen gels regarding changes in position, cellular extensions, and relation to the margin of the gel.

MATERIALS AND METHODS

Fibroblast cultivation. Fibroblasts from human foreskin (AGO1518A) were obtained from the cell culture repository at the National Institute on Aging, which is part of the Coriell Institute For Medical Research (Camden, NJ). The cells were cultured in Eagle's minimal essential medium (EMEM) with 20% heat-inactivated newborn calf serum, 2 mmol freshly added L-glutamine, 50 IU penicillin per ml, and 50 μg streptomycin per ml. Cultures were maintained in an incubator with a humidified atmosphere at 37° C in 5% CO_2 and 95% air. Cells were grown to confluence before subcultivation. The experiments were done at Passages 15–20.

Collagen lattices. Collagen type I was extracted from rat tail tendons as described by Bell et al. (4), with minor modifications. Ligaments from six rat

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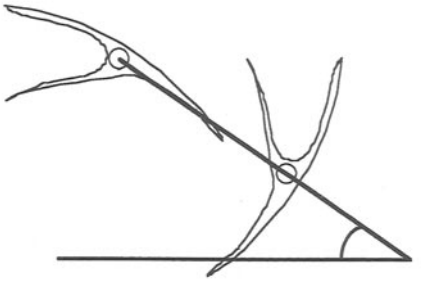

FIG. 1. A drawing showing two cells with cellular extensions and centrally placed nuclei. A line is drawn through the nuclei and another line drawn parallel to the tangent of the periphery of the lattice. The angle between these lines and the distance between nuclei were measured to estimate movements of the cells within the lattices. A similar technique was used to estimate angular changes of cellular extensions.

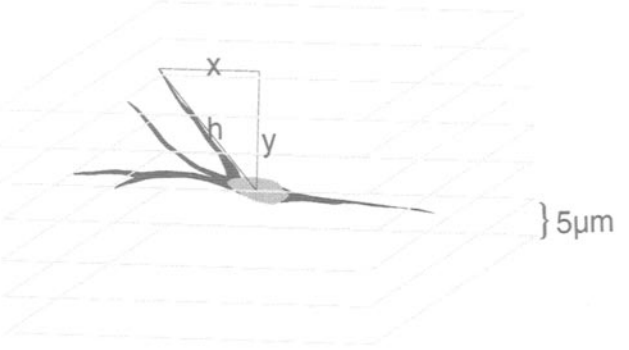

FIG. 2. A drawing showing the three-dimensional reconstruction of a fibroblast in a collagen gel from optical sections 5 µm apart. The length of an extension was calculated according to the geometry of a right-angled triangle ($h^2 = x^2 + y^2$) where h denotes the length of the extension, x the length of the projection of the extension in the horizontal plane and y in the vertical plane (y = number of sections × 5 µm).

tails were placed in 300 ml diluted acetic acid (1/1000). The mixture was stirred for 48 h at 4° C and centrifuged at 26 100 rpm for 1 h. An aqueous stock solution was prepared and the collagen concentration adjusted to 25 mg/ml. The collagen lattices were prepared by adding to 1 ml chilled serum-free medium, 0.5 ml chilled collagen, 0.5 ml chilled freshly trypsinized fibroblasts (mixed with serum-free medium to a concentration of 1×10^5 cells/ml) and 20 mmol HEPES buffer in 35-mm petri dishes. All samples of serum and media were purchased from Labdesign, Täby, Sweden. For the main experiments, five fibroblast-populated collagen lattices (10^5 cells/lattice) were allowed to polymerize for 15 min after which they were detached from the wall and the bottom of the dish (floating lattices). Another five lattices were prepared in the same way as the floating lattices except that they were not detached from the wall and the bottom of the dish (anchored lattices).

To assess if the measuring procedure influenced lattice contraction, the areas of four lattices were estimated at 14 h, at the end of cellular measurements (21 h), and after 48 h. Images of the lattices were captured with a Hamamatsu CCD video camera connected to a Macintosh computer, and the area of the lattice was estimated with the image analysis program NIH Image 1.58 (by Wayne Rasband at U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nih.gov.).

