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Compressive Deformation and Damage of Muscle Cell Subpopulations in a Model System

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Abstract—To study the effects of compressive straining on muscle cell deformation and damage an *in vitro* model system was developed. Myoblasts were seeded in agarose constructs and cultured in growth medium for 4 days. Subsequently, the cells were allowed to fuse into multinucleated myotubes for 8 days in differentiation medium, resulting in a population of spherical myoblasts (50%), spherical myotubes (35%), and elongated myotubes (15%) with an overall viability of 90%. To evaluate cell deformation upon construct compression half-core shaped constructs were compressed up to 40% strain and the resulting cell shape was assessed from confocal scans through the central plane of spherical cells. The ratio of cell diameters measured parallel and perpendicular to the axis of compression was used as an index of deformation (DI). The average DI of myoblasts decreased with strain level (0.99 ± 0.03, 0.70 ± 0.04, and 0.56 ± 0.10 at 0%, 20%, and 40% strain), whereas for myotubes DI decreased up to 20% strain and then remained fairly constant (0.99 ± 0.06, 0.55 ± 0.06, 0.50 ± 0.11). The discrepancy in DI between spherical myoblasts and myotubes at 20% strain was explained by the relative sensitivity of the cell membrane to buckling, which is more pronounced in the myotubes. Sustained compression up to 24 h at 20% strain resulted in a significant increase in cell damage with time as compared to unstrained controls. Despite differences in membrane buckling no difference in damage between myoblasts and spherical myotubes was observed over time, whereas the elongated myotubes were more susceptible to damage. *© 2001 Biomedical Engineering Society.* [DOI: 10.1114/1.1349698]

Keywords—*In vitro* model, Muscle cell, Compression, Cell deformation, Cell damage.

INTRODUCTION

Studies of soft tissues under mechanical loading have shown that muscle tissue is highly susceptible to localized compression, eventually leading to tissue degeneration in the form of pressure sores.[1,4,5,14,32] This tissue degeneration starts at the cellular level and is characterized by nuclear pyknosis and an early disintegration of the contractile proteins in the cells, followed by inflammatory reactions.[7,10,20,32] Although it is clear that both the magnitude and duration of compression affect cellular breakdown, the underlying mechanisms whereby tissue compression results in cell damage are poorly understood. Muscle tissue can be conceived as consisting of three major components, which are differently affected by mechanical loading: the muscle cells, the surrounding extracellular matrix, and blood and lymph vessels. Previous studies on the etiology of pressure sores have focused on the last two components, which determine the mechanochemical environment of the cell. From these studies it was hypothesized that tissue damage occurs as a consequence of impaired perfusion and transport of metabolites to and from the cell. However, this theory can only partly explain the onset of tissue damage and has, to date, not been fully verified.[14,15,21,40] We hypothesize that sustained deformation of the muscle cells in the tissue plays a significant role in the damage process. Indeed cell deformation triggers a variety of effects, such as local membrane stresses, volume changes, and modifications of cytoskeletal organization, which may be involved in early cell damage, whereas changes in the mechanochemical environment of the cell may induce additional damage. It has been shown that when subjected to tensile or shear strains, the response of muscle cells to deformation is of major importance to mechanotransduction, i.e., the process by which muscle cells detect and respond to their mechanical environment.[11,38] A comparable response might be expected for compressive straining.

As it is clearly not possible to examine the effects of sustained cell deformation during compressive strain in intact tissue independently of other factors (such as blood perfusion), an *in vitro* system involving cultured muscle cells has to be developed. Such a system enables improved control of experimental conditions and offers the potential for reproducible, well-characterized constructs. An appropriate system must allow application of clinically relevant levels of strain, preferably in a non-

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contact manner to avoid influencing cell damage. *In vivo* studies have demonstrated damage to muscle tissue at compressive strains of up to 40%.[13,34] In addition, it is important to use a culture system that mimics the three-dimensional (3D) tissue environment of the cells. Under *in vitro* condition muscle cells can be maintained in monolayer but, in this state, the typical 3D ''tissue'' configuration is absent. Finally, the system must allow quantification of cell deformation during compressive loading. The present study utilizes an *in vitro* 3D model system consisting of cultured muscle cells embedded in agarose gel. Agarose has been successfully used as a construct for the study of strain-induced chondrocyte deformation.[17,27,23–25] Strain applied to the translucent agarose gel will result in deformation of the living cells seeded within it, which can be visualized using confocal laser scanning microscopy. The gross material behavior of agarose/chondrocyte constructs has been characterized as viscoelastic in nature up to large strains.[22,24,27] Therefore, the gross behavior of the agarose/muscle cell constructs was also assumed to be viscoelastic. The aims of the present study were to characterize the newly developed system, to assess cell deformation within the system at clinically relevant strains, and to study the consequences of sustained cell deformation in terms of cell damage.

