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The stiffness of bone marrow cell–knit composites is increased during mechanical load

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Abstract

A novel device for mechanical stimulation of primary adult rat bone marrow cells cultured on three-dimensional knitted textiles has been prototyped. A method has been developed ensuring a well-defined, high-density, and reproducible cell seeding on the knitted fabric. After culturing for 18–52 days the cell–knit composites were subjected to uniaxial 2% stretching and relaxation. The frequency was altered between 0.1 Hz (196 min, loading phase) and 0.01 Hz (360 min, resting phase). Identically treated knits without cells exhibited a slight stiffness reduction, whereas the stiffness of knits with cells increased from cycle to cycle. The stiffness increase was found to depend on the duration of the culture period before mechanical loading. Our data suggest that the extracellular matrix deposited by the cells on the knit and intact microtubuli of living cells cause the observed stiffness increase. In comparison to the unstrained static cell–knit composites cell proliferation and bone cell differentiation were reduced by the mechanical load.

Keywords: Mechanical load; Culture; Bone marrow; knit; Stiffness; Strain

1. Introduction

Mechanical loading governs the extent of growth and differentiation of many tissues, including bone. The mechanical strain window and the effects on bone formation and remodeling are determined by the frequency and the magnitude of the strain [1,2]. The mechanism behind the sequence from sensing the strain up to cell reaction is not fully understood [3]. In vitro investigations are especially predestinated to elucidate mechanisms of specific processes. Among others this knowledge is needed in designing culture conditions for generating in vitro bone tissue. This engineered tissue can be potentially used to augment bone or to fill critical size bone defects.

In order to study effects of mechanical loading on bone marrow cells a three-dimensional model system was developed, which enables to stimulate cells mechanically under well-defined conditions. Knitted textiles as porous scaffolds provide not only optimal spatial and nutritional conditions for engineering of biological tissues, but are capable of transferring mechanical load to cells. Furthermore, knits have the advantage that cells can migrate to sites at which optimal nutritional and mechanical loading conditions are present [4]. Generally, cells are cultivated as two-dimensional monolayers. Comparative studies of behavior and performance of three- and two-dimensional cultured cells showed essential differences, with three-dimensional cultured cells behaving much more in vivo like [5–7]. The advantage of three-dimensional textile superstructures is that cells can be cultured and mechanically loaded in an in vivo like three-dimensional fashion. Therefore, a knit pulsating system was developed for uniaxial, cyclic stretching of knit–cell composites. The premise of the use of knits as scaffolds for mechanical loading studies is that cells are homogeneously covered by cells at a rather high cell density.

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Abbreviations: ALP: Alkaline phosphatase; RBMC: Rat bone marrow cells; PBS: Phosphate buffered saline; TRAP: Tartrate-resistant acid phosphatase

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Therefore, before starting the mechanical loading experiments, a cell seeding technique had to be developed that enables a homogeneous high cell density coverage of the knits.

Under physiological conditions maximal strains of about 2000 με have been assessed in humans with a maximal strain rate of about 40,000 με/s [8]. The macroscopic strain (ε) is defined by the ratio of the induced increase in length to the original length. Strains above 5000 με are thought to be present in the pathological overload situation. In these cases woven bone is formed similar to a bone repair reaction [9]. Strains above 10,000 με are assumed to occur in vivo after a bone fracture. However, only strains below 50,000 με are known to induce intramembranous bone [10]. Different effects of strains on osteoblast cell proliferation and differentiation in vitro have been reported [3,11,12]. Usually, strains above 4000 με are adequate to modify osteoblast-like cell function, generally expressed by a change in cell shape. In the present study a situation was chosen to mimic where in vivo intramembranous bone is formed. Therefore, the investigations described here use a macroscopic strain application of 20,000 με.

The aims of the present study were: (i) to develop an optimized knit pretreatment and cell seeding technique, (ii) to assess the difference in mechanical-properties-evolution of the knit–cell composite with and without cells as a result of mechanical stimulation, (iii) to reveal data to explain the latter obtained difference and (iv) to determine the influence of mechanical loading on the proliferation and differentiation of bone cells in comparison to the unstrained reference.

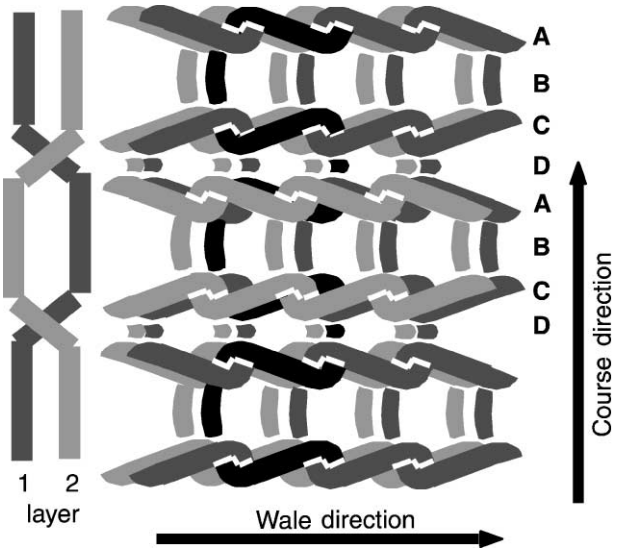

Fig. 1. Schematic representation of the knit used. The yarns are composed of 24 filaments drawn for reasons of simplification as a single thread with only one filament. The knit is composed of 2 layers which are alternating on the left and right side, changing sides between position C and A (for the sake of clarity one yarn is drawn black).

