Measurement of Mechanical Forces Generated by Skin Fibroblasts Embedded in a Three-Dimensional Collagen Gel

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Mechanical activities developed by cells play a significant role in the embryogenesis, development, and physiopathology of pluricellular organisms. A technique is described to measure in vitro the traction force developed by cells seeded into a three-dimensional polymeric collagen lattice. It is based on the use of strain gauges generating an electrical signal upon tension that is amplified and recorded. The intensity of the signal depends on the number and type of cells, cytoskeleton integrity, concentration of collagen in the lattice support, and fetal calf serum in the culture medium. Skin fibroblasts from humans and animals produce traction forces ranging from 100 to 1000 mg per million cells. In the gel under tension, the cells are in mechanical dynamic equilibrium with their support. It is suggested that the mechanical activity of fibroblasts and the control of the tension that they operate on the lattice support participate in the structural organization of the dermis and in its physiologic tension.
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The mechanical activity of cells plays a major role in tissue organization during development. The traction generated by cells plays an obvious role in organogenesis, as in palate development [1] or tooth eruption [2], which are examples of the many activities mediated by this function. In vivo, many types of cells are able to perform mechanical work, such as the muscle cells involved in the locomotory function and contraction of all hollow organs. The cells of connective tissues that are closely connected to a support of polymeric macromolecules also display mechanical function, such as during the process of wound contraction [3].

Most cell types, epithelial, endothelial, mesenchymal, and neural, display a mechanical activity in culture. This mechanical activity is expressed by a locomotory behavior, a process involving the participation of the cytoskeleton. The forces generated by cells can be visualized in vitro by the formation of stretch lines between colonies of fibroblasts cultured on a retractable surface [4]. Such mechanical activity is also clearly expressed in the model of Bell et al [5] in which cells retract a floating network of collagen fibrils. We have developed a device allowing the measurement of forces produced by cells in such a collagen gel [6,7]. Our technique can be used in vitro as a tool to investigate the significance and the mechanisms involved in mechanical activity. In this paper, we report the influence of various parameters and pharmacologic agents on the mechanical forces developed by normal human and animal skin fibroblasts, as well as by diseased human skin fibroblasts.

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Abbreviations:
c-AMP: adenosine 3′-5′ cyclic monophosphoric acid
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethylsulfoxide
DS: dermatosparactic calf skin fibroblasts
EDS: Ehlers-Danlos syndrome type I fibroblasts
FCS: fetal calf serum
HEPES: 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
NCS: normal calf skin fibroblasts
NHS: normal human skin fibroblasts
PSS: scleroderma fibroblasts

MATERIALS AND METHODS

Normal and diseased human and calf skin fibroblasts were obtained by outgrowth from skin explants [8] in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Brussels, Belgium), glutamine (292 mg/l), ascorbic acid (50 mg/l), sodium bicarbonate (2.1 g/l), penicillin (100 U/ml), and streptomycin (100 U/ml).

Normal human skin-derived cells (NHS, five strains) were collected from infants and young patients undergoing excisional or plastic surgery. Scleroderma skin-derived cells (PSS, five strains) were obtained in our department from adult patients suffering from systemic scleroderma [9] or were provided by Dr. Th. Krieg (Ludwig-Maximilians University, Munich, Germany) [10]. The cell strains of Ehlers-Danlos Syndrome Type I (EDS, five strains) [11] were issued from the skin of patients diagnosed according to the clinical signs and criteria described by Ihme et al [12]. Animal skin fibroblasts from normal (NCS, one strain) and dermatosparactic calves (DS, two strains) were described previously [8].

Culture in Collagen Gels Collagen type I was purified and sterilized as described previously [8]. Sub-confluent fibroblasts in monolayer on plastic were detached with 0.25% trypsin and suspended in 5 ml of DMEM supplemented with 10% FCS. After

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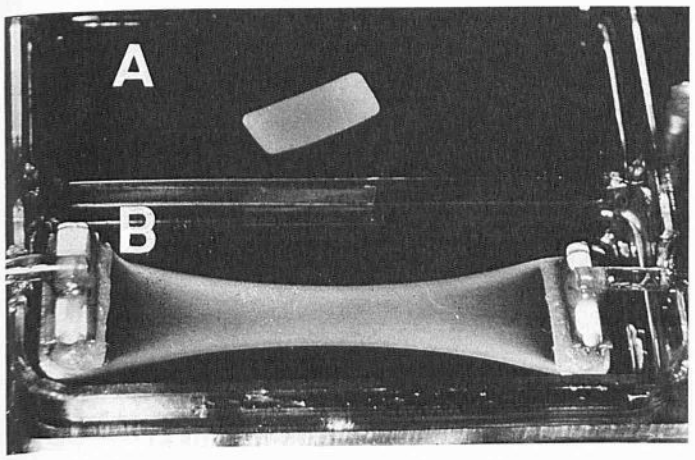