MATERIALS AND METHODS

Muscle Cell Seeded Agarose Constructs

The established C2C12 mouse skeletal myoblast line (ECACC, Porton Down, UK) was used for the study. The myoblasts were seeded at low densities ($2-3\times10^4$ cells cm^{-2}) and grown in monolayer culture in tissue culture flasks (Falcon, Becton Dickinson, Oxford, UK). The cells were cultured in a 37 °C, 5% CO_2 humidified incubator and fed with fresh growth medium every 3 days. Growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Paisley, UK) supplemented with 15% ($v\ v^{-1}$) heat-inactivated fetal bovine serum (Gibco BRL), 200 mM L-glutamine (ICN Biomedicals, UK), 1% ($v\ v^{-1}$) nonessential amino acid solution (×100, Gibco BRL), 20 mM HEPES (Gibco BRL), and a penicillin/streptomycin dual antibiotic solution at 5000 IU/5 mg ml (ICN Biomedicals). The myoblasts were maintained in continuous passage using trypsinization of subconfluent cultures (~70%–80% confluency, $\sim6\times10^4$ cells cm^{-2}). The experiments reported were performed utilizing myoblasts of passages 10–14, which retain the ability to differentiate into mature myotubes.[2]

To enable the application of compressive strains, myoblasts were seeded within agarose constructs. A 6% low gelling temperature agarose suspension (Type VII, Sigma Chemical Co., Poole, UK) in Earl's balanced salt solution was melted by autoclaving and subsequently cooled to 37 °C on rollers. The agarose suspension was then added to an equal volume of a cell suspension at a concentration of 4×10^6 myoblasts ml^{-1} in growth medium to yield a final concentration of 2×10^6 myoblasts ml^{-1} in 3% agarose. The agarose/cell suspension was plated in sterilized Perspex moulds and gelled at 4 °C for 20 min. Using a specially made punch, the gel was cored into identical cylinders (4 mm diameter×5 mm high), which were incubated in growth medium for 4 days to allow the cells to divide and proliferate. Subsequently, the constructs were cultured in a differentiation permissive medium, consisting of DMEM plus 2% ($v\ v^{-1}$) Horse Serum (Gibco BRL), to induce fusion and myogenic differentiation of the cells into multinucleated myotubes.[2] The differentiation medium was replaced every 3 days. After 8 days in differentiation medium, equivalent to day 12 in culture, the final cell number due to proliferation and subsequent fusion was approximately 1×10^6 cells ml^{-1}. By then the constructs contained a mixture of myoblasts and multinucleated myotubes. The latter either rounded up in the agarose gel or adopted an elongated, rod-shaped morphology. The rounded multinucleated cells are hereafter referred to as ''spherical myotubes,'' whereas the rod-shaped cells are referred to as ''elongated myotubes.'' The final cell concentration allowed the assessment of cell deformation of single cells, without interference of adjacent cells.

Cell differentiation and morphology was monitored by light and phase contrast microscopy using an Olympus IM microscope (Olympus Optical, UK). Cell viability in the model system was determined using the trypan blue exclusion test.

Assessment of Cell Deformation

At different stages of the culture protocol the morphology of two of the isolated muscle cell subpopulations (spherical myoblasts and spherical myotubes) within the agarose was examined at gross construct strains of 0%–40%. For this purpose cell/agarose cylinders were cut longitudinally to produce half-core shaped constructs, each with a flat surface suitable for microscopy. The cells within the agarose were stained with the viable fluorescent probe Calcein-AM (Cambridge BioScience, Cambridge, UK). Calcein-AM diffuses across the cell membrane into the cytoplasm of viable cells where it is hydrolyzed by intracellular esterase to release the fluorescent Calcein. The excitation and emission spectra of Calcein are similar to those of fluorescein, with an optimal excitation wavelength of 490 nm and an optimal emission wavelength of 520 nm. The muscle cells were stained en bloc by incubating the specimens in a 5 μM solution of Calcein-AM in either growth medium (day 4) or differentiation medium (day 8, day 12)

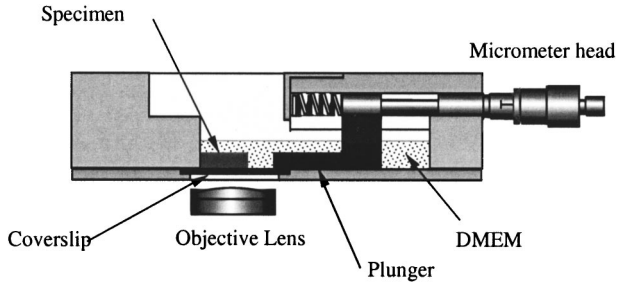

FIGURE 1. Schematic representation of test rig used to compress cell seeded agarose constructs.

at 37 °C, 5% CO_2 for a minimum of 4 h. The specimens were subsequently rinsed in fresh DMEM in preparation for microscopy.

The half-core constructs were subjected to unconfined uniaxial compression, using a specially designed test rig that allows the cells within the agarose to be visualized using confocal microscopy.[23] The rig, represented schematically in Fig. 1, consists of a stainless steel frame, which rests on the stage of an inverted microscope, and a plunger for compression of specimens in a direction perpendicular to the microscope axis. A single half-core shaped construct was placed on a coverslip in the rig with the flat surface down and the longitudinal axis parallel to the axis of the plunger. The plunger is controlled by a micrometer head that is graduated at intervals of 0.01 mm, corresponding to a strain of 0.2% for the 5 mm long specimens. To prevent dehydration and shrinkage, the construct is maintained in a bath of DMEM.

