Modulation of *in vitro* myogenesis induced by different polymer substrates

O. PETILLO, S. MARGARUCCI, G. PELUSO*
Institute of Proteins Biochemistry and Enzymology, C.N.R., Via Toiano 6, 80072 Arco Felice, Naples, Italy

A. BARBARISI
Institute for Clinical Surgery, II University of Naples, Naples, Italy

M. A. B. MELONE
2nd Division of Neurology, School of Medicine, II University of Naples, Naples, Italy

L. AMBROSIO, L. NICOLAIS
Department of Materials and Production Engineering, University of Naples, Italy
E-mail: peluso@peg.ibpe.na.cnr.it

The understanding of substrate dependence of cellular differentiation is important in the surface design of biocompatible artificial devices as well as cell-incorporated tissue engineered devices. In an attempt to understand some of the genetic and epigenetic aspects of the control of cell differentiation in the presence of two different materials, Chronoflex (CH) and plasma treated Chronoflex coated with Hyaluronan (CH-HA), we used primary cultures of human myogenic cells, a model that encompasses cell proliferation, migration, fusion, and differentiation dependent gene activation. By testing both the material samples on the growth of human myoblasts in primary cultures, we demonstrated that both CH and CH-HA substrates were able to support the cell growth since they did not affect cell count and DNA synthesis. On the contrary, the degree of myoblast differentiation, assessed as a function of creatine phosphokinase (CPK) activity on living cells, was completely different on the two biomaterials. Indeed, the amount of CPK increased on CH-HA cultured cells as a result of myotube formation, while CH grown myoblasts remained unfused and displayed no increase on the CPK activity even after 12 days culture. Moreover, the expression level of MyoD and myogenin mRNA, both related to myogenic cell differentiation, appeared extremely low in CH-grown cells, while they were rapidly induced in CH-HA cultured myoblasts.

1. Introduction

The understanding of substrate dependence of cellular differentiation is important in the surface design of biocompatible artificial devices as well as cell-incorporated tissue engineered devices [1, 2]. For example, tissue engineering of skeletal muscle on biomaterial scaffolding requires artificial substrates able to support not only an initial adhesion, spreading and proliferation of myoblasts, but also fusion and differentiation of individual myoblasts into myotubes as well as alignment of myotubes and subsequent maturation into terminal myofibers [3]. The successful generation of differentiated muscle tissue *in vitro* and *in vivo* is under strict control of tissue-specific gene expression. Indeed, the myogenesis can be defined as the transition from myoblasts to the myotube stage through three main events: the cessation of DNA synthesis in the G1 phase of the cell cycle, the coordinate expression of a battery of muscle genes and the fusion process. Indeed myoblasts, the precursors of skeletal muscle fibers, must express muscle specific regulatory factors of the MyoD family to differentiate into multinucleated muscle fibers [4]. These factors belong to the family of basic helix–loop–helix DNA binding proteins and can act as transactivators of muscle genes [5].

In an attempt to understand some of the genetic and epigenetic aspects of the control of myogenesis in the presence of biomaterials, we used primary cultures of human myogenic cells, a model that encompasses cell proliferation, migration, fusion, and differentiation dependent gene activation. For this purpose two complementary strategies were used: sub-cloning and the manipulation of the culture conditions. By these assays we have defined the role of artificial substrates

*Author to whom correspondence should be addressed.

such as Chronoflex (CH) and plasma treated Chronoflex coated with Hyaluronic acid (CH-HA) on myoblast proliferation and differentiation.

Finally, the characterization of compatible materials able to induce a generation of skeletal muscle tissue *in vitro* will be beneficial for reconstructive surgery and will allow for more effective treatment of muscular disorders.

2. Materials and methods

2.1. Materials used

The materials used were films of aliphatic polycarbonate polyurethane Chronoflex AL 80 A, (CH) supplied by Polymedica Inc., and plasma treated Chronoflex coated with hyaluronan (CH-HA). The polymer was coated as follows: amino groups were introduced on the substrate surface using a proprietary plasma (glow discharge) treatment. Covalent linking of the hyaluronan to the substrate surface was obtained by the reaction of the polysaccharide carboxyl groups with the amino groups of the substrate [6].