2. Materials and methods

2.1. Scaffold preparation

The scaffold used (Fig. 1) was a knitted polyethyleneterephthalate (PET) multifilament yarn. Each thread consists of 24 highly textured filaments, having a diameter of 20 μm. The number of stitches per cm was 15.2 in the course and 15.5 in the wale direction. The thickness of the knit in relaxed state is 1.0 mm. The size used was 7.0 cm × 4.0 cm. In order to optimize cell adhesion and a homogeneous cell distribution within the knits the effect of inclusion of three different knit treatment steps was tested. They consist of a cleaning step (step 1), a prestretching step (step 2) and a plasma treatment step (step 3). They were performed one day before cell isolation. Regarding the cleaning step, knits were cleaned by subsequently bi-distilled water, sonicated for 120 s in acetone and ethanol. In step 2, knits were prestretched by fixing the knits on two opposite sides in a way that they could be stretched for 10% in the course direction. The knits were autoclaved in this position afterwards. Step 3 represented a plasma activation of the knit surface. For this, knits were air plasma treated for 60 s at 2 mbar, 10 mHz and 60 W (Tissue cleaner PDC-32G, Harrick, USA). After the plasma treatment the knits were transferred into cell culture flasks (80 cm^2 substratum surface, Nunc, CH) containing 25 ml α-MEM (Nunc, CH). After 30 min the medium was replaced by cell culture medium containing 89% α-MEM, 10% heat inactivated fetal calf serum and 1% antibiotics (PSN, Gibco, CH).

2.2. Preparation of the cell cultures

Rat bone marrow cells (RBMCs) were obtained from tibiae and femora of male adult Wistar rats (250–300 g). The rats were killed by decapitation. The bones were excised, freed from soft tissue, without the cartilage ends transferred into isolation medium [13] and broken afterwards. RBMCs were dispersed by repeated pipetting. Pieces of bone, coagula and cell clusters were removed by filtration over a 200 μm mesh polyester (PET) filter. The cells were sedimented by centrifugation (14 min at 30*g*) and resuspended in phosphate buffered saline (PBS). After recentrifugation pellets were resuspended in cell culture medium. The RBMC density was assessed using a coulter counter (Coulter Z1, IG AG, Zürich) (counting window 7.5–21.7 μm). Knits with 20×10^6 RBMC were incubated in a cell culture incubator (5% CO_2, 95% humidity) under gyratory shaking for 18–52 days. In the first experiment some knits were incubated without gyratory shaking. This period is termed preincubation period. For development and optimization of the cell seeding and knit pretreatment procedure, living cells on the knits

were stained by treating the cell–knit composite by adding thiazolylbluetetrazolium-bromide (MTT) to the culture medium. The staining intensity and distribution of the differently treated composites were compared by light microscopy after incubating the composites under cell culture conditions for 1 h.

2.3. Testing conditions

To be able to mechanically stimulate and to follow on-line mechanical properties of the cell–knit composites after the preincubation period, knits were transferred into the knit pulsating device (Fig. 2A and B). It consists of two lamellae made from PEEK between which the cell–knit composite is fixed. One of these lamellae is connected to a sensitive force sensor (quartz crystal sensor; Kistler Instrumente AG, CH), the other to a rotator responsible for the stretching and relaxation of the cell–knit composite. The tops of the lamellae are connected to the module frame by flexible metal sheet membranes.

Of the knits the central 3 cm of the knit (course direction) was used and the remaining parts were removed as