To study the influence of culture medium and cell fusion on cell dimensions at 0% strain separate constructs, cultured to day 4, day 8, and day 12, were mounted in the test rig in the unstrained state. In each construct the dimensions of 15 myoblasts and 15 spherical myotubes were measured. In a second experiment, a construct cultured to day 12 was subjected to a gross compressive strain of 20%, applied at a rate of approximately 20% min^{-1}. A 10 min period of stress relaxation of the construct was permitted, prior to measurements of cell dimensions on a group of 15 myoblasts and 15 spherical myotubes (group approach). This procedure was repeated on a separate construct compressed by 40% strain. In a third experiment a single cell approach was adopted in which changes in cell shape were followed in individual cells. A construct cultured for 12 days was mounted in the test rig and the dimensions of a single myoblast or myotube were determined in the unstrained state. The construct was then subjected to incremental strains of 10%, 20%, 30%, and 40%. The same cell was measured to determine its dimensions at each strain level, after a 10 min period of relaxation. This protocol was repeated using further constructs until a total of four myoblasts and four myotubes were measured. All deformation measurements were performed at room temperature. Since these were relatively short-term measurements the effect of temperature on cell viability, agarose properties, and cell shape was expected to be negligible.

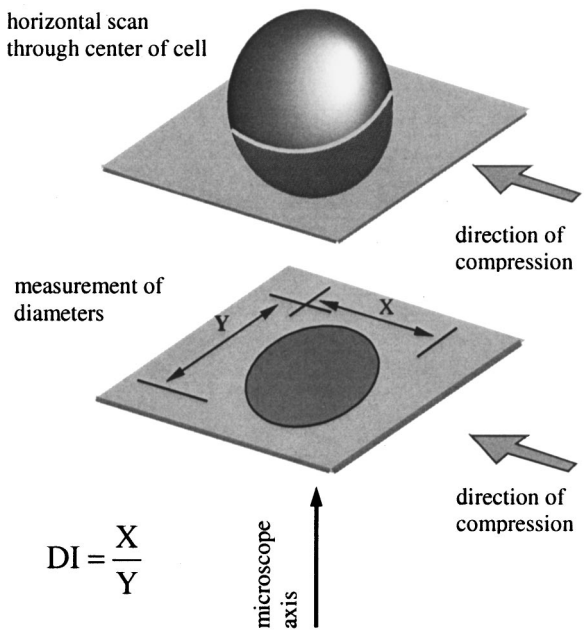

FIGURE 2. Assessment of deformation of muscle cells using confocal microscopy (see Ref. 23).

A confocal laser scanning microscope and its associated image analysis software (Molecular Dynamics Ltd., Sevenoaks, UK) was used to visualize and measure the fluorescent cells within the constructs. The confocal system is based around an inverted microscope (Diaphot 1000, Nikon UK Ltd.) using a ×40 oil immersion, 0.55 numerical aperture objective. The argon ion laser excitation was set at 19 V and either 3% or 30% full power at 488 nm, with emission recorded above 510 nm. A confocal pin hole aperture of 50 μm was used with a photogain of ×2 or ×4 and a photomultiplier tube detector voltage of 600 V.

Individual muscle cells were visualized at a depth of 50–100 μm into the agarose construct using a previously described confocal technique described by Knight *et al.*[23] To review briefly, a single horizontal scan was made through the center of a fluorescently stained cell. A half-maximum intensity threshold was applied to the confocal image and the full width half-maximum (FWHM) diameters (in μm) of each cell were measured parallel (X) and perpendicular (Y) to the compression axis of the test rig. Measurements were made with a precision of 0.13 μm corresponding to one pixel. Subsequently, the ratio of the cell X and Y diameters was determined as a deformation index (DI), as indicated in Fig. 2 and employed in previous studies.[17,23,27] Alternatively, cell de-

formation was quantified in terms of cell strain (percent) in the directions parallel and perpendicular to compression. In this case cell strains are given by

$$\epsilon_x = \frac{X_u - X_s}{X_u} \times 100$$

and

$$\epsilon_y = \frac{Y_u - Y_s}{Y_u} \times 100,$$

where X_u and Y_u are the diameters of the unstrained cell and X_s and Y_s are the diameters of the strained cell. Since the measurement of cell strain requires the diameters of the same cell under both strained and unstrained conditions, it is only applicable in the third deformation experiment using a single cell approach. Both techniques produce accurate and highly reproducible measures of deformation for spherical cells in a simple way.[23] Deformation of nonspherical cells, i.e., the elongated myotubes, was not quantitatively assessed in the present study, but since the spherical cells predominate in this system (see Results section), the measurements on spherical cells provide a good estimation of cell deformation in response to gross straining of the construct.