For the study, all samples were cylindrically shaped (15.5 cm in diameter, 3 mm thickness) and were sterilized by antibiotics for 2 h before experiment.

2.2. Cell culture model

Muscle samples were obtained from healthy donors ($n = 10$) undergoing corrective orthopaedic surgery. Fragments smaller than $3\,mm^3$ were taken from the muscular area being surgically operated on, excess connective tissue was removed and the muscle fragments seeded onto Falcon dish in a small volume of Dulbecco's modified Eagle's medium (DMEM) with a high concentration (40–50%) of fetal calf serum (FCS). Once the fragments become adherent to the plastic surface, the first outgrowth of fibroblasts usually followed. When the cells covered at least 50% of the growth surface, the explants were picked off from the center of the outgrowth, minced to obtain fragments smaller than $1\,mm^3$ and transferred to fresh 60-mm plates, coated with either 0.01% human type I collagen or 1% gelatin. Myoblasts were allowed to grow until 60–70% (~ 3 weeks) in DMEM nutrient mixture supplemented with $3.7\,g\,l^{-1}$ sodium bicarbonate, 20% FCS, 2 mM glutamine, 0.5% chick embryo extract (CEE), $100\,U\,ml^{-1}$ penicillin, $100\,\mu g\,ml^{-1}$ streptomycin, and $0.25\,\mu g\,ml^{-1}$ amphotericin B. The 20% FCS contained 2 nM insulin and 5 mM glucose. To subculture, cells were trypsinized and passaged at a density of 1×10^4 cells in 35-mm coated culture plates. The primary monolayer was designated the first cell population doubling.

For all experiments the myogenic cells were plated at a density of $5 \times 10^4\,ml^{-1}$ onto discs of Chronoflex and Chronoflex-HA plasma treated material that were placed in 24-multi-well plates of the same internal diameter as that of the discs, completely covering the bottom of the wells. All multi-well plates were incubated at 37 °C in 5% CO_2/air for the indicated time. Controls were performed using plastic alone.

2.3. Cytofluorimetry

Cultures obtained from muscle explants were washed three times with warm medium, drained well, and incubated with 5 ml of 0.6 mM ethylenediamine tetra-acetic acid (EDTA) (1/5000 Versene) at 37 °C for 13–15 min. The adherent cells were then vigorously washed off the plates, washed in medium, and resuspended to 10^6cells ml^{-1} in cold phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumin (PBS–BSA) for fluorecence-activated cell sortina (FACS) analysis, as previously described [7], with some modifications. Briefly, cells were examined in a Becton Dickinson cytofluorograph using the 488-nm emission of an argon laser; to determine the gating parameters for the final sorting procedure, an aliquot of the cell suspension was analyzed as regards the forward light scatter (size), perpendicular light scatter (nucleocytoplasmic ratio), and propidium iodide staining (dead cells). Live cells were those without bright propidium iodide staining and with sufficient forward scatter to exclude remaining cellular debris. Thereafter, the viable cells were displayed on a graph combining forward versus perpendicular light scatter. Isodensity lines of the contour plot defined two populations of cells, small cells with a lower nucleocytoplasmic ratio, and large cells with a higher nucleocytoplasmic ratio.

2.4. Immunofluorescent labeling

In order to determine whether one of these two populations of cells, small and large, might correspond to muscle satellite cells (SCs), 25 µl of fixed and permeabilized cells and 10 µl of fluoroscein isothiocyanate (FITC)-conjugated human monoclonal antibody Leu19 (MoAb-Leu19) was incubated for 30 min at 4 °C, washed three times with PBS–BSA at 4 °C and resuspended in 100 µl of the same buffer. The specificity of Leu19 for an antigen of SCs, which are the stem cells of muscle regeneration, has been already described [8].