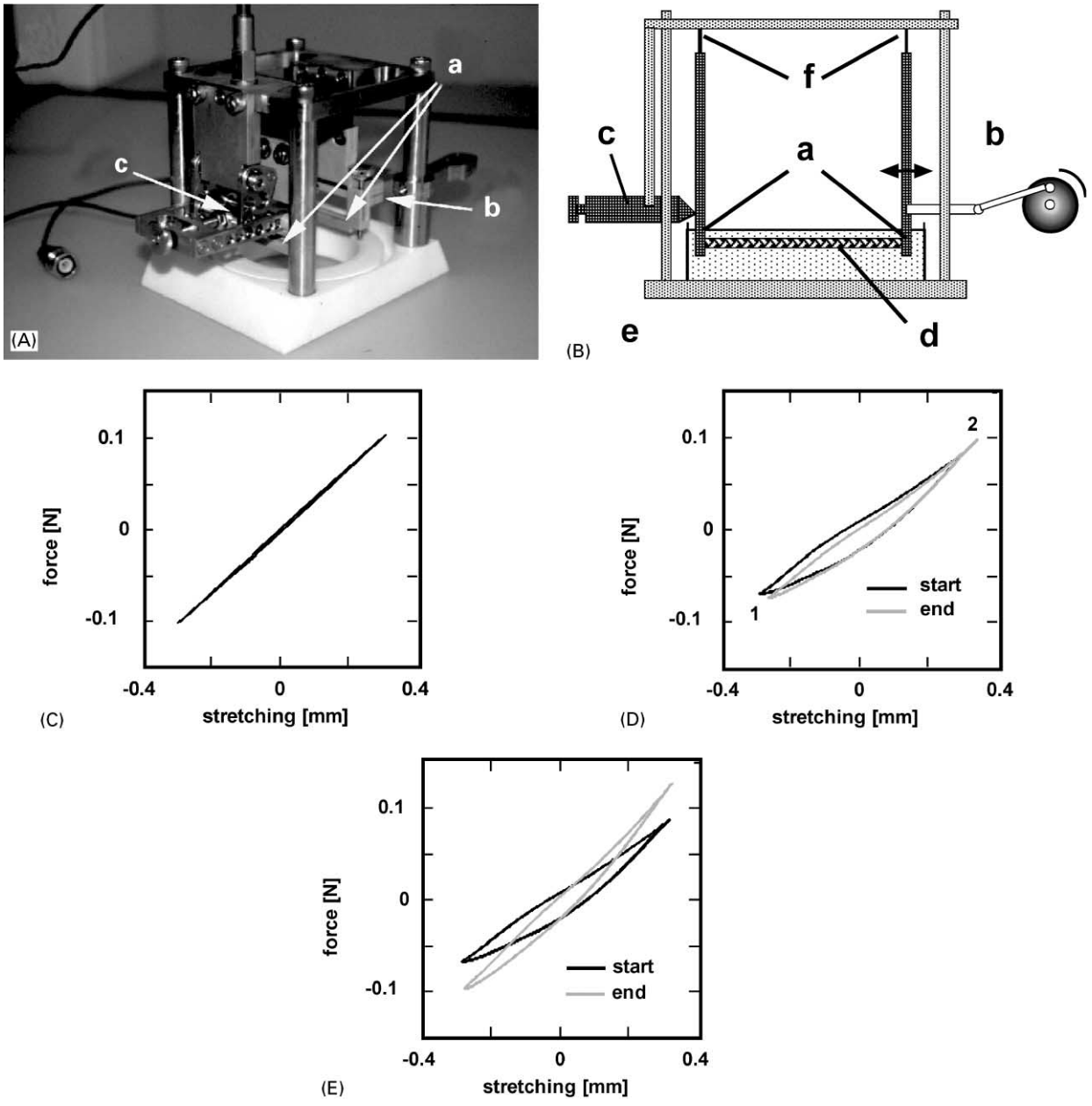

Fig. 2. Picture (A) and schematical representation of the mechanical loading device (B). The force–strain hysteresis of a spring (C) and the knits without (D) and with (E) cells at start and end of the mechanical loading experiments. 1 and 2: a knit stretched for 0% and 2%, respectively. a: location where knits are fixed; b: part of the loading device which will be connected to the stretching motor; c: force sensor; d: cell–knit composite; e: culture dish with medium; f: flexible metal membranes.

far as not needed for fixing into the device. The knits were mechanically loaded under cell incubator conditions. In the present experiments they were alternately stretched by 2% (20,000 με or 20 mε, simulating bone fracture conditions), in the course direction, sine wave, for 196 min at 0.1 Hz or 2000 με/s (termed loading phase) and for 360 min at 0.01 Hz or 200 με/s (termed resting phase) (Fig. 2B). The mechanical strain of each single cell is dependent on the location within the knit ranging from zero to above 50 mε as a result of the complexity of the knit used. As concluded from light microscopy images of knits loaded with and without 20 mε strain, in the course direction maximal strains above 20 mε were observed at D position in Fig. 1 measuring the distance between C and A, whereas at B, measuring the distance between A and C, strains of only 0–10 mε could be observed. Simultaneously, the width of layers C and A was reduced by 0–7% corresponding to a strain of 0–70 mε. Furthermore, as a result of the elongation, a contraction of the knit perpendicular to the course direction, i.e., in the wale direction, was observed. The contraction was almost zero at the position where the knit was connected to the device, but around 5% in the central part resulting in 50 mε strain.

To uncover the evolution of the knit stiffness and effects on cell performance, identically treated knits without living cells were used for comparison. Furthermore, static knits with cells (not shifted, not stretched) and knits with cells that were cyclic shifted instead of stretched were included in this study. The shifted knits were connected to the knit pulsating device only at one side instead of two sides, i.e., the knits were connected to the side without the force sensor. In the shifted knits it may be assumed that the cells will predominantly be subjected to fluid shear forces. Since latter cell–knit composites are not connected to the force sensor, the stiffness evolution cannot be assessed in these composites.

The current study consists of 9 independent experiments of which 8 use knits with cells and one knits without cells but preincubated with serum containing medium for 35 days. The knits with cells were preincubated for 18 (3 experiments), 35, 38, 39, 45 and 52 days. The cells on the 39-day preincubated knits were killed by formalin vapor prior to the mechanical loading experiment. Serum incubated knits and knits with dead cells were included in the present study to discriminate between unspecific effects and effects induced by living cells.

The prepared cell–knit composites were prestretched within the mechanical loading device, giving rise to a spring constant of 0.33 N/mm verified by the piezoelectric force sensor. The well-defined starting conditions guarantee reproducible mechanical stimulation. Subsequently, the mechanical stimulation was performed using a total strain of 20 mε. After the run-in period of 12 h, the force was in situ monitored continuously during each loading phase for 76 h (with the exception of one reference measurement).