Assessment of Cell Damage

To study cell damage in response to sustained compression cylindrical constructs cultured up to day 12 were subjected to 20% gross strain for periods of 1, 2, 4, 12, and 24 h. This level of strain, corresponding to a normal construct stress of about 4.3 kPa, was chosen because of the difference in membrane buckling between myoblasts and myotubes under these conditions (see Results section) and the clinical relevance for pressure sore development.[1,14,20,32]

For each time period 16 constructs were placed upright in a central position in appropriate wells of a 24-well testing plate (Costar Corporation, Cambridge). This testing plate was positioned in a specially designed cell straining device (Dartec, Stourbridge, UK).[28] The device consists of an incubator (Herraeus Instruments, Wolverhampton, UK) within a loading frame and enables individual constructs to be loaded via 11 mm long flat-ended indenters, either statically or dynamically, in unconfined compression. A standard load cell and linear variable displacement transducer are used to control load application via a central rod, attached to a removable mounting plate incorporating 24 indenters. Movement of selected indenters can be restricted by a clamping mechanism, whereas other indenters are allowed to move freely. In this way eight constructs were subjected to 20% compressive strain (20% min^{-1}). The other constructs were treated as unstrained controls, subjected only to a tare strain of 0.8% due to the mass of the indenters (2 g). Each cylinder was incubated at 1 ml differentiation medium and kept under standard culture conditions during the straining protocol. At time zero and at the end of each time period, constructs were removed and fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate. The constructs were then dehydrated through a series of alcohol and xylene concentrations, cut vertically through their center and embedded in wax. Four 5-μm-thick slices, about 200 μm apart, were taken from both halves of two representative constructs. These slices were stained with Harris's haematoxylin and eosin and examined for cell damage using an Olympus BH-2 light microscope under a random blind procedure. Cell damage was assessed from evidence of membrane disruption or nuclear pyknosis or fragmentation.[7,10] The number of viable and damaged cells was counted across the field of view in a raster formation and expressed as percentage of total cell number. Cells were further categorized as myoblasts, spherical myotubes, elongated myotubes, and the relative damage in the respective subpopulations calculated as a percentage of the total number of cells per subpopulation. For each time period a minimum of 200 cells were evaluated in this way for both strained and unstrained constructs.

Data Analysis

Deformation Studies. The shapes of spherical myoblasts and myotubes were assessed from the FWHM diameters of each cell. Although it is accepted that the edge identification in the confocal images is not well defined in all cells (Fig. 4), it is anticipated that any inaccuracies in diameter measurements were limited by the use of group averages and a single observer. In the first two experiments the DI values for both myoblasts and myotubes were normal in distribution. Hence, differences in DI between the two cell types were tested with an unpaired Student's t tests. Analysis of variance (ANOVA) was used to determine the effect of culture medium and cell fusion on cell shape in the unstrained state on day 4, 8, and 12 constructs. The effect of strain level on DI of spherical myoblasts and myotubes was also assessed with ANOVA. In the third deformation experiment individual cells were tracked to study cell shape in response to increased compression. Because of the limited number of cells that was examined in this experiment, comparisons between DI, ϵ_x, and ϵ_y of different cells and in different constructs were not subjected to statistical inferential tests.

Damage Studies. ANOVA was used to test the effect of straining period on the total percentage of cell damage as

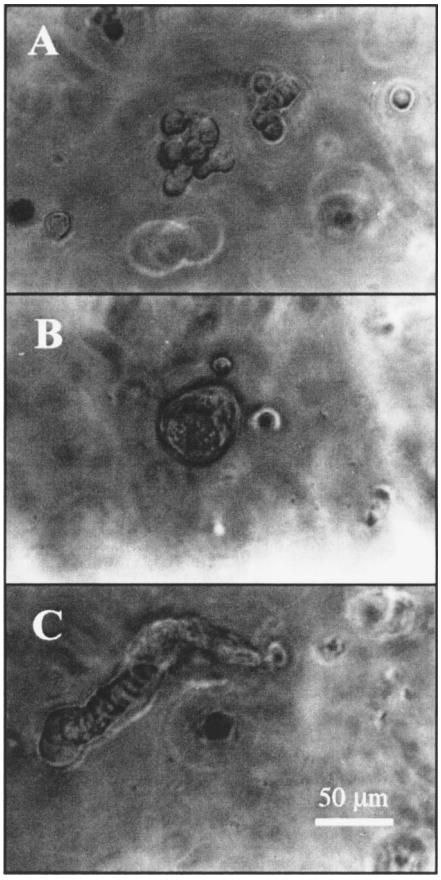

FIGURE 3. Phase contrast photomicrographs of muscle cells in agarose at different stages of the cultural protocol. (A) Individual myoblasts and clusters of myoblasts (day 4), (B) spherical myotube (day 8), and (C) snaked-up elongated myotube (day 12). Magnification: ×600.

well as the differences in cell damage between the three subpopulations. Unpaired Student's t tests were used to compare damage in strained and unstrained constructs. In all cases the 0.05 level of significance was used.

RESULTS

Cell Seeded Agarose Constructs, Effect of Culture Period

The culture protocol for muscle cell seeded agarose constructs was performed using single cell suspensions with viabilities of at least 96%. During the 12-day culture protocol cell viability gradually decreased, but always exceeded a value of 90%. Representative phase contrast micrographs of muscle cells in agarose at different stages of the culture protocol are presented in Fig. 3. Constructs up to day 4 contained only spherical myoblasts, although an increasing number of cell clusters were observed. By day 4, the constructs generally consisted of 50% individual myoblasts and 50% clusters of up to 20 myoblasts [Fig. 3(A)]. After switching the culture medium to differentiation permissive medium, the cell clusters gradually started to fuse into spherical [Fig. 3(B)] and, less frequently, elongated or rod shaped myotubes [Fig. 3(C)]. By day 12, the constructs contained approximately 50% myoblasts, 35% spherical myotubes, and 15% elongated myotubes. A horizontal scan through the center of a myoblast and a spherical myotube in an unstrained construct is shown in Fig. 4(A).