The fluorescence was calibrated to standard sensitivity using fluorescent polystyrene microspheres. A flow rate of 1000 cells s^{-1} was optimal for our purposes. Control cell samples not stained with specific antibody allowed determination of the proper FACScan gates to distinguish positive from negative cells in each staining procedure. The percentages of fluorescence-positive cells and the mean fluorescence and scatter values of the fluorescence-positive cells were determined using Lysis II software. The proportion of each cell type obtained was determined by integration over selected regions of the multiparameter data space. We concluded, from this method, that small cells corresponded to SCs, whereas large cells were fibroblasts, according to the data of Baroffio *et al.* [9].

2.5. [^{3}H]thymidine ([^{3}H]Thd) incorporation

One milliliter of quiescent cell suspension (5×10^4 cells) was seeded onto discs of CH and CH-HA materials placed in a 24-well microtest plate, and followed by incubation at 37 °C. During the 24 h culture period cells were exposed to 1 µCi ^{3}H-Thd ($2\,Ci\,mmol^{-1}$ Amersham) by 2 h pulses. Finally, cells were harvested and washed on glass fiber filters and radioactivity determined.

2.6. DNA content by flow cytometry

To confirm results of [^{3}H]Thd uptake, DNA content per cell was assessed by flow cytometric measurement of propidium iodide (PI) fluorescence. Fraction of cells in S phase was then estimated based on DNA content [10]. Cells were passaged and harvested as described above for growth assays. At a cell density approximating the 80–85%, quiescence was induced ($=$ day -2), by serum starvation (0.5% FCS). At day 0, medium was changed to 20% FCS. Treated cells were placed into test tubes at a concentration of 5×10^5 cells ml^{-1}. Chicken erythrocytes (CRBC) were added in amounts equal to 20% of the tested cells as internal biological standards. Samples were centrifuged at 200 ***g*** for 5 min and the cell pellets resuspended in PBS, gently vortexed and washed twice. Finally, 1 ml of staining solution, (0.01 M Trisma base, 700 units l^{-1} RNAse, 0.1% Nonidet P40, 10 mM NaCl and 7.5×10^{-5} M PI), was added per test tube and vortexed gently. After 30 min incubation in ice water, the nuclear suspensions were fixed by addition of 100 µl of 2% paraformaldehyde per test tube, vortexed, and stored in the dark at 4 °C overnight until flow cytometric analysis. The CRBC also served as a threshold for acceptable fluorescence intensity; events having less fluorescence than CRBC nuclei were considered debris. In all samples, a total of 10 000–20 000 nuclei were recorded and results were analyzed using the LYSYS software.

2.7. Determination of myogenic cell fusion rate

Fusion of myogenic cells was assessed by the formation of plurinucleated myotubes and expressed as the number of nuclei included in each myotube per square centimeter. Entire dish area was counted from day 4 to 8 and only half the dish area for day 10. So, at least 2000 nuclei from myotubes were counted at each determination at day 10.

2.8. Determination of biochemical myogenic cell differentiation

Myogenic cell differentiation was assessed biochemically by determination of muscle creatine kinase (CK) activity in cells collected and sonicated in 15 mM NaCl and 1 mM EDTA, pH 7.5. CK activity was determined with CK *N*-acetylcystein-activated monotest.

2.9. RNA extraction and semiquantitative–polymerase chain reaction (RT–PCR)

Total RNA was extracted from the cell cultures using RNAzol reagent according to the protocol of the manufacturer. The mRNA sequences of MyoD, and Myogenin retrieved from the databank ''Lasergene'' (DNAstar Inc., Ohio, USA), were used to select primer pairs for RT–PCR amplification, utilizing OLIGO.4 software. The mRNA levels of the genes under analysis were then measured by RT–PCR amplification. Appropriate regions of hypoxanthine guanosine phosphoribosyl transferase (HPRT) were used as control of RT–PCR experiments. Amplifications, carried out for 28–30 cycles, were as follows: 94 °C for 1 min, 57 ßC for 1 min and 72 °C for 1 min. Each RT–PCR experiment was repeated at least three times. Amplification products were electrophoresed on 2% agarose gel in $1 \times$ TAE buffer. Semiquantitative analysis of mRNA levels was carried out by ''GEL DOC 1000 UV FLUORESCENT GEL DOCUMENTATION SYSTEM'' (Biorad Company).