Fig. 2C represents the device calibration by the use of a coil spring. The spring constant is given by the slope, and the negligible hysteresis reveals the elastic behavior of the spring. Contrary to the spring, both the knit and the cell–knit composite exhibit significant hysteresis, which corresponds to the dissipation of mechanical energy during one cycle of stimulation (compare Fig. 2D and E). The stiffness, as used in the present communication, corresponds to the spring constant defined by the turning points 1 and 2.

To evaluate the importance of ECM in the stiffness increase of the cell–knit composite in one selected experiment a cell–knit composite (35 days preincubated) was treated 3 days after start of the mechanical loading by a mixture of arabinofuranosylcytosine (ara-C, 0.1 mM) and colchicine (0.1 mM). A second cell–knit composite was treated by a vehicle. Ara-C is known to kill proliferating cells [13], e.g., progenitor cells, fibroblastic cells and preosteoblasts, whereas colchicine inhibits microtubule polymerization. As tested in bone marrow cell cultures cultivated in 24 well plates and treated by this mixture for 2 and 3 days all proliferating cells died. Only cells exhibiting osteoblast- and osteoclast-like morphology were not affected. The mechanical loading and force measurements proceeded for 56 h.

To determine the influence of mechanical loading on the proliferation and differentiation of bone cells in comparison to the unstrained reference, after termination of 6 of the experiments, knits with living cells were cut into two identical pieces. One piece was used to measure MTT-conversion as an index for cell activity [13]. The other piece was used to determine cell mass (total cell protein) and DNA, alkaline phosphatase activity (ALP) (differentiation of progenitor cells into (pre-)osteoblasts) and tartrate acid phosphatase activity (TRAP) [14]. Active osteoclasts are known to synthesize high quantities of TRAP. Significant effects ($p < 0.05$) were determined using the ANOVA two factorial Dunn-Bonferroni test. Some representative knits were morphologically analyzed using a scanning electron microscope.

3. Results

3.1. Optimization of knit pretreatment and seeding technique

Seeding under static conditions resulted in a high cell coverage of the culture flask substratum with nearly no cells present on the knits. In contrast, by culturing the knits under gyratory shaking conditions no cells attached to the flask substratum but high cell densities were found on the knits. Without the knit cleaning and plasma treatment cells were present in clusters that were irregularly distributed on the knit. By including both steps

a homogeneous cell coverage of the knits was achieved at least in the area of the knit used for mechanical loading. Inclusion of the plasma treatment step gives rise to increased cell densities as judged after 18 days in culture (Fig. 3). The inclusion of the prestretching step reduced significantly the stiffness decrease of cell-free knits that normally occurs during mechanical loading when using unstretched cell-free knits.

3.2. Effects of mechanical loading on the stiffness of the cell–knit composite

The stiffness of the knit–cell composite increased during mechanical loading, whereas the stiffness of knits without cells was slightly reduced (Figs. 2 and 4). The increase in stiffness was found to depend on culture period before mechanical loading. Slight increase was found in cell–knit composites preincubated for only 18 days. Maximal stiffness increase was found for cell–knit composites preincubated for at least 35 days (Fig. 4). The observed stiffness increase correlates with the quantity of protein present on the knits, representing extracellular matrix (ECM) and cell protein, at the end of the mechanical loading experiment (Fig. 5A). Other biochemical parameters do not correlate with the stiffness increase although in the case of MTT-dehydrogenase activity (Fig. 5B) the mean value found for 38–52 preincubation cell–knit composites was 195% of the mean value found for 18 days preincubated composites. Regarding cell differentiation, mean ALP and TRAP activity values were virtually the same in cell–knit composites preincubated for 18 or for 38–52 days.

As mentioned above the knits were stretched alternately for 196 min at 0.1 Hz and for 360 min at 0.01 Hz. In knits preincubated for 35–52 days the stiffness increased