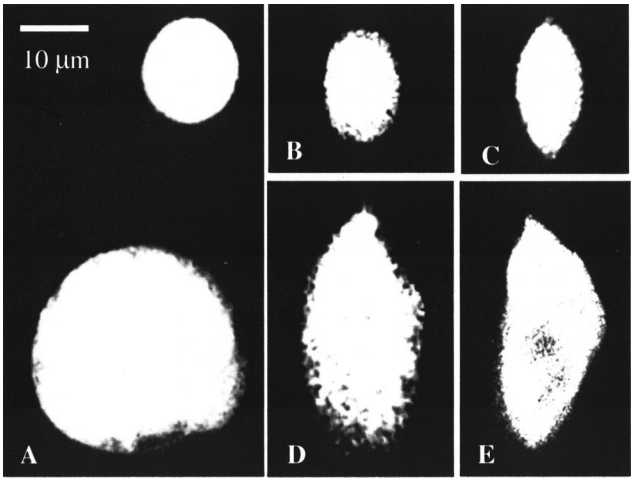

FIGURE 4. Horizontal confocal scans through the center of muscle cells. Note the buckling at the equators of the cells during straining. (A) Myoblast and rounded myotube in an unstrained construct, (B),(C) myoblasts at 20%, and 40% construct strain, and (D),(E) myotubes at 20%, and 40% construct strain.

The mean cell diameters and DI values of spherical myoblasts and myotubes in unstrained constructs at days 4, 8, and 12 are given in Table 1. All DI values were close to unity and there were no group differences between DI values at the three times in culture. This suggests that both cell types adopted a spherical shape in the unstrained state. Myoblast diameters ranged from 8.9 to 15.8 μm and there was a slight, but nonsignificant increase in diameter between day 4 and 12. Spherical myotubes were consistently larger than myoblasts and grew significantly with time spent in culture ($p<0.01$). Between days 8 and 12 spherical myotubes had approximately doubled in diameter. Although no quantitative assessment was performed, the elongated myotubes appeared to be slightly longer in day 12 constructs compared to day 8, with lengths ranging from about 50 to 150 μm.

Cell Deformation

Figure 4 shows typical examples of horizontal confocal scans through the center of muscle cells at 0%, 20%, and 40% construct compression. The scans suggested that all myoblasts and some myotubes deformed from a

TABLE 1. Diameters (X, Y) and DI of myoblasts and spherical myotubes at 0% strain at different stages of the culture protocol (mean±sd).

	Myoblasts			Myotubes		
	X (μm)	Y (μm)	DI	X (μm)	Y (μm)	DI
Day 4	11.0±1.5	11.3±1.6	0.97±0.04	N/A[b]	N/A	N/A
Day 8	12.2±2.0	12.2±2.0	1.00±0.04	22.8±3.2[a]	23.6±4.2[a]	0.97±0.08
Day 12	12.3±1.8	12.3±1.8	0.99±0.03	48.0±11.9[a]	48.9±11.9[a]	0.99±0.06

[a]Significant difference between diameters of myoblasts and myotubes.
[b]N/A: not appropriate.

spherical to a more ellipsoid morphology when the constructs were compressed to 20% strain. About 90% of the myotubes, however, showed localized buckling at this level of strain, appearing as a pointed apex at the equators of the horizontal cell sections. Further, three-dimensional reconstruction from a series of horizontal scans revealed that buckling of the myotubes also occurred along the midmeridian running between both equators. Hence the cells presented a UFO-shaped morphology. At 40% strain all myotubes and most myoblasts had adopted this morphology.

Changes in cell shape were quantitatively assessed in groups of cells using the DI. For myoblasts DI decreased significantly with the level of strain, reaching mean (±standard deviation) values of 0.70±0.04 and 0.56 ±0.10 at 20% and 40% strain, respectively (Fig. 5). In myotubes a marked, significant decrease in DI was observed when the construct was compressed to 20% strain (0.55±0.06), but little further decrease was measured when the strain was increased to 40% (0.50±0.11). At 20% strain the DI of myotubes was significantly smaller

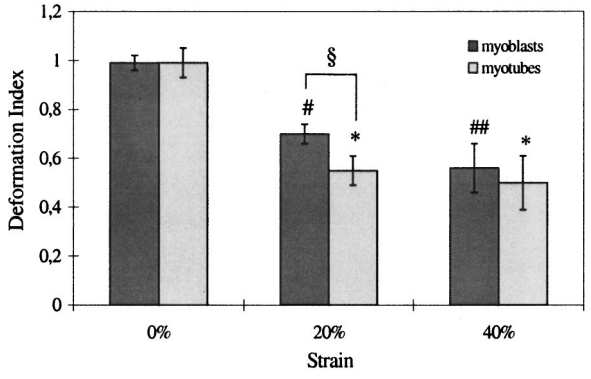

FIGURE 5. Deformation indices (mean±standard deviation) of myoblasts and myotubes measured in 12 constructs compressed to 0%, 20%, and 40% strain. #: DI of myoblasts at 20% strain differs significantly from DIs at 0% and 40% strain ($p<0.05$), ##: DI of myoblasts at 40% strain differs significantly from DI at 0% strain ($p<0.01$) and DI at 20% strain ($p<0.05$), *: DIs of myotubes at 20% and 40% strain differ significantly from DI at 0% strain ($p<0.05$), and §: significant difference between DIs of myoblasts and myotubes at 20% strain ($p<0.05$).

than that of myoblasts, whereas at 40% strain no significant difference in DI between the two cell types was found (Fig. 5).