2.10. Scanning electron microscopy (SEM)

Samples were fixed for 15 min with 2.5% (v/v) gluteraldehyde in 0.1 M cacodylate for 10 min. Samples were then dehydrated and dried using critical point CO_2 method and finally sputter-coated with gold–palladium, before observation with a Hitachi microscope.

3. Results and discussion

The three main developmental steps of human satellite cells in culture are proliferation (day 4 to day 6), alignment of the cells (day 8) and fusion, i.e. formation of plurinucleated myotubes (about day 10). To standardize results from many cultures (at least three for each experiment) we assigned the value 100 for the control at day 10 for proliferation, morphological and biochemical differentiation.

Testing both the material samples on the growth of human myoblasts in primary cultures, in order to assess their effects on DNA synthesis and on cellular proliferation, we demonstrated that: (1) CH and CH-HA did not negatively affect myoblast proliferation and DNA synthesis; and (2) compared with plastic control both materials seem to be more powerful in stimulating G1 to S-phase passage, and DNA synthesis, being more efficacious for completion of mitosis, so as in anticipating the plateau phase of cells (Fig. 1a, b).

When grown on CH-HA the myoblast cells have a high tendency to form myotubes, after 6 days of culture whereas fusion could be partially detected on CH, where percentage of fusion varied between 10 and 20%

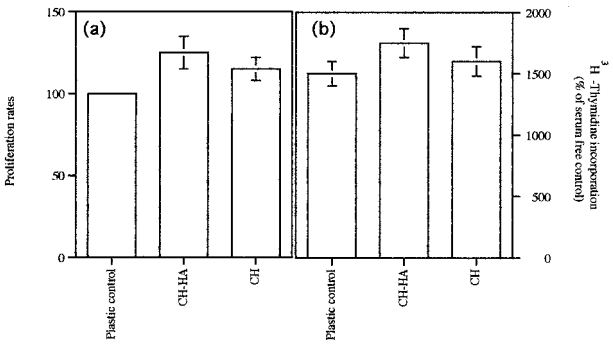

Figure 1 (a) Proliferation rates observed at day 4 of culture after seeding on the different artificial substrates. Results are expressed as a percentage (mean $\pm$ SEM) of the result in controls (plastic material). (b) [^{3}H]Thymidine incorporation into myoblast cells cultured on different materials. [^{3}H]Thymidine was added 2 h before harvesting. [^{3}H]Thymidine incorporation in unstimulated myoblasts was 325 ± 25 d.p.m. Data are expressed as the mean $\pm$ SEM (each point in quadruplicate).