Laser scanning microscopy and cellular measurements. The lattices were kept on a prewarmed microscope stage covered by a plastic hood and the temperature was kept at 37.5° C. The lattice was kept in the same position on the microscope stage during the entire measurement procedure. One area populated with 2–5 fibroblasts close to the margin of the lattices was studied in each lattice. At each h, during the 7-h period of measurements, a set of 16–26 images 5 µm apart was captured and stored in a computer. The microscope was a Zeiss LSM 410 inverted confocal laser scanning microscope. The confocal pinhole device was not used in these measurements, and the scanned image was captured in a photomultiplier tube registering transmitted phase-contrast light. The microscope was equipped with three laser beam sources: 488, 543, and 633 nm. The quality of the phase-contrast images were similar with either of the three lasers and the 633-nm helium neon laser (5 mW), which has the lowest photon energy, was used. Measurements were made with a 40× long distance dry LD-objective (N.A. = 0.6) and each image captured measured 512 × 512 pixels.

In each set of images, the distance between nuclei (calculated in the same way as cellular extensions, *see* below) and the angle between a line drawn between two nuclei and the tangent at the periphery of the lattice were estimated (Fig. 1). These calculations were made to assess any movements of the cell bodies. The length of the cytoplasmic extensions and the distances between the nuclei (cell bodies) were measured according to the geometry of a right-angled triangle (Fig. 2). The length of an extension h or the distance between two cell nuclei was calculated according to the formula: $h^2 = x^2 + y^2$, where x denotes the length of the projection of the extension in the horizontal plane and y the length of its projection in the vertical plane (y = number of sections × 5 µm). These calculations were performed with the image analyzing program NIH Image 1.58 (*see* above). The changes in length of the cellular extensions from h to h gives the velocity of forward and backward movements of the extensions. The formation and withdrawal of extensions as well as contacts between them were also registered.

The measurement error, including method and operator error, for the length of cellular extensions was 1.0 ± 0.6 µm (mean ± SEM). This value was obtained by measuring the same extension 10 times and closing the image between each measurement.

Confocal microscopy. Two gels were fixed with 4% paraformaldehyde and stained with phalloidin-Bodipy® (Molecular Probes, Eugene, OR) for 20 min after 48 h of contraction. These gels were then used to assess the cellular network characteristic of late contraction. We studied actin fluorescence after activation at 488 nm using the confocal mode (*see* Fig. 6).

Statistics. The Wilcoxon rank-sum test was used to assess the significance of the difference between groups at a probability of less than 0.05.

RESULTS

The changes of the areas of the collagen gels during contraction are shown in Fig. 3. During the 7-h period of measurements, the areas of the lattices decreased 9% ($P < 0.02$). After these 7 h on the microscope stage, the lattices continued to contract when put back into the incubator, which shows that the cells were still alive and active. The distance between the cell nuclei in floating lattices decreased by a mean of 6.77 ± 1.5 µm ($P < 0.001$) during the time of measurements. On the other hand, the mean distances between nuclei in anchored lattices did not change significantly and measured 0.41 ± 2.48 µm ($P > 0.88$). The angle between the tangent of the margin of the lattice and the line drawn between two nuclei remained almost constant in both groups, the changes of the angles being 0 ± 0.15 degrees in floating and 0.3 ± 0.07 degrees for anchored lattices.

A total of 20 cells with 62 cellular extensions and 16 cells with 44 extensions were studied in floating and anchored collagen lattices, respectively (Table 1). The diameter of the extensions was in the order of 1–2 µm. In both groups the cells formed new extensions which lengthened, whereas others shortened and eventually disappeared (Tables 1,2). The velocity (increase and/or decrease) of the movements of the extensions was slightly higher in the anchored group but the difference was not statistically significant (Table 2). Also the angles between the extensions and the tangent of the margin of the lattices varied from h to h in both groups, but no distinct