Experiments on single cells confirmed the findings identified using the group approach. Thus, some myotubes were observed to buckle at 20% compressive strain, whereas the onset of buckling in myoblasts occurred at higher levels of 30% and 40% strain. Further, values for DI measured in single cells follow a similar trend to that using the group approach. Table 2 summarizes the mean cell strains and deformation indices of individual cells followed during gross compression of the construct up to 40%. Cell strains in the direction of compression (ϵ_x) correlated well with the applied construct strain, for both myoblasts and myotubes. By contrast, strains in Y direction (ϵ_y) were variable for both cell types and did not correlate with the level of construct strain.

Cell Damage

For all straining periods the total percentage of cell damage was significantly higher in strained constructs than in unstrained controls. Cell damage in strained constructs increased significantly with time of compression from 43.4% (1 h) to 96.2% (24 h), whereas cell damage in unstrained controls remained constant over time with an average value of 28.5%±2.5% (Fig. 6). At time zero the percentage damage was 23.8%±3.0%. When corrected for damage in unstrained controls, the mean increase in damage due to sustained compression was 12.5%, 23.9%, 30.8%, 44.6%, and 67.2% for the respective time periods.

Table 3 summarizes cell damage in the three subpopulations, calculated as a percentage of the total number of cells. For all three subpopulations the percentage damage was significantly higher in strained constructs than in unstrained controls. Damage to the spherical myoblasts and spherical myotubes increased fairly linearly with period of compression and, with the exception of constructs strained for 2 and 12 h, no significant difference in the amount of damage was found between these two sub-populations. After 24 h of straining nearly

TABLE 2. Cell strains (ϵ_x,ϵ_y) and DI of myoblasts and spherical myotubes in agarose at five different compressive strains (mean±sd).

	Myoblasts			Myotubes		
Strain (%)	ϵ_x (%)	ϵ_y (%)	DI	ϵ_x (%)	ϵ_y (%)	DI
0	0	0	0.99±0.04	0	0	1.01±0.08
10	9.1±2.0	−5.0±2.0	0.84±0.03	9.3+1.1	−4.1+0.9	0.85±0.10
20	20.4±3.2	−10.3±0.8	0.69±0.05	19.8±4.2	−32.6±1.0	0.55±0.07
30	28.7±7.2	−30.4+10.1	0.54±0.07	29.6±3.8	−44.4±9.1	0.51±0.06
40	36.9±10.4	−39.6±11.9	0.50±0.08	37.2±6.4	−47.8±8.5	0.48±0.08

all spherical cells were damaged. The elongated myotubes were particularly sensitive to damage. Up to 4 h of straining the percentage damage to these cells was significantly higher than in the other subpopulations and after 12 h of compression all elongated myotubes were damaged.

DISCUSSION

Muscle tissue consists of three components, the muscle cells, the surrounding extracellular matrix, and blood and lymph vessels, each responding distinctly to mechanical loading. Muscle degeneration in the form of pressure sores starts at the cellular level and it is hypothesized that sustained deformation of the muscle cells during tissue compression plays a major role in this process. To evaluate the effects of sustained cell deformation independently of other factors a novel three-dimensional *in vitro* model system, consisting of muscle cells seeded and cultured in agarose was developed. Characterization of the model system indicated a high proportion of cell viability throughout the culture protocol, although most of the myoblasts and multinucleated myotubes adopted a spherical shape in the agarose gel. In addition, the typical cross-striated appearance and spontaneous contractility often seen in monolayers of C2C12 cells[31,35] was not observed. In alternative systems where fully differentiated day 8 or day 12 myotubes obtained from monolayers were seeded directly into agarose similar, mostly spherical, cell morphologies were observed, but at significantly lower cell viabilities (<70%) (unpublished results). The rounding-up of the muscle cells in agarose is possibly due to the absence of fibers, such as found in collagen gels, which are required for cell anchorage. Collagen gel, however, is not suitable as scaffold for maturating muscle cell cultures.[29,37] Therefore, alternative scaffolds based on the work of Vandenburgh *et al.*[39] are being developed for future studies.[8]

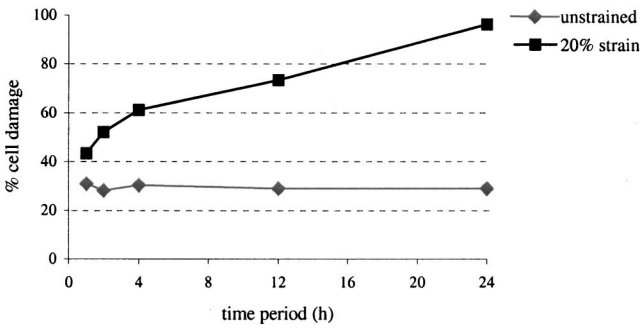

FIGURE 6. Percentage cell damage in uncompressed and compressed (20% strain) cell seeded agarose constructs.

