Use of rhBMP-2 Activated Chitosan Films To Improve Osseointegration

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Received November 10, 2005; Revised Manuscript Received January 12, 2006

Considering the design and development of biomaterials used in tissue engineering, not only is it important that they are biocompatible but also that they induce the desired cellular response for tissue regeneration. Chitosan, a biocompatible and bioresorbable polymer of *N*-acetylglucosamine and glucosamine is used in our work combined with recombinant human BMP-2 (rhBMP-2), a potentially useful activation factor for bone repair. In this way, we try to combine the biological and filmogenic properties of this biopolymer with the osseoinductive ability of the rhBMP-2. Results showed that the chitosan films employed, without and with rhBMP-2 activation, are able to support cellular growth and proliferation on them and that only the rhBMP-2 activated ones are able to differentiate from a myoblastic mouse cell line (C2C12) toward osteoblastic phenotype. Osseoinduction properties of rhBMP-2 activated films persist for a long storage time. The in vivo experiments performed confirm the expectative created by the in vitro results obtained and are an indication that rhBMP-2 activated chitosan films could be a very attractive biomaterial for the enhancement of osseointegration of surgical prostheses and implants and for the purpose of tissue engineering bone regeneration.

Introduction

Chitosan, a copolymer of *N*-acetylglucosamine and glucosamine obtained from chitin through its deacetylation, is now well-known for its numerous and interesting biological properties as a biocompatible, bioresorbable, and bioactive polymer.[1−4] Some chemically modified chitosans have been described as osseoinductive materials.[5,6] Nevertheless, this kind of activity for the unmodified chitosan has not been described.

Unmodified chitosan has excellent filmogenic properties; its biodegradation kinetics is slower than that showed by other biocompatible polymers (i.e., collagen), due to the fact that there is not any mammalian known specific enzymatic activity directed toward its degradation, like chitosanases found in bacteria, fungi, and plants.[7,8] It has also great chelating and adsorption ability of different chemicals and biological compounds, mainly due to its cationic nature. All of these properties make this biomaterial a very attractive carrier for bioactive molecules for some applications in tissue engineering.[9]

To promote not only the initial cellular adhesion but also the subsequent cell proliferation is an important consideration for the design and development of biomaterials used for tissue regeneration.[10−13]

On the other hand, bone morphogenetic proteins (BMPs), a group of proteins that are members of the transforming growth factor β (TGF-β) superfamily, have been identified as cytokines that induce ectopic bone formation.[14−18] Bone morphogenetic protein-2 (BMP-2) is one of the proteins originally identified as a factor present in bone extract that triggers ectopic bone formation at nonskeletal sites in vivo.[19,20] Recombinant human BMP-2 (rhBMP-2) is potentially a useful activation factor for bone repair, when combined with a matrix like collagen or decalcified bone.[21,22]

The above-mentioned filmogenic properties make chitosan a suitable biodegradable biopolymer for covering complex surfaces, like those occurring in dental or surgical implants, and the incorporation in such a transient cover of true osseoinduction factors such as BMPs could speed up the osseointegration of the implanted device. This is the main objective of the work performed in our laboratory.

Nevertheless, we have to face several main requirements in the development of an osseoinductive biodegradable chitosan surface for clinical implants. First, the cells not only have to be able to adhere to the chitosan film, but also the film has to promote, or at least to permit, its proliferation on it. If this is the case, the rhBMP-2 target cells initially adhered to the films can be differentiated toward osteoblastic lineage and later proliferate on the biodegradable film surface. Second, the rhBMP-2 adsorbed on the film surface shall remain active on it and should not be inactivated by the adsorption process. Finally, for a true practical utility of this kind of bioactivated chitosan films, the protein should remain active on the film after a long-term storage.

To evaluate the chitosan films performance with respect to the two last requirements, it is a prerequisite to have chitosan films than fulfill the first requirement outlined, i.e., chitosan films with good cellular proliferation characteristics. To date, the studies performed on chitosan films as a biomaterial have

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10.1021/bm050859e CCC: $33.50
Published on Web 02/15/2006

focused on the in vitro investigation of the role of the acetylation degree on some biological properties of chitosan films toward different cell lines,[23,24] but published results suggest also that the cellular proliferation depends on the cell line used.[23,25] Bearing in mind these previous results, we developed different chemical approaches that would not only promote the cell attachment but also cell proliferation.[26]

In this work, we chose C2C12 (muscle myoblast from mouse) cell line to evaluate the influence of the chitosan film on the viability, adherence, and cellular proliferation: It is a multipotent cell line able to differentiate toward different phenotypes under the action of some chemical or biological factors. In particular, this cell line is used for the testing of rhBMP-2 in vitro activity, through the appearance of alkaline phosphatase activity in this cell line concomitant with its evolution toward osteoblastic lineage. It is noteworthy to say that the ability to check in vitro the efficiency of chitosan films as BMP carriers relay on the development of such a kind of film with good adherence and proliferation characteristics for this cell type.

The present work focused on the evaluation of the osseoinductive properties induced by the incorporation of rhBMP-2 in such kind of films using the cell line C2C12. Finally, also other important goals were to perform a preliminary evaluation of the stability of the bioactivated films upