The effects of collagen type I topography on myoblasts *in vitro*.

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Background

The process of muscle growth is a central issue in the business of producing animals for meat. At the most fundamental level, the process of muscle development and growth is a complex sequence of events whereby muscle cells respond to a number of stimuli in order to form organised muscle tissue. Cells respond to a variety of cues from their environment, which can include chemical, mechanical and topographical signals. The differentiation of myoblasts requires a combination of signals. It is well documented that myoblast fusion is strongly influenced by the chemical nature of the surrounding matrix[1] and can be effected by mechanical stimulation[2]. Studies have additionally shown that a large variety of cell types are also influenced by surface topography of a substrate[3]. There is evidence that cells can recognize and interact with surface topographies as small as 30nm[4]. It is known that cells grown on a collagen-coated surface differentiate more readily than those grown in the absence of the extracellular matrix protein[5], but there is little knowledge as to whether the effects of myoblast interaction with collagen are due solely to chemical interactions, or whether topographical signals play a role as well.

Objective

The purpose of this study was to investigate the behaviour of myoblasts on biologically relevant surfaces with varying topography. The specific aim of this work was to determine whether the longitudinal 67 nm periodicity of collagen fibers may provide topographical signals that affect the growth and differentiation of myoblasts during the development process.

Methods

Fibrous collagen surfaces were prepared using Collagen I from fetal bovine dermis that was acid-extracted purified by differential salt precipitations, dialyzed into 5mM acetic acid, and frozen. Glass squares cut from microscope slides were ultra-sonicated in water and cleaned by UV/Ozone. For fibril formation, thawed collagen solution was mixed on ice with a neutralising buffer, yielding pH 7.4, and concentrations of 30 mM phosphate, 150 mM NaCl, and 200 µg/ml collagen. Glass samples were quickly coated with a thin liquid film, equalling 20 ng collagen/mm², and incubated at 34°C and 100% humidity. After 20h, freshly reconstituted fibrils were deposited on the glass surface by drying. Molecular collagen type I surfaces were prepared by incubating the glass squares with 100 mg/ml acid soluable collagen typeI for one hour.

C2C12 cells from ATCC (American Type Culture Collection, Manassus, VA), were maintained under a 5% CO_2 stream at 37°C, plated at 7,500 cells/cm² on surfaces coated with either molecular or fibrous collagen type I and grown to confluence in DMEM containing 10% foetal calf serum (FCS).

AFM analysis was performed on a Digital Instruments Dimension 3000 operating in tapping mode. Images were recorded using Super Sharp Silicon cantilevers (Nanosensors, Wetzlar, Germany). Scanning proceeded at a rate of 0.5 lines/s and with a sampling density of 512 x 512 points/image. Height image is supplemented with 20% artificial illumination.

For cell morphology and immunostaining assays,cells were fixed with 4% paraformaldehyde, permeablised with saponin and stained with rhodamine phalloidin (Molecular Probes, Eugene OR), DAPI (Sigma, St. Louis, MO) and anti talin monoclonal or polyclonal anti collagen I antibody (Chemicon, Temecula, CA), followed by Alexa 488 secondary antibodies (Molecular Probes, Eugene Oregon). Images were obtained using a Leica DmIRB inverted microscope (Leica, DK) couples to a Coolsnap digital camera (Roper Scientific, DE). Images were obtained and analysed using the Image Pro Plus system (Image House, DK). Cell proliferation in each culture was assayed by counting cell nuclei numbers after staining with DAPI.

The activity of the enzyme creatine phosphokinase (CPK) increases as myoblasts fuse into myotubes. The level of CPK was therefore assayed as a measure of cell differentiation and myotube formation in the different culture conditions, using the CPK assay kit (Sigma, St. Louis, MO), on cell suspensions whose total protein concentration was determined using a BCA kit (Pierce, Rockford, IL), on homogenised cell suspensions.

Results and Discussion

Cells plated on fibrous collagen type I exhibit an altered morphology as compared to cells on molecular collagen. C2C12 cells which have been plated on fibrous collagen (Fig. 1B) spread more extensively than on molecular collagen surfaces (Fig 1A) and contain numerous small filopodia. Each of these cellular extensions interacts directly with a collagen fibre by forming prominent adhesion plaques (Fig 1C). These adhesion sites are not seen in areas of the cell overlying regions of molecular collagen.

AFM analysis of these cells on fibrous collagen shows that the myoblasts line up along individual collagen fibres (Fig 2A). At higher magnifications, it is seen that the individual filopodia spread along a collagen fibre longitudinally (Fig 2B). The membrane of the filopodia appears wrapped around the collagen fibre (Fig2C).

Adhesion and proliferation analysis show that myoblasts spread more readily on fibrous collagen (Fig 3A) and proliferate more rapidly (Fig. 3B). There was no significant difference in the differentiation rates of cells plated on the two collagenous surfaces (Fig 3C).