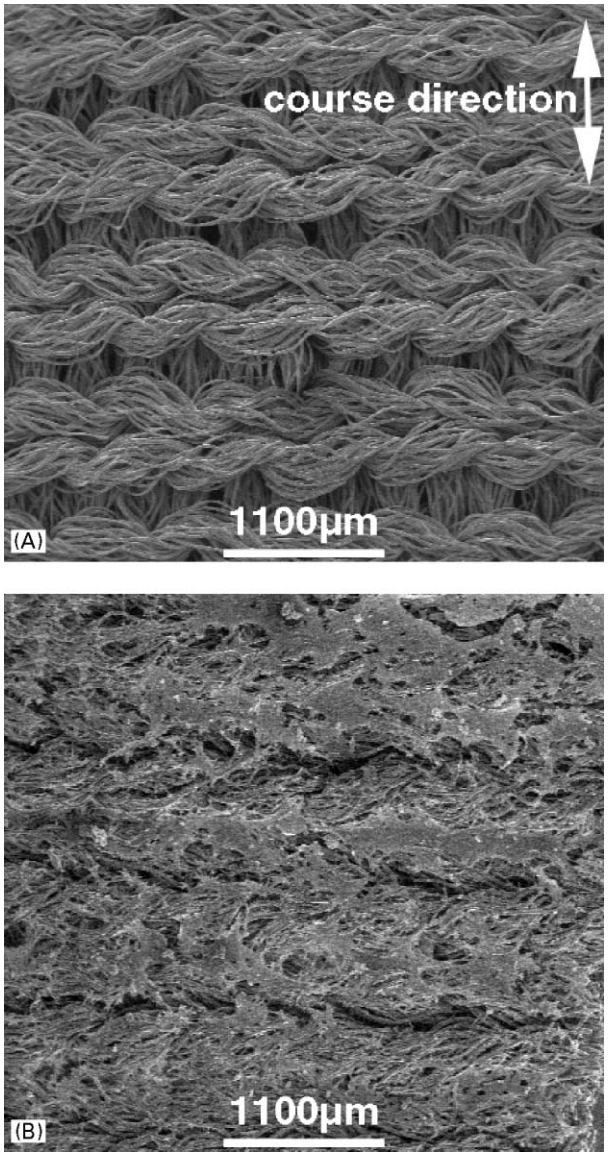

Fig. 3. Scanning electron microscopic pictures of a knit without (A) and with (B) cells after 18 days in culture. Please note that the biological material (ECM with cells) is predominantly present at locations with highest filament density.

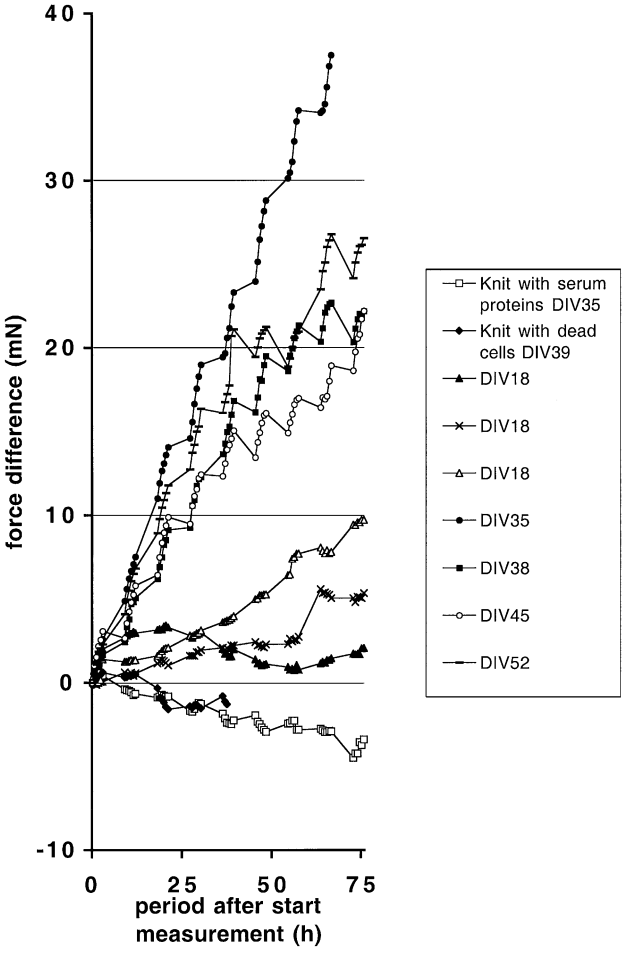

Fig. 4. Time evolution of force difference between relaxation and 2% stretching relative to start of the measurements of 9 independent experiments.

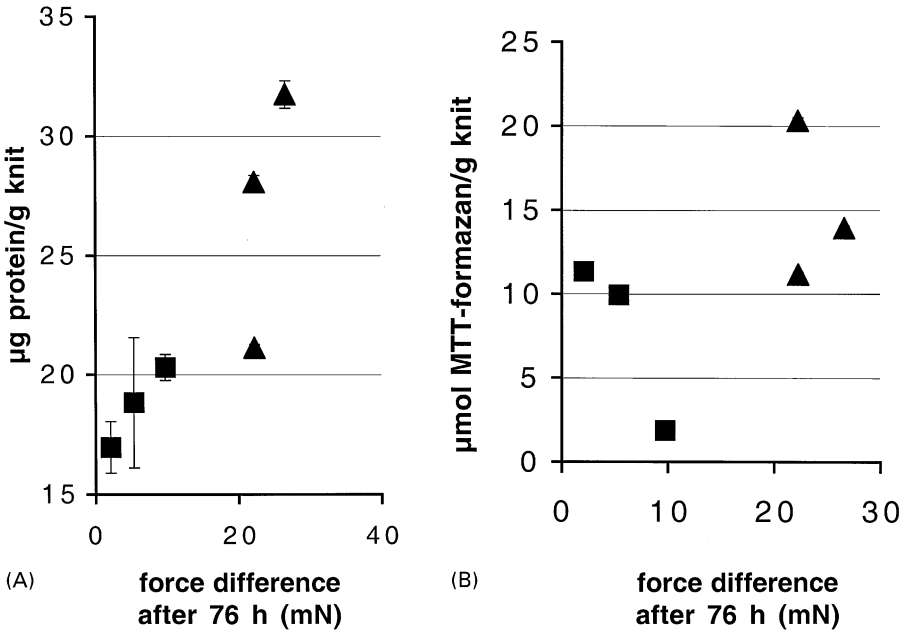

Fig. 5. Correlation between stiffness increase and protein content (A) and MTT-formazan formation (B) at the end of the mechanical loading experiment in cell–knit composites that were preincubated before start of the mechanical loading for 18–52 days (mean experimental values ± SD of triplicate measurements are shown). The knit–cell composites shown were preincubated for 18 (■) or 35 days and more (▲).

nearly exclusively during the loading phase. Such a feature was not detected for 18 days preincubated knits. For these knits an almost continuous stiffness increase was observed (Fig. 4).