Figure 1. Retracted and restrained collagen lattices. *A*: Free-floating retracted lattice. *B*: A similar lattice is restrained at both extremities by polymerizing collagen on immobilized glass rods covered with Velcro.

counting and centrifugation, the suspension was brought to the desired cell density in 5 ml DMEM buffered by 30 mM HEPES and supplemented with the adequate concentration of FCS. The lattices were prepared following a variant of the procedure described in detail previously [8]. Briefly, 4.5 ml of a 1.5× concentrated medium buffered with 30 mM HEPES and 10 mM NaOH was added to 2.25 ml of a solution of type I collagen (3 mg/ml) in 0.1% acetic acid, and to 8 ml of the suspension of fibroblasts in DMEM. This mixture was poured in rectangular Falcon dishes that had been made hydrophobic by treatment with Sigmacote (Sigma, St. Louis, MO), washed with sterile distilled water, and dried. The gel polymerized within 1 min at 37°C.

Measurement of Retraction The progressive reduction in size of free-floating collagen gel (Fig 1*A*) was estimated as a function of time by measuring the length of the rectangular gel.

Measurement of the Forces Developed by Fibroblasts Rectangular gels were polymerized around and between two glass rod captors covered by Velcro and positioned at the extremities of the dish (Fig 1*B*). The gels were placed in the thermostatic chamber of the measuring device (Fig 3) immediately after preparation. One captor was connected to a mobile vernier and the other to strain gauges. The gel was set to minimal tension by adjusting its length with the vernier. The electrical signals generated by strain gauges upon development of tension were magnified 100 times by a stabilized amplifier and recorded continuously for 24 h. The response of the measuring device was linear over the range of 0–5 grams.

Morphologic Studies Punch biopsy samples were taken from collagen gels, fixed in 4% buffered saline formaldehyde, and 20-μm–thick sections were used for immunoperoxidase staining. Antibodies against bovine collagen type I and human fibronectin were used as previously described [13,14]. Their specificity was ascertained by the methods of Foidart and Reddi [15], Elisa [16], and Western-blot [17] as described in previous experiments.

Pharmacologic Studies Cycloheximide (2×10^{-5} M), colcemid (2×10^{-5} M to 1×10^{-4} M), and cytochalasin B (10^{-7} to 2×10^{-6} M) were diluted in DMEM and added to the gel immediately before polymerization. Betamethasone dipropionate in DMSO (0.1% final concentration) was diluted in DMEM and used between 5×10^{-6} and 10^{-5} M.

RESULTS

The free-floating collagen gel progressively retracted as a function of time (Fig 1*A*). The kinetic characteristics of this retraction have been previously described [5,11]. Under usual experimental conditions (type I collagen 1 mg/ml, 10^6 human skin fibroblasts), the