Although the muscle cell/agarose system is a limited representation of *in vivo* muscle tissue it represents a simple, reproducible model to test our hypothesis on the damaging effects of compressive strain-induced muscle cell deformation. Since high levels of cell viability are critical in these studies, the system is very useful for this specific purpose. A major advantage of mounting muscle cells in a scaffold such as agarose is that the constructs can be compressed to clinically relevant levels of strain, using the specially designed test rig, which enables living cells to be easily visualized and measured with confocal microscopy. Moreover, the system allows deformation of cells without contacting the cells, which is not possible in alternative systems for compressive straining of cells.[19,33,36,41]

Evaluation of the culture protocol showed that myoblasts started to fuse and differentiate into myotubes after a change of culture medium. This method of cell fusion has been described for C2C12 myoblasts in monolayers by Bains *et al.*,[2] although the fusion rate is lower in the present 3D system. This is probably due to the lower degree of cell confluence in the agarose constructs. The diameters of the multinucleated myotubes increased significantly from day 8 to day 12 in culture (Table 1), but remained stable thereafter (results not presented). In addition, the relative amounts of muscle cell subpopulations remained unchanged after 12 days in culture. Therefore, day 12 was chosen as the end stage of the culture protocol.

The mechanical stability of the agarose gel with time in culture was not evaluated in the present study. Agarose forms hydrophilic gels and water uptake and swelling may possibly change the overall mechanical proper-

TABLE 3. Cell damage in the three muscle cell subpopulations in the model system, calculated as percentage of the total number of cells per subpopulation in strained and unstrained constructs.

	% damaged myoblasts		% damaged spherical mytobes		% damaged elongated myotubes	
Time (h)	Unstrained	Strained	Unstrained	Strained	Unstrained	Strained
0	23.6	N/A[a]	28.4	N/A	42.1	N/A
1	28.2	43.9	35.7	44.0	63.2	83.3
2	36.6	44.7	26.3	61.5	50.0	87.0
4	25.0	61.0	24.3	67.7	35.0	92.9
12	26.4	72.7	32.1	93.1	43.5	100.0
24	26.3	95.7	29.7	95.0	37.5	100.0

[a]N/A: not appropriate.

ties of the material. Previous studies, however, have demonstrated that the mechanical properties of 3% agarose constructs remain stable for periods up to several weeks and are not affected by the presence of chondrocytes within the agarose.[9,17] It seems reasonable therefore to assume that the muscle cell/agarose constructs remain mechanically stable over the complete culture period. Nevertheless care must be taken when making absolute comparisons with other studies on cell deformation in agarose. The heterogeneous character of the muscle cell/agarose constructs due to the presence of different cell morphologies and myoblast clusters will give rise to a different local mechanical environment than evident in chondrocyte/agarose constructs.[18] The description of the complex mechanical environment around the muscle cells in response to a relatively homogeneous construct strain requires the development of a multiscale finite element model.[8] By prescribing a damage threshold for muscle cells, obtained from the present experiments and others, it will be possible to predict the amount and location of cell damage in response to gross compressive strain.

In the experiments examining cell deformation both the single cell approach and the group approach resulted in similar cell shapes and hence similar values of DI at equivalent strain levels. When the agarose constructs were compressed, the seeded muscle cells exhibited a marked deformation. In myoblasts an increase in gross construct strain of up to 40% produced a significant decrease in DI (Fig. 5, Table 2). This trend is consistent with previous studies on the deformation of spherical chondrocytes in agarose.[17,23,27] The DI of myotubes, by contrast decreased significantly up to 20% strain, but remained stable thereafter. The marked deformation of myotubes at 20% strain, associated with a lower mean DI value compared to myoblasts (Fig. 5), might be attributed to the early onset of buckling at the equators of the myotubes. This process is indicated by the large increased value for ϵ_y associated with a decrease in the DI at 20% strain (Table 2). By contrast myoblasts demonstrated buckling at strains above 30%. Hence, at the highest construct strains, both cell types demonstrated buckling and their DI was equally affected by the changes in cell shape and localized buckling. Thus, the use of the deformation index should be limited to the assessment of cell shape from a sphere to an oblate ellipse. When assessing more complex cell shapes and deformations, including those in buckling cells and elongated myotubes, techniques such as digital image correlation and 3D image reconstruction need to be applied.[6]

If the cells were to be assumed to behave like incompressible solids, any change in cell diameter in the direction of compression (X) would be equal to the square of the reciprocal of the change in the mutually orthogonal directions (Y and Z). This assumption was calculated to be appropriate for the X and Y diameters of myoblasts and myotubes at the level of 10% construct strain, prior to any bucking of the cells (Poisson's ratios approximately 0.5). At 30% and 40% construct strain Poisson's ratios were larger than 1.0, albeit with a limited data set, for both cell populations. This questioned the earlier assumption at these higher strain levels.