By adding a mixture of ara-C and colchicine after 3 days of mechanical loading of the treated knit about 22% of the total cell number died as concluded from the reduction in MTT-dehydrogenase activity in treated versus untreated composites at the end of the experiment. In parallel the stiffness increase was reduced by 70%. The stiffness of the untreated cell–knit reference composite increased further on with the same pattern as seen at the previous 3 days.

3.3. Effects of mechanical loading on cell morphology and cell performance

Mechanical loading of the cell–knit composites resulted in a change in cell plus ECM distribution as suggested by scanning electron microscopy analysis (Fig. 6). Mechanical loading resulted in a preferential alignment of the ECM plus cells along the single filaments. Less bridging of ECM plus cells between the filaments was observed in the mechanical loaded knits. The scanning electron microscopy images, however, do not allow for differentiation between cells and ECM. In contrast, no differences in cell distribution in strained and unstrained knits were seen in cell–knit composites by light microscopy after staining cells with MTT.

Regarding cell function a significant reduction in cell proliferation (DNA content) in stretched and non-stretched but cyclically shifted (dislocated) knits in comparison to static knits was found (23% and 31% reduction, respectively). Also most other parameters were significantly reduced in comparison with the static knits (TRAP activity: 23% and 28%; ALP activity: 31% and 17%; cell protein: 7% and 20%; MTT conversion: 1% (not significant) and 16%, respectively) (Fig. 7). The cells on stretched and those cyclically shifted but unstretched knits behaved similarly. No correlation seems to exist between the mechanical load induced change in cell performance (as defined by DNA and cell protein content, ALP and TRAP activity) and the length of the culture period before loading.

4. Discussion

In the present study, the effects of mechanical stimulation of cells cultured in a three-dimensional organized cell–knit composite were investigated, using primary long bone marrow cells of adult rats. The main parameters of the study were cell–knit composite stiffness and cell function. This is the first study where cells are cultured on a knitted fabric and thereafter subjected to mechanical load.

Since the differentiation of bone marrow cells containing mainly non-anchorage dependent progenitor cells to anchorage dependent cells takes a few days, with current techniques it was impossible to seed knitted scaffolds reproducibly with bone marrow cells. Xiao and coworkers found that in contrast to static conditions

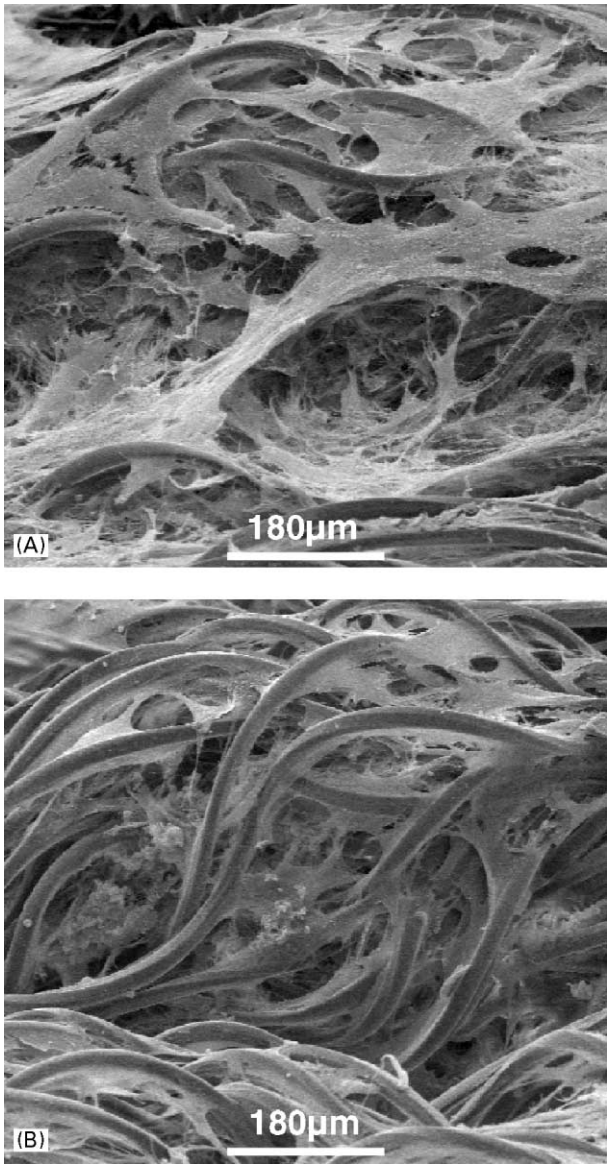

Fig. 6. Effects of mechanical loading on rat bone marrow cell morphology. Scanning electronmicroscopy images of a static knit (A) and a loaded knit with cells at the end of the mechanical loading experiment (B). Note that in (B) the biological material (ECM plus cells) is predominantly aligned along the filaments. The knit–cell composites shown were preincubated for 18 days.

dynamic seeding of fibroblasts onto porous scaffolds leads to efficient as well as homogeneous cell attachment [15]. A homogeneous cell coverage is a prerequisite to successfully perform reproducible mechanical loading experiments. In agreement we found that nearly no cells attached to the static knits. However, by cultivating the knits under gyratory shaking conditions, cells were not able to attach to the flask surface anymore but to the knit. By including the cleaning and plasma treatment steps a homogeneous high-cell-density coverage of the knits was achieved. The cleaning step removes filament sizing, which may reduce serum protein adsorption, whereas plasma treatment increases surface charge. The inclusion of the prestretching step reduced significantly the change in the hysteresis figure of cell-free knits during mechanical loading. Thus by including all three knit pretreatment steps and by culturing the cell–knit composites under gyratory shaking conditions optimal prerequisites were revealed for starting the mechanical loading experiments.