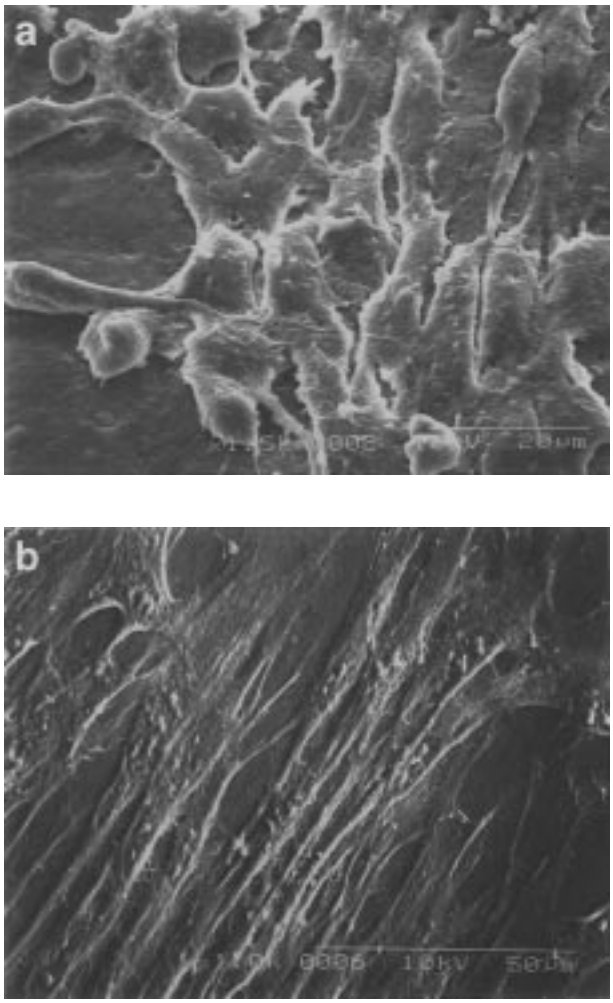

Figure 2 Morphological characteristics of CH (a) and CH-HA (b) grown myoblasts at day 12 of culture

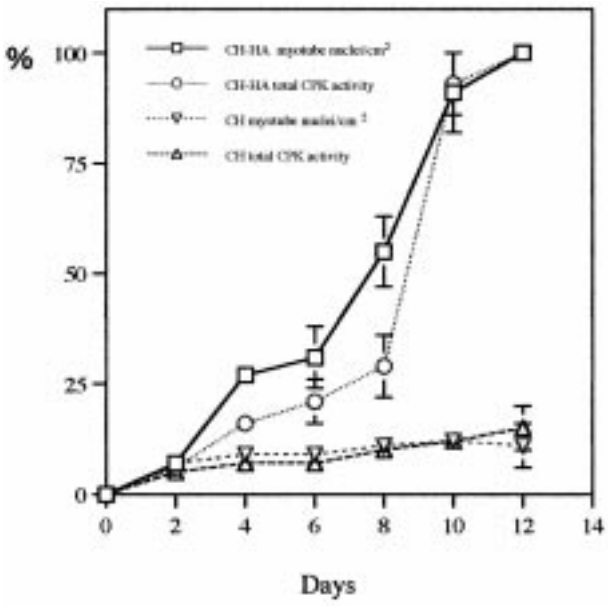

Figure 3 Rates of fusion (myotube nuclei/cm^2) and total CPK activity in human muscle cells cultured on different artificial substrates. Results are expressed as a percentage (mean $\pm$ SEM) of the result in controls (at least nine parameters) at day 12 (100%).

(Fig. 2a, (b)). Since differentiated myoblast cells possess a high level of CPK activity (biochemical differentiation), the degree of differentiation was at first assessed as a function of CPK activity on living cells during growth on either CH or CH-HA

Until day 4, cells proliferated at the same rate on both artificial substrates, and no significant level of CPK was observed. Between day 4 and day 6 the level of CPK increased 30-fold on CH-HA, whereas no increase could be observed with cells grown on CH surface. At day 8 after seeding, the amount of CPK had again increased on CH-HA-cultured cells as a result of myotube formation, while CH-grown myoblasts remained unfused and displayed no increase in the CPK activity even after 12 days (Fig. 3).

Hence, it is clear that in human myoblasts the expression of the muscle-specific biochemical program is greatly influenced by the artificial substrates. Moreover, HA seems to exert some positive influence on the differentiation of human myoblast cells, as estimated by the maximum level of CPK activity reached in comparison with the plastic control.

After 6 days growth on CH, cells were labeled for 2 days with [^{3}H] thymidine. Our data indicate that less than 1% of the cells passed through S phase during this time. By labeling nuclei with PI and analyzing DNA content by flow cytofluorimetry we determined that about 90% of the nuclei of myoblasts were in the G1 phase. We have also shown that one doubling of cell population occurs 24–26 h after replating the CH grown myoblasts on CH-HA materials. These results indicate that, following their 6 days growth on CH, the myoblasts are stopped in G1, but are not irreversibly withdrawn from the cell cycle. Following this arrest, myoblast cells neither fused nor activated the synthesis of muscle-specific proteins.

This confirms other findings with mouse myoblasts that even a prolonged arrest in G1 is not sufficient to trigger terminal myoblast differentiation, although the probability that a myoblast will differentiate increases with the duration of the G1 phase. Thus, one must assume that the events that are essential for terminal differentiation do not occur when myoblast cells are grown on CH.