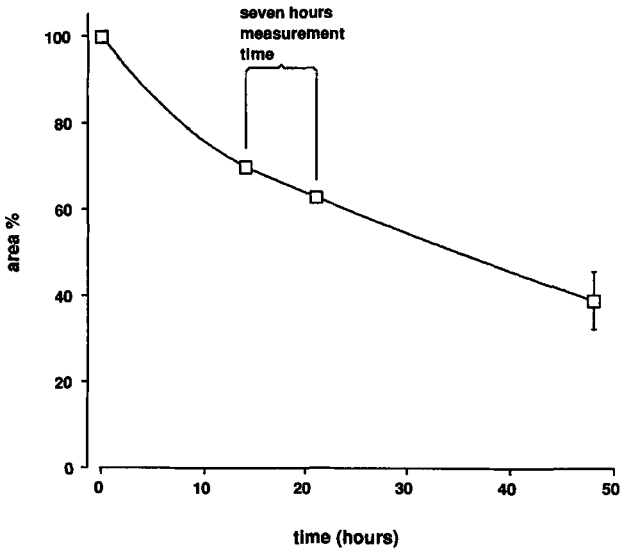

FIG. 3. Contraction curve showing the percentage of area reduction in four lattices during 48 h (mean ± SEM). The 7-h time of cellular measurements between 14 and 21 h is indicated.

TABLE 1

NUMBER OF EXTENSIONS AND CONTACTS IN FLOATING AND ANCHORED COLLAGEN LATTICES

	Floating lattices (n = 5)	Anchored lattices (n = 5)
No. of cells measured	20	16
Extensions per cell (at start)	3.1	2.8
New extensions per cell	1	0.6
Dissolved extensions per cell	0.9	0.7
Contacts (at start)	3	5
New contacts	14	5
Interrupted contacts	4	2

TABLE 2

VELOCITY (MEAN ± SEM) OF THE MOVEMENTS OF CELLULAR EXTENSIONS AND THE NUMBER OF EXTENSIONS CHANGING DURING THE OBSERVATION PERIOD

	Floating lattices	Anchored lattices
Extension velocity (μm/hour)	20.8 (3.8)	27.5 (4.2)
Shorter (n)	20	7
Longer (n)	5	7
Unchanged (n)	5	2

pattern of movements was seen and there was no tendency for the extensions to run parallel to the periphery of the lattice. The pattern of motility, as described above, of two fibroblasts during the measurement period is shown in Fig. 4. Bleb-like formations shown in Fig. 5 could sometimes be found at the end of the extensions.

Contacts between cells were formed and dissolved, both between extensions and between extensions and the body of the cell. The net result of newly formed contacts was 10 in the floating and 3 in the anchored lattices (Table 1). After 48 h the extensions were seen to form a network of intercellular connections (Fig. 6).

DISCUSSION

Cellular movements have mostly been studied mostly by two-dimensional time-lapse cinemicrography with cells cultured on the bottom of culture dishes. However, cells may behave differently in a more natural, three-dimensional environment than when spread out on a flat surface. For instance, the mitogenic effect of epidermal growth factor on smooth muscle cells and NRK cells was inhibited by transforming growth factor-beta in subconfluent cell monolayers but stimulated when the same type of cell was cultured in soft agar (3). The distribution of actin microfilaments has also been shown to differ between fibroblasts cultured on a flat surface or incubated in a collagen lattice. In collagen lattices actin is distributed along the inner surface of the entire plasma membrane whereas on surfaces, actin distribution is restricted to the part of the membrane in contact with the flat surface (13). In collagen lattices used for contraction studies *in vitro*, the fibroblasts operate in a three-dimensional environment and their movements cannot be followed and recorded with conventional two-dimensional time-lapse techniques. The present study concerns a new application of phase-contrast laser scanning microscopy to study cellular events in three dimensions over time. Three-dimensional reconstructions of cells and tissues has long been done in cell biology but, to our knowledge, not applied in time-lapse studies. Using this technique, we found a decrease in the distance between cell nuclei in floating but not in anchored lattices. The cellular extensions were also shown to shorten and extend continuously, and an increasing number of intercellular contacts were established with time. No distinct pattern of arrangement of the extensions in relation to the gel margin was seen.