One of the main goals of the present study was to assess the difference in mechanical-properties-evolution of the knit–cell composite with and without cells as a result of mechanical stimulation and to reveal data to explain the latter obtained difference. Our study demonstrates that during mechanical loading cell–knit composite stiffness is only increased if living cells are present on the knit (Fig. 4). This increase in stiffness including its evolution with time was found to depend on the culture period before the cell–knit composites were transferred to the loading device. The increase in stiffness may be due to (i) the release of ECM components, (ii) the increase in cell mass, (iii) tensional reactions of the cells together with the increase in cell number. (iv) All of these may greatly be affected by the cell type composition and the degree of differentiation of the cells present on the knit.

Cell differentiation: Since ALP and TRAP activities (as index for osteoblast and osteoclast differentiation, respectively) were similar in the various mechanical loaded cell–knit composites, one may conclude that the observed differences in stiffness increase cannot be traced back to an altered degree in cell differentiation at least on the osteoblast and osteoclast level.

ECM and cell number: Of the parameters tested only the protein content of the cell–knit composite correlates with the extent of stiffness increase. The proteins present on the knits represent ECM and cell proteins. The total quantity of cell protein is determined by cell size and cell number. Since no relationship exists between cell activity (MTT-dehydrogenase activity as an index for active living cell number) and increase in stiffness, it is hypothesized that the increase in ECM quantity is predominantly responsible for the observed stiffness increase.

Tensional reactions: The importance of ECM in the observed stiffness increase is partly put in question by a preliminary experiment where a cell–knit composite was treated 3 days after start of the mechanical loading by a mixture of ara-C, killing proliferating cells, and of colchicine, destroying microtubuli. Since the reduction in stiffness surpasses the decrease in the number of living cells taking MTT-dehydrogenase activity as an index by at least 3.2-fold we hypothesize that an important part of the stiffness increase besides that by the ECM is based on intact microtubuli of living cells and not on cell number per se. Regarding tensional cellular reactions, it is known that cyclic mechanical loading of fibroblasts results in a modification in cell orientation and cell mediated