We also studied the expression levels of MyoD and myogenin mRNA in the myoblast cells cultured on CH or CH-HA substrates (Fig. 4). In actively proliferating myoblasts, MyoD transcript was not detectable while its level in confluent cells cultured on CH-HA was about seven-to nine fold over that in exponentially growing myoblasts.

Three days after the induction of myogenesis, the expression level of MyoD transcript in CH-HA cultured cells was maximal while it slowly peaked in CH grown myoblasts, reaching only 25% of the level of the control cells.

Again, we examined in our cell cultures the myogenin mRNA level that appeared extremely low in each cell culture during myoblast proliferation before differentiation. In CH-HA cultured cells, myogenin transcript were rapidly induced and reached maximal level at the third day after the induction of differentiation. In contrast,

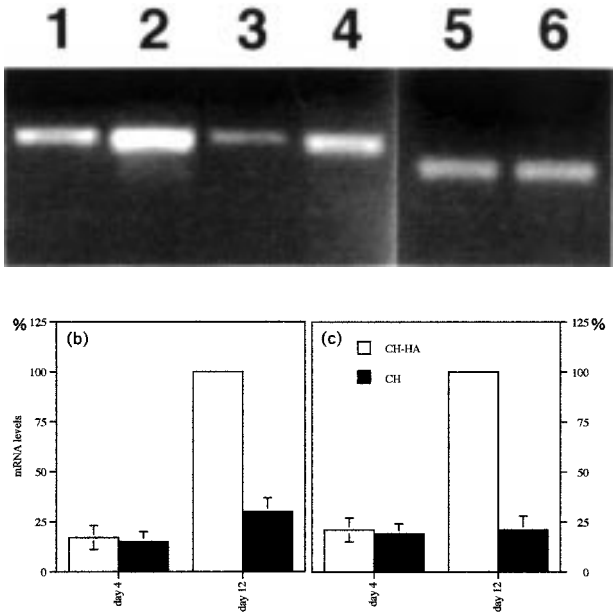

Figure 4 (a) Agrose gel electrophoresis analysis (a) of RT-PCR products in myoblasts cultured on different artificial substrates after 3 days from the begining of differation: Lane 1: CH MyoD mRNA; Lane 2: CH-HA MyoD mRNA; Lane 3: CH Myogenin mRNA; Lane 4: CH-HA Myogenin mRNA; Lane 5: CH HPRT mRNA; Lane 6: CH-HA HPRT mRNA. (b) MyoD/HPRT and (c) Myogenin/HPRT mRNAs ratio in CH or CH-HA grown myoblasts at day 4 and 12 of culture. Results are compared to the data found in plastic control cultured cells evaluated at day 12 (100%) for both MyoD and myogenin

myogenin mRNA was practically at the basal level in CH cultured myoblasts at all times evaluated.

To determine whether CH or CH-HA could modify mRNA stability in myoblasts, the HTPR gene transcript was studied. These results demonstrated that the HTPR mRNA level was not modified by culture conditions, thus suggesting that the decrease in MyoD and myogenin transcript levels in CH-cultured myoblasts were the consequence of changes in their transcription rates.

Next, a time-course analyses of muscle gene expression was performed, starting from homogeneous populations of CH grown cells arrested in G1, which were further stimulated to differentiate by culturing on CH-HA.

After culturing for 6 days on CH, myoblasts did not accumulate muscle transcripts typical of the differentiated state such as MyoD and myogenin. Accumulation of these transcripts was first detected 24 h after the replating on CH-HA substrate, then increasing rapidly between 24 h and 48 h to reach a plateau.

Because myogenic differentiation is often associated with the transfer of the cells from a highly to a poorly mitogenic medium, as is the case in experiments where the cells were transferred from a 20% FCS-containing medium into a medium containing early 2% FCS, we analyzed consequences of such treatment in cultures grown on CH-substrate. Interestingly, this shift from high (20%) to low (2%) FCS containing medium did not correlate with the increase of MyoD whose transcripts remained undetectable.