A proposed mechanism for contraction of connective tissue is rearrangement of collagen fibrils by moving fibroblasts (1,5,8). Originally it was assumed that the cell pulled itself forward by retaining and elongating its shape, with the cellular extensions moving like tank treads (8). Hay et al. (9) suggested on the basis of ultrastructural findings that fibroblasts within type I collagen migrate by continuously synthesizing a new front end, while the rear end of the cell pulls and aligns collagen fibrils trailing the cell and finally breaks the contact with the cell. We could not see such synchronized growth of the front end of the cellular extension and dispersion of the rear end. Cells have also been reported to migrate from the interior region of a collagen lattice during contraction and eventually form a layer that encapsulates the gel (1,2) and possibly to form an actin cable around the gel (18). Such centrifugal movement of cells could not be confirmed in our system when lattices were reincubated up to 48 h after experimentation. No movement of cell nuclei could be detected in anchored gels during the 7-h period of observation, although such gels are known to build up large tension forces (5,21). The reduced distances between cell nuclei in floating lattices were presumably the result of an increase in cellular density as the volume of the lattices diminished. Also, if the cells had been moving around in the gel, the angles between the periphery of the lattice and the line drawn between two nuclei would have changed. The conclusions from these

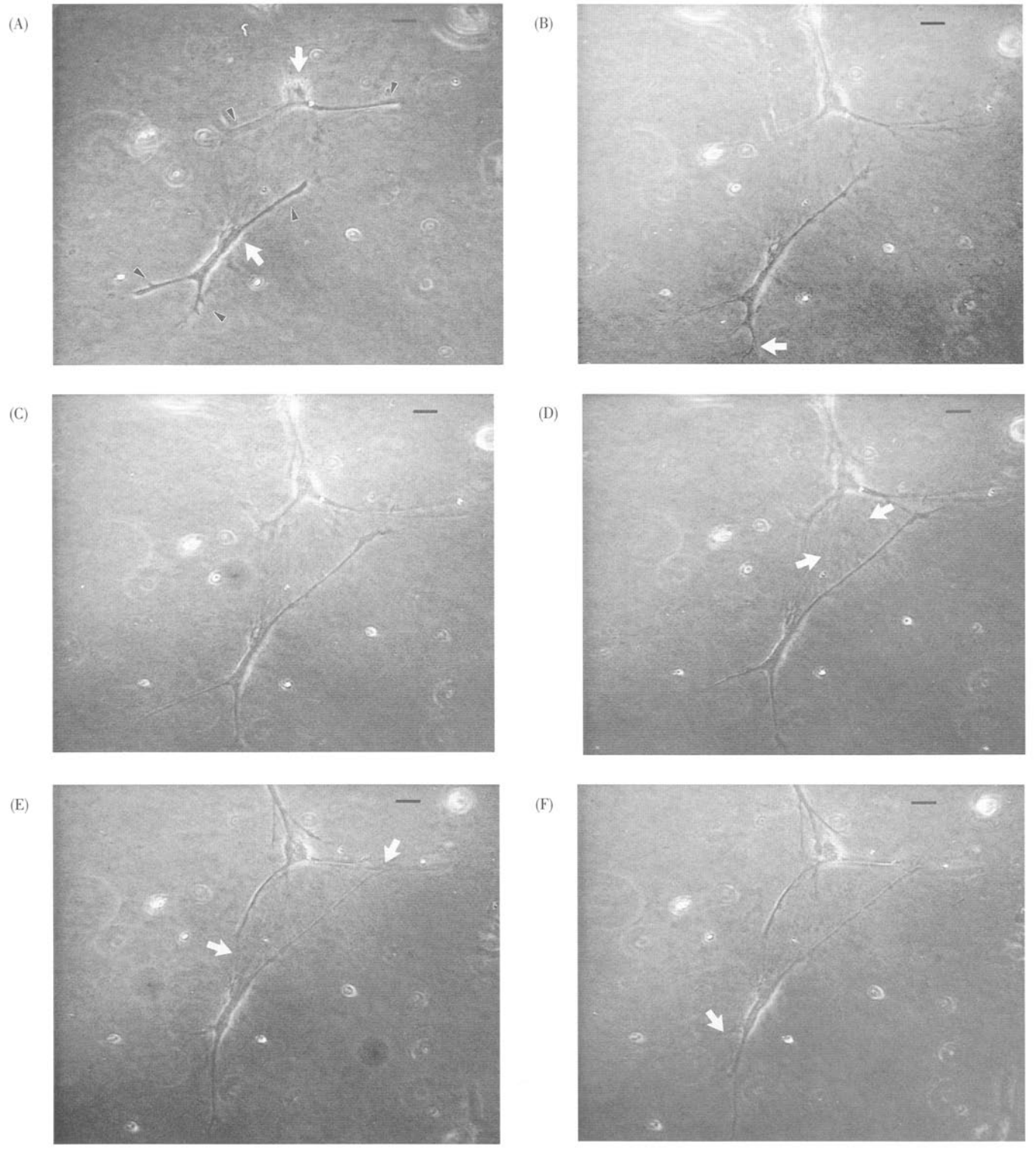

FIG. 4. PHOTOMICROGRAPHS OF PHASE-CONTRAST IMAGES OF TWO FIBROBLASTS AND THEIR EXTENSIONS CAPTURED DURING A 7-H OBSERVATION PERIOD (*BAR* = 20 μm). (*A*) shows two fibroblasts (*arrows*) with three cellular extensions (*arrowheads*) in the lower cell and two in the upper, 1 h after start of measurements. No intercellular contacts are seen. (*B*) After 3 h one new extension has developed from the lower cell (*white arrow*) and the other extensions have lengthened. In (*C*) the extensions have lengthened more after 4 h. (*D*) *Note* the aligning of collagen fibrils between the two cells (*arrows*) after 5 h. (*E*) After 6 h, two contacts have been established between the cells (*arrows*). (*F*) After 7 h, one extension of the lower cell has almost disappeared (*arrow*).

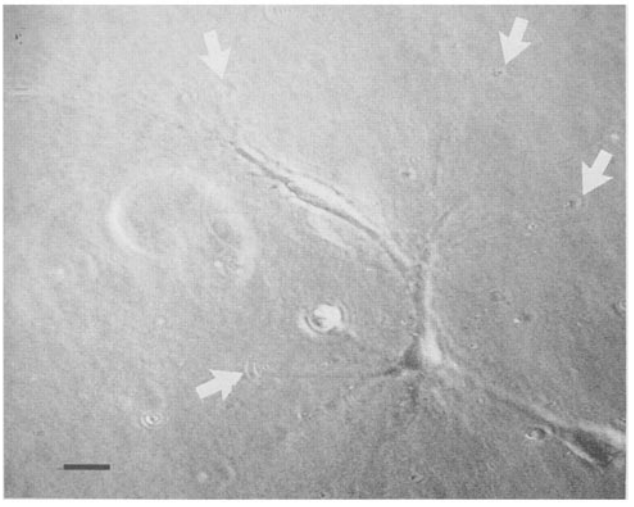

FIG. 5. Photomicrograph of a phase-contrast image of a collagen lattice showing bleb-like formations at the ends of some cellular extensions. (*bar* = 20 μm).

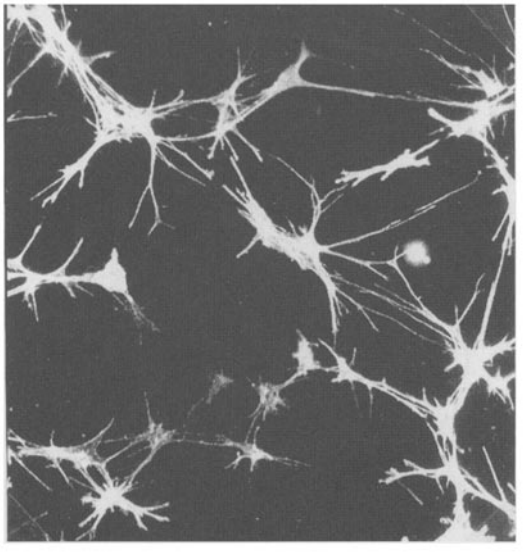

FIG. 6. Photomicrograph of five superimposed images of focal planes 10 μm apart captured in the confocal microscope. F-actin of the cells is stained with phalloidin-Bodipy®, excitation wavelength 488 nm. Numerous intercellular contacts are seen after 48 h of incubation and the cells form a complex cellular network.

results is that whole cells do not move around in the collagen matrix of floating lattices during contraction.