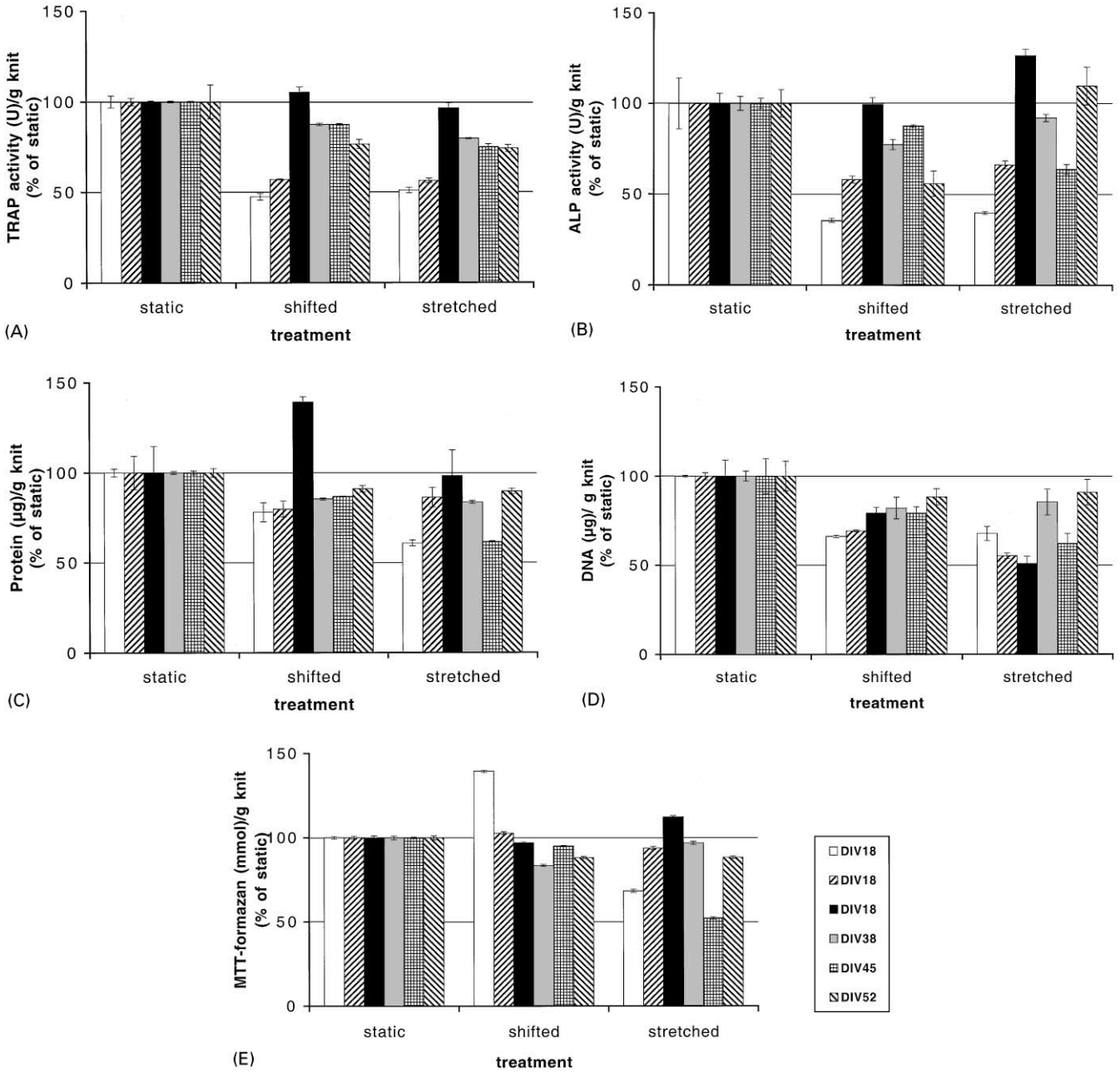

Fig. 7. Effects of mechanical loading on TRAP (A) and ALP (B) activities, cell protein (C) and DNA (D) content, and on MTT-dehydrogenase activity (E) using cell–knit composites that were cultured for different periods before stimulation (mean ± SD of triplicate measurement of each single experiment).

contractile tension [16–18]. Whereas microfilaments are related to the mechanism of signal transduction in response to mechanical stimulation [11], microtubules are a potential target for translating changes in externally applied mechanical stimuli to alterations in cellular phenotype [19], e.g. cell shape and orientation. Thus, our results suggest that ECM and intact microtubuli of living cells also play a key role in the observed stiffness increase.

In summary, the increase in stiffness is not based on a single but on multiple factors including ECM and cell tensional forces.

Under the mechanical strain regimen chosen results indicate that a slight mechanical loading (dislocation of the knit without active stretching) affects cell performance, e.g., proliferation and differentiation. These effects are found to be comparable to those of stretched knits although regarding DNA, protein content and TRAP activity these effects were more pronounced in the 20 mε strained knits. In analogy, modification of cell performance has been reported for in vitro studies using osteoclast- or osteoblast-like cells that are slight mechanical loaded with strains within the range of the overuse

window defined as 2–5 mε or by strains between 10 and 170 mε being within the pathological overload window [20–24]. In knits that were only shifted the cell stretching was minimal, whereas the fluid shear forces were comparable to those that were stretched. Thus fluid shear forces seem to be mainly responsible for the induced modification in cell performance. Several research groups suggested that fluid flow is the primary stimulus by which bone cells respond to mechanical strain [25, 26].

In line with the study of Matsuda and coworkers [27] we found that cell proliferation was diminished compared to the static control. In analogy to our study Matsuda and coworkers stimulated the cells at a frequency of 0.1 Hz and a strain within the overuse window (90 mε). Most studies describing no effect have been performed at frequencies between 0.1 and 0.25 Hz [28], and those describing a positive effect on cell proliferation have been performed at frequencies between 0.25 and 1 Hz [29]. In analogy to these in vitro findings in vivo stimulation of non-fractured rat tibiae resulted in increase in bone formation at frequencies at and above 0.5 Hz but not at 0.2 Hz and below [26]. So far it is not known, which part of the stimulus, e.g., frequency, duration, and rate of the applied stimulus, is decisive for the induction of cellular responses. It has been reported that following mechanical loading at a frequency of around 0.1 Hz osteoblasts react with cell membrane depolarization whereas at frequencies above 0.2 Hz cell membrane is hyperpolarized [30]. Thus, the induced membrane potential (hyperpolarization or depolarization) may play a crucial role in the type of cell response. In line with this hypothesis, electric field stimulation of semi-monolayer osteoblastic cell cultures known to reduce transmembrane potential has been described to reduce cell proliferation [31]. Additional experiments performed at different mechanical loading frequencies are needed to verify the current hypothesis.

Besides a reduction in cell proliferation we also detected a reduction in differentiation. This finding is in line with other studies of mechanical stimulation of osteoclast-like cells [24] but is in contrast to those using osteoblast-like cells. Regarding ostoblast-like cells, generally, no, or cell differentiation promoting, effects were reported [20,22,28,29]. In addition to differences in stimulation regimen, this effect may be explained by the type of scaffold (filaments versus plain membrane surfaces) and the dimensionality of the culture used for mechanical stimulation. It is generally accepted that mechanical stimulation is transduced into a cellular response by induction of various biochemical signals such as growth factors that regulate further cellular reactions. These signals may promote an inhibition of cell proliferation. Generally, two-dimensional cultures on a flat, membrane-like surface have been used for mechanical stimulation. In this environment signaling factors that are released, are diluted before contacting neighboring cells compared to cells that are organized in close contact with each other in a three-dimensional fashion.

In conclusion, optimal conditions for knit pretreatment and cell seeding were developed. It is shown that during cyclic uniaxial mechanical loading of a three-dimensional knit–bone marrow cell composite the stiffness of the knit–cell composite is increased. Evidence was found that ECM plays a key role in the observed stiffness increase. As a result of the mechanical loading cell function, regarding cell proliferation, protein synthesis, ALP and TRAP activity, of cells cultured on these knits are reduced.

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