Human myoblasts exhibit a high tendency to differentiate when cultured on control plastic. In order to study the effect of other artificial substrates on the transition from myoblast to myotube, we analyzed materials, such as CH and CH-HA with different physicochemical surface characteristics, for their ability to affect the myogenesis. Moreover, the presence of HA on the substrate provided high hydrophilicity and minimize non-specific cellular adhesion [11].

Among the two materials, CH, while permitting extensive proliferation of cells, proved to be the most adverse for differentiation; indeed when cultured on CH myoblast cells did not express the myogenic program. Moreover, myoblasts regain the ability to fuse where replated on CH-HA or on control plastic. Thus, not only do complex interactions occur between cells and artificial substrates, but these interactions are able to modulate the myoblast behavior.

The mechanism by which HA acts to promote myogenesis remains to be elucidated. The fact that in the human fetus HA is widely distributed in distinct developing organs, including skeletal muscle, indicates that HA may well participate in myogenesis *in vivo* [12, 13]. Furthermore, epigenetic control of myogenic differentiation has been postulated in the case of *in vitro* cultured myoblast cells derived from muscle at various stages of embryo development [14].

4. Conclusions

In light of the differences that human myoblasts exhibit in growth on different artificial substrates, human myoblast cultures would be useful for a more complete biocompatibility evaluation of artificial materials, by characterizing those regulatory events, such as appearance of transcription factors, changes in the chromatin conformation, and modifications of the pattern of DNA methylation, involved in the control of muscle-cell differentiation [15]. More precisely, it will be of interest to determine whether biomaterials, which, according to previous criteria, induce the differentiation of human cells, are also able to promote the *in vivo* integration of prostheses in specific tissue sites of implantation.

References

1. B. D. RATNER, ''Comprehensive polymer science'' (Pergamon, Oxford, 1989) p. 201.
2. N. ZIATS, K. MILLER and J. ANDERSON, *Biomaterials* **6** (1988) 396.
3. W. D. STEIN and F. BROWNER, ''Cell shape: determinants, resultation and regulation role'' (Academic Press, New York, 1989).
4. E. N. OLSON, *Dev Biol.* **154** (1992) 261.
5. C. P. EMERSON, *Curr. Opin. Genet. Dev.* **3** (1993) 265.
6. O. PETILLO, A. BARBARISI, S. MARGARUCCI, M. MORRA, L. AMBROSIO, L. NICOLAIS in Proceedings of 13th European Conference on Biomaterials, Goteborg, Sweden, 4–7 September 1997, p. 93.
7. C. WEBSTER, G. K. PAVLATH and D. R. PARKS, *Exp. Cell Res.* **174** (1988) 252.
8. W. SCHUBERT, K. ZIMMERMANN and M. CRAMER, *Proc. Natl Acad. Sci. USA* **86** (1989) 307.
9. A. BAROFFIO, J. P. AUBRY and A. KAELIN, *Muscle & Nerve* **16** (1993) 495.
10. J. W. GRAY, P. N. DEAN and J. C. FUSCOE, *Science* **238** (1987) 323.
11. D. CAMPOCCIA, J. A. HUNT, P. J. DOHERTY, S. P. ZHONG, M. O'REGAN, L. BENEDETTI and D. F. WILLIAMS, *Biomaterials* **17** (1996) 963.

12. S. RAGANATHAN and K. DAHA, *Cell Biol. Int.* **19** (1995) 161.
13. B. BRAND-SABERI, A. J. GAMEL, V. KRENN, T. S. MULLER, J. WILTING and B. CHRIST, *Dev. Biol.* **178** (1996) 160.
14. N. K. WHITE, P. H. BONNER, D. R. NELSON and S. D. HAUSCHKA, *ibid.* **44** (1975) 346.
15. S. SCARPA, M. LUCARELLI, F. PALITTI, D. CAROTTI and R. STROM, *Cell Growth Differ.* **7** (1996) 1051.

Received 1 December 1998
and accepted 9 April 1999