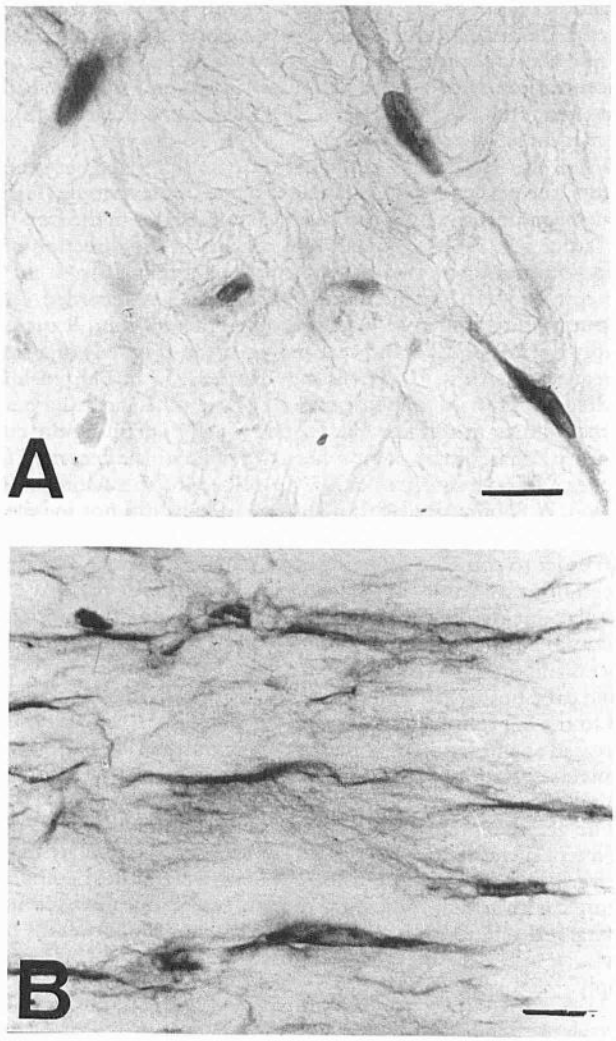


Figure 2. Organization of the cells and collagen polymers in retracted and restrained lattices. Immunoperoxidase staining of formaldehyde-fixed sections as described in *Materials and Methods*. *A:* Retracted lattices as in Fig 1*A* reacted with antifibronectin antibodies. The fibroblasts are elongated and oriented in all directions and the collagen fibers have no preferential orientation. *B*: A rectangular restrained lattice as in Fig 1*B* reacted with anti-type I collagen antibodies. The fibroblasts are organized parallel to the collagen fibers and aligned along the long axis of the gel. *Bar*, 10 μm.

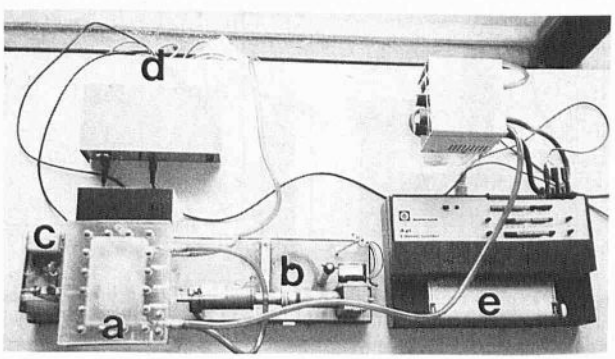


Figure 3. Photograph of the measuring device. The gel is placed in the thermostatic chamber (a). One captor is connected to a vernier (b) and the other to strain gauges (c) and the gel is set to minimal tension by adjusting its length with the vernier. The electrical signals generated by strain gauges upon traction is amplified (d) and recorded (e).

reduction in size of the gel reached 50–80% after 24 h (not illustrated). The retraction of the gel was proportional to the number of cells, to the concentration of FCS, and was inversely related to the concentration of collagen. Cells, initially round, rapidly became elongated, attached to collagen fibers as observed earlier [8], and oriented in all directions (Fig 2*A*).

When the extremities of the gel were immobilized between the captors, the gel acquired a paraboloid shape in a few hours (Fig 1*B*). In such conditions, fibroblasts became elongated as in the free-floating lattice but aligned parallel to the fibers in the direction of the main vectorial force, the long axis of the gel (Fig 2*B*).

In presence of living fibroblasts, tension was recorded by the measuring device (Fig 3) within the first 30–60 min. It increased rapidly during 6–12 h and reached a plateau (Fig 4*A*) or a slowly increasing slope (Fig 4*B*), persisting for at least 12 h. The tension (F) reached after the initial phase varied between 0.1 and 1.0 g per 10^6 normal human or calf fibroblasts. The time (T) defining the end of the initial phase is the point where the slope of the recorded force reaches an angle lower than 10° in respect to the stabilized final tension. A polymerized gel in absence of cells did not induce any tension signal.

In order to compare different cell strains and various experimental conditions, a "unit" of mechanical activity was devised. We took into account the two main parameters that define the mechanical activity, i.e., the tension (F) at the end of the initial phase of the process and the time required to reach it (T). The function F/T was found to be linearly related to the number of cells in the gel (Fig 5*A*) and to the concentration of FCS up to 2% (Fig 5*B*). It also linearly increased as a function of collagen concentration (Fig 6*A*), whereas an inverse relation was observed in a freely retracting lattice (Fig 6*B*).

The gel under tension behaved as a visco-elastic material. When the traction was increased (Fig 7*A*) or released (Fig 7*B*) by moving the vernier, the starting tensional values were almost completely restored within 1 h, indicating that an equilibrium existed in the mechanical relationship of the cells with their support. This property was lost after the cells had died or exhausted their energy supply.