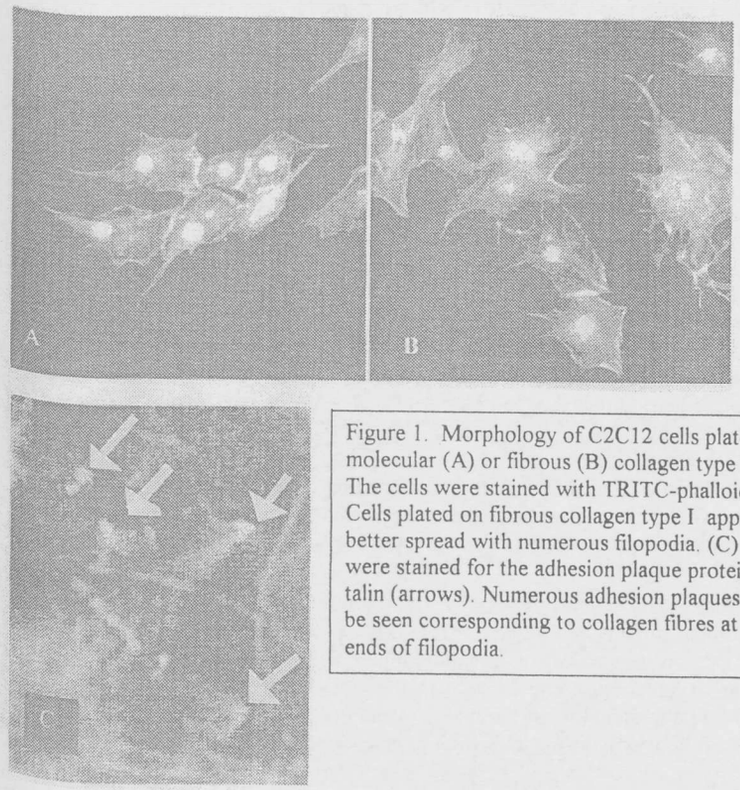

Figure 1. Morphology of C2C12 cells plated on molecular (A) or fibrous (B) collagen type I. The cells were stained with TRITC-phalloidin. Cells plated on fibrous collagen type I appear better spread with numerous filopodia. (C) Cells were stained for the adhesion plaque protein talin (arrows). Numerous adhesion plaques can be seen corresponding to collagen fibres at the ends of filopodia.

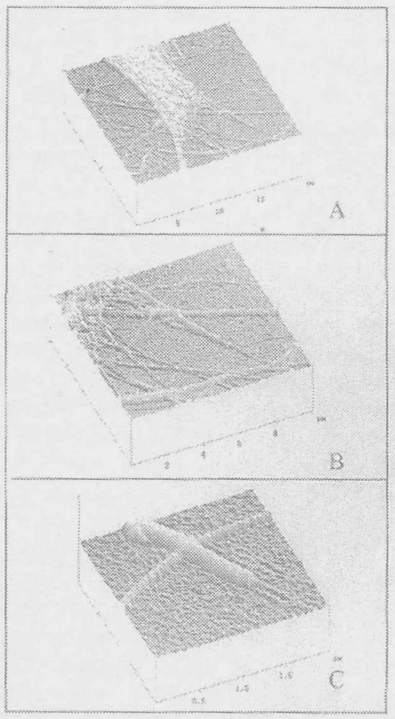

Figure 2. AFM images of a region of a C2C12 cell plated on fibrous collagen I. (A) Low magnification: The cell itself appears to align itself lengthwise along collagen fibers. (B) Higher magnification: Individual projections can be seen aligned along the length of the fibers. (C) The end of the filopod wraps itself around the collagen fibre.

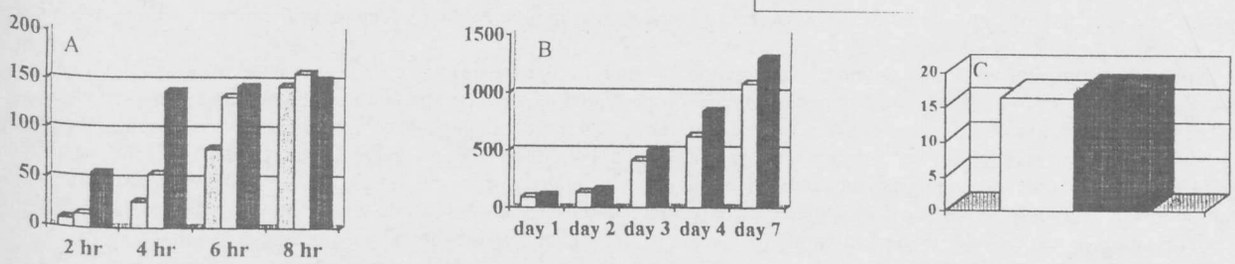

Figure 3. Behaviour of cells on collagen surfaces. Grey- uncoated; White – molecular type I collagen; Black – fibrous type I collagen. (A) Cell adhesion (number of cells/field). Myoblasts adhere to fibrous collagen surfaces within 4 hours after plating while cells on molecular collagen are not fully adherent until 6 hours. (B) Cell proliferation (number of cells/field). Cells proliferate more rapidly on fibrous collagen surfaces. (C) Cell differentiation (CPK activity). There is no significant difference in CPK activity in cells plated on the different collagen surfaces.

Conclusions

Our results show that cells plated on fibrous collagen surfaces exhibit a more spread morphology with numerous small surface pseudopods. Additionally, cells adhere to, and spread on, fibrous collagen surfaces more readily than molecular collagen surfaces. The interactions between myoblasts and fibrous collagen surfaces lead to higher proliferation rates than interactions with molecular collagen, but the differences seen in the interactions of cells with fibrous collagen do not lead to significant differences in cell differentiation rates. Therefore, cells recognize and react to the surface topography of collagen fibres, but these topographical signals do not lead to large differences in cell differentiation, a defining step in the development and growth of muscle.

References

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