The present work unequivocally showed that the fibroblasts were in continuous motion by extending and withdrawing cellular extensions. A hypothesis of such cellular actions during contraction has earlier been inferred from studies on collagen rearrangement in lattices during contraction (22). The velocity of this process was slightly more marked in anchored than in floating lattices but the difference was not statistically significant.

It could be hypothesized that the cellular extensions reached out for collagen fibers to "pull them in" and arrange them in long sprays aligned parallel to the long axis of the cell extensions. Such aligning of collagen fibers into linear tracts has also been described by others (8,17,19,22). We also observed aligning of collagen fibers along the axis of extensions and between cells, and possibly the occasionally observed blebs at the end of extensions could be attachment sites for collagen fibers. Ultrastructural observations have implicated cell–collagen and cell–cell interactions in controlling fibroblast-mediated contraction of collagen gels (2,7,12). In granulation tissue (17) extracellular filaments (fibronectin) and actin microfilaments form close transmembranous associations on the surface of the fibroblast as shown by electron microscopy. In collagen lattices contraction has been shown to be mediated by $\alpha_2\beta_1$ integrin (11,15).

Fibroblasts generate tension in anchored lattices which results in transformation of fibroblasts to myofibroblasts with thick cytoplasmic stress fibers rich in actin (5,21). Fibroblasts in anchored lattices synthesize more collagen and release more collagenase than cells in floating lattices (14). On release of the attached lattice, it contracts within min and loses its stress fibers (21). Rapid contraction is followed by slower contraction which is analogous to the contraction of the floating collagen lattice (4). Despite the fact that the contraction of a floating lattice is a different process from the one seen during the rapid contraction of a released anchored lattice, the mechanism by which the tension is generated in an anchored lattice seems to be similar to that involved in the contraction of a floating lattice. We found no significant differences in the number of new extensions or contacts formed during the 7 h of measurements and no difference in the velocity of movements of extensions between floating and anchored lattices. During the contraction process intercellular connections are formed (1,7) that with time will constitute a network by intercellular connections (2). It has been stated that the stabilization of the intercellular attachments initiates aggregation of cytoplasmic microfilaments to form thick stress fibers which denote cessation of cell locomotion (5,21). During the time of study we noticed an increasing number of intercellular contacts, and after 48 h they were forming a network. We think that the cell-to-cell connections function as a stabilizing unit for the contracted tissue, which also has been proposed by others (5).

Three-dimensional reconstructions of cells from optical serial sectioning is an established technique in cell biology and is usually made of images captured in confocal microscopes or of regular microscopical images by deconvolution image analyses techniques. However, phase-dependent imaging of relatively transparent objects can give optical sections that appear to be somewhat thinner than with fluorescence imaging in the presence of confocal optics (10). This means that parts of a cell that are in the focal plane in the different sections of the z-series can easily be identified and marked for further 3-D reconstruction. No adverse effects were observed in cell morphology or function during and after the experimentation. Normal contraction of the collagen gels was seen both during the experiment and after the experiment (Fig. 2) and the contraction curve was similar to what has been obtained earlier (6). Serum-free culture medium was used in the collagen lattices, as we have previously shown that serum can inhibit their contraction (6).

In conclusion, the results of the present study show that advancing and retracting movements of cellular extensions is an important feature of collagen gel contraction and that cellular locomotion appears to be minimal. Furthermore, three-dimensional reconstruction of images captured with laser scanning microscopy over time was shown to be a useful technique for three-dimensional time-lapse studies *in*

vitro in order to analyze cell behavior during collagen gel contraction. From the presented data, we suggest that the continuous movement of the cellular extensions is a fundamental part of the mechanism for contraction of collagen gel lattices.

ACKNOWLEDGMENTS

The study was supported by grants from the Swedish Medical Research Council (Project No. 9881), Swedish Society of Medicine (Project No. 94/953.0), and County Council of Östergötland, Sweden (Project No. 96/058).

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