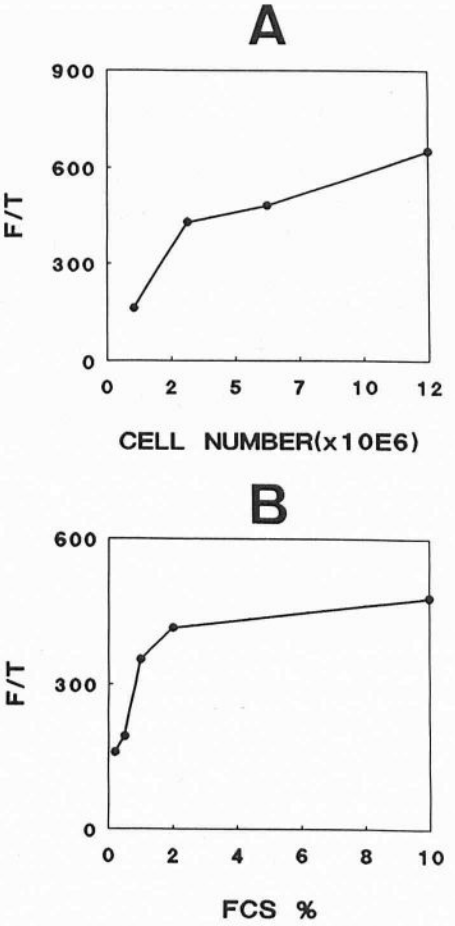


Figure 5. Mechanical activity (F/T, in mg/h) of calf skin fibroblasts as a function of the cell number (*A*). *B*: The concentration (in volume %) of fetal calf serum in the medium (3×10^6 fibroblasts).

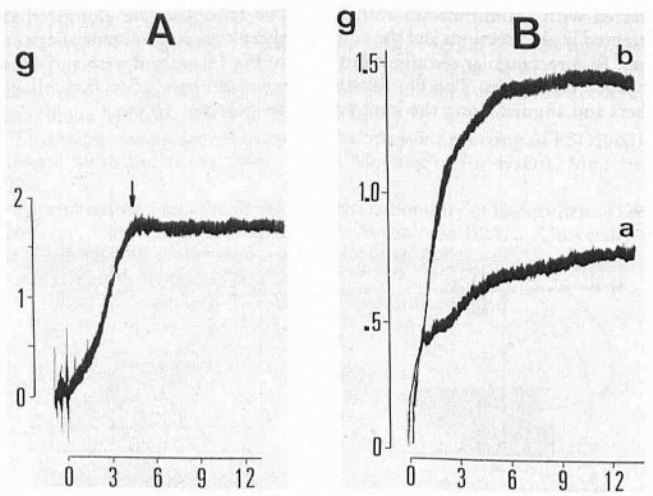


Figure 4. Typical recording of the force generated by skin fibroblasts. *A*: The force generated by 3×10^6 calf skin fibroblasts in presence of 10% FCS embedded in a collagen lattice was measured as a function of time (hours). In this particular case, the calf skin fibroblasts developed a force (F) of 1.8 g in 5 h (T at the *arrow*), which corresponds to a F/T of 360 mg/h or 120 mg/h/10^6 cells. *B*: The force developed by 3×10^6 (a) and 6×10^6 (b) normal human skin fibroblasts was recorded as a function of time (hours). In this strain, the F/T for the (a) curve is 700 mg in 9 h or 78 mg/h or 26 mg/h/10^6 cells and for the (b) curve it is 160 mg/h or 27 mg/h/10^6 cells.

Forces Generated by Human and Calf Skin Fibroblasts in some Connective Tissue Diseases The traction force developed by fibroblasts from involved skin in systemic scleroderma or from EDS I patients did not differ significantly from that produced by normal human fibroblasts. The mean values of F/T recorded in seven experiments performed with various numbers of PSS fibroblasts and in nine experiments performed with EDS I fibroblasts were, respectively, 87 ± 16% and 103 ± 39% of the mean values recorded with normal human skin fibroblasts (n = 12).

As illustrated in Fig 8, dermatosparactic calf skin fibroblasts displayed a significantly decreased capacity to develop mechanical tension as compared to normal calf skin fibroblasts.