Despite the difference in sensitivity to buckling at 20% strain no clear difference in cell damage between myoblasts and spherical myotubes was observed over time. Buckling probably occurs as a consequence of high local stresses in the cell membrane. The higher sensitivity of myotubes to buckling compared to myoblasts may be due to either their larger diameters, the reorganization of nuclei within them or the associated mechanical inhomogeneity. It has been shown that, given an equal membrane thickness, the sensitivity of fluid filled spheres to localized buckling increases with sphere diameter.[16] Liu *et al.*[30] performed extensive studies on the deformation of fluid-filled spherical microcapsules with diameters comparable to the spherical myotubes (65 μm). The authors used a theoretical model consisting of a nonlinear elastic membrane filled with incompressible fluid (Mooney–Rivlin material behavior) to calculate the tension profile in the membrane, expressed as stress per unit

length of the deformed surface. Experiments showed that compression up to 60% strain of individual microcapsules between two parallel plates caused buckling and finally bursting of the polymeric membrane at the equators of the cell. Membrane rupture was evident when tension was highest at the equators, i.e., at the bursting points, reaching a value of 1.04 $N\,mm^{-1}$. It is conceivable therefore that buckling at the equators of deformed muscle cells may eventually lead to membrane rupture. However, the level of 20% strain used in the present cell damage experiments might not be sufficient to cause any direct damage to the membrane. Furthermore, cell damage in the present study was assessed from evidence of membrane disruption and nuclear pyknosis or fragmentation, but no distinction was made between these two markers. Hence, a direct relationship between buckling and membrane damage still needs to be established.

In addition to membrane properties, the local deformability of biological cells such as buckling depends on the integrity of the cytoskeleton.[33] As the typical cross-striated appearance of muscle cells could not be detected in all three subpopulations in the present study it might be argued that these cells are devoid of a bundled microfilament organization in agarose. Further, it is most likely that the integrity of the cytoskeleton is affected by the absence of an extracellular matrix. This aspect must be considered when interpreting the damaging effects of sustained cell deformation.

The percentage of cell damage in the developed model system was significantly enhanced in strained constructs and increased with time of compression for all three subpopulations. This increase in cell damage with compression period is consistent with well-established relationships between tissue comparison and damage derived from animal models.[7,14,20,32] Despite differences in experimental setup and degree of compression, these models indicate significant tissue damage after 2 h of sustained compression, which increases thereafter. Damage assessment in these studies typically involves histology of excited tissue to examine for signs of cellular necrosis, whereas the markers used to assess cell damage in the present study may involve both apoptosis and necrosis.[12]

In the damage experiments the percentage of viable cells in unstrained controls ($71.5\% \pm 2.5\%$) was lower than in the model characterization experiments (viability $>90\%$). This is probably due to the fixation process of the constructs in the damage experiments and the different markers used for assessing cell viability in the respective studied. There was no evidence that the tare strain applied to the control constructs led to any significant decrease in cell viability, since cell viability in unstrained controls was not different from that in time zero values ($76.2\% \pm 3.0\%$).

The level of 20% construct strain used in the cell damage experiments corresponds to a gross agarose stress of about 4.3 kPa. This level of compression has been considered as a causal factor for pressure sores, since it is traditionally quoted as the capillary closing pressure.[26] Surface pressures applied to the skin in excess of this value will produce a degree of ischaemia and, if applied for a sufficient period of time, may lead to damage in the form of pressure sores.[3,14,20,32] Several arguments, however, challenge this theory. Capillary closure depends on local stresses and strains inside the tissue and not just on global pressures applied to the skin. Second, although relationships between surface pressures and blood flow or oxygen tension have been studied, threshold levels associated with tissue damage are not well established.[1,14,15,20,32] In addition, soft tissues can remain viable for extended periods of time without blood supply during surgical procedures. We therefore hypothesized that sustained deformation of the cells inside the tissue is an important component of cell damage regardless of the level of nutrient and oxygen supply. The significant differences in cell damage between strained and unstrained controls in the present study support this hypothesis. Since predisposing factors for cell damage, such as oxygen and nutrient supply to the constructs, were equal for strained and unstrained construct, the deformation of the constructs and the associated cells was considered as the trigger for cell damage. The mechanisms whereby deformation causes cell damage, however, remain unclear. Apart from direct trauma to cellular structures, such as the cell membrane, diffusion through the deformed construct and across the deformed cell membrane may be impaired, particularly when considering large molecules.[28] To obtain an improved insight into the etiology of compressive strain-induced cell damage future studies should focus on structural, mechanical, and biochemical changes in response to sustained cellular deformation.

In conclusion, we have described a simple model system for the study of compressive strain-induced muscle cell deformation and damage under *in vitro* conditions. The system is easy to produce and highly reproducible. Due to its mechanical stability over time it is suitable to study the effects of sustained cell deformation. At levels up to 20% strain the deformation of spherical cells can be determined with relatively simple two-dimensional measurements of the deformation index, whereas other techniques need to be developed to assess deformation at larger strains and in more complex cell shapes. Although the system is at present a limited representation of *in vivo* muscle tissue, it is valuable for studying compressive strain-induced cell damage such as evident in pressure sores.

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