Pharmacologic Modulation of the Traction In the presence of cycloheximide (5 μg/ml), the force developed by human skin fibroblasts attained 20% of the control cultures. A dose-related inhibition of the traction developed by 3×10^6 human fibroblasts was observed in the presence of colcemid, cytochalasin B, and betamethasone dipropionate (Fig 9).

DISCUSSION

A technique to measure the mechanical force generated by cells in a three-dimensional collagen lattice was preliminarily described [6,7]. This technique has been further refined to allow a clear demonstration that traction on the support is a physiologic property of connective tissue cells in culture. In order to produce this force, cells required the expression of a privileged cell-matrix interaction, an organized cytoskeleton, and energy.

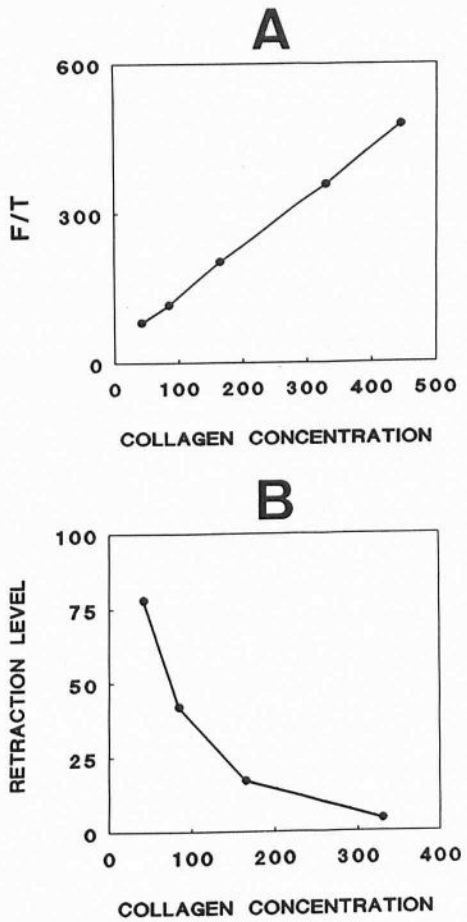


Figure 6. Comparison of F/T and the rate of retraction of a free-floating lattice as a function of collagen concentration. *A*: The mechanical force (F/T, mg/h) developed by 3×10^6 calf skin fibroblasts increases linearly with the concentration of collagen (μg/ml). *B*: The rate of retraction of a floating lattice after 24 h by 3×10^5 calf skin fibroblasts decreases as a function of the concentration of collagen (μg/ml).

In both the free-floating and restrained lattices, normal fibroblasts were elongated and attached to the collagen fibers as they appear in vivo. Cell attachment is required to allow the development of force [8,14]. According with the microcinematograpic observations of Nishiyama et al [18], fibroblasts in the free-floating lattice actively pull and retract filopodia attached to collagen fibers to collect the fibrils around the plasma membrane. This results in a contraction of the gel while the cells remain elongated along the polymers with a random orientation. In the rectangular lattice immobilized at its extremities, the developing tension will organize the collagen polymers in the direction of the long axis and the cells in parallel, as demonstrated in Fig 2*B*. This orientation of fibers and cells allows an effective transmission of the forces. The efficiency of this mechanism increases as a function of collagen concentration (Fig 6*A*) by either enhancing the contacts between the cells and their support and/or improving the rigidity of the collagen network. The opposite relationship is observed in the free-retracting gel (Fig 6*B*) because the density of the collagen fibrils conditions the resistance opposing to retraction.

The magnitude of the forces developed by calf skin fibroblasts is in the same order as that recently reported for dog periodontal ligament fibroblasts [19]. The human skin fibroblasts, as well as the human periodontal ligament fibroblasts,* are less efficient.

* Mashshini J, Jason-Mashshini F, Lefèbvre P (personal communication).

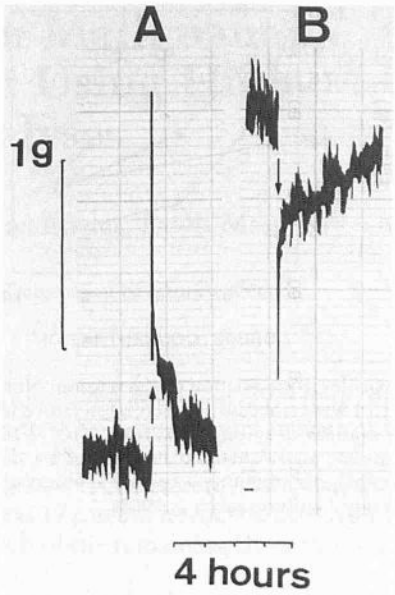


Figure 7. Effect of traction and relaxation on the gel. When the maximum tensional force developed by 10×10^6 human skin fibroblasts was attained *(A)* a traction (↑) or *(B)* a relaxation (↓) of ± 2 g was imposed by moving the vernier to which is fixed one extremity of the lattice. In both cases, the initial tension was almost completely restored within 1 h.

The mechanical activity of the cells requires protein synthesis and the functional integrity of the cytoskeleton. Cycloheximide significantly reduces the traction. Cytochalasin B and colcemid, agents disrupting the cytoskeleton, suppress the mechanical activity. The reduction of traction observed by depleting the culture medium in FCS might be related to the reduced concentration of TGF-β and PDGF, which are growth factors known to control the retractile activity of fibroblasts in the free-floating lattice [20,21].

The defective capacity of dermatosparactic fibroblasts to develop a tension on their support is obviously related to a disturbance in the capacity of attachment of these cells to the matrix [8,22]. In this disorder, collagen bundles are not organized in vivo and skin is fragile [23]. No difference, as compared to normal fibroblasts, was observed in cells from patients with EDS I, supporting previous observations [11]. Scleroderma fibroblasts produced forces comparable to normal skin-derived cells. Disturbed mechanical activity is not the physiopathologic mechanism of the abnormal rheologic properties of skin in these diseases.

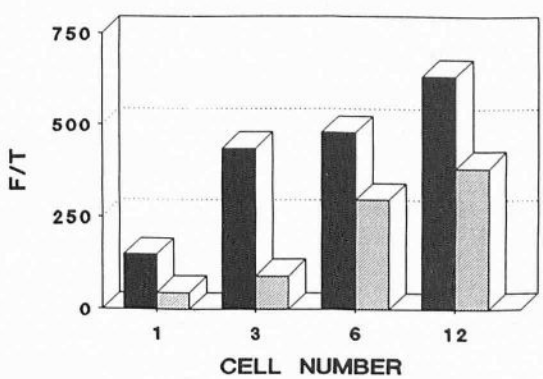


Figure 8. Mechanical activity of normal and dermatosparactic calf skin fibroblasts. Increasing number (from $1-12 \times 10^6$) of fibroblasts isolated from normal (■) and dermatosparactic (▨) calf skin was tested for their mechanical activity.

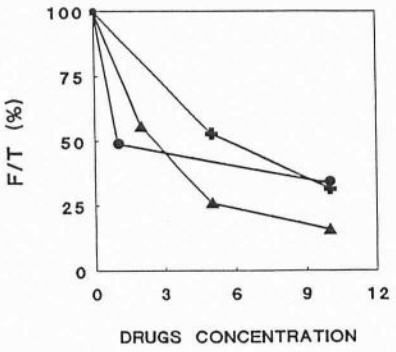


Figure 9. Pharmacologic modulation of the tension. Normal human skin fibroblasts (3×10^6) were embedded in a collagen lattice in the presence of increasing concentrations of colcemid (▲, from $2.10^{-5} - 10^{-4}$ M), cytochalasin B (●, from $10^{-7} - 10^{-6}$ M) and betamethasone dipropionate (✚, from $5.10^{-6} - 10^{-5}$M). An inhibitory activity was observed for these agents as compared to control cultures taken as 100%.

The recording of forces in the restrained lattice might be a more "physiological" parameter than the measure of the rate of retraction of the free-floating lattices. Cells in normal tissues are included in a matrix that resists traction. Fibroblasts in the dermis might be considered as being in equilibrium with their support, as observed in Fig 7. In the growing child and young adult, skin tightly fits the underlying volume. This property might be related to the mechanical activity of the cells producing and organizing a framework of polymers under optimal tension. The expression of this tension is Langer's lines. Cells also react to mechanical forces that are known to operate a pre-translational regulation of their phenotype.*

Some pathologic conditions characterized by sclerosis and reorganization of the architecture of the dermis might benefit from the action of drugs able to modulate the mechanical activity of fibroblasts. The rate of retraction of a free-floating collagen lattice provides an interesting model for pharmacologic studies and has been previously used to investigate the effect of inhibitors of protein synthesis disrupting the cytoskeleton [5], and the activity of corticosteroids or other drugs [24,25]. The screening of such drugs might also be effectively performed using the technique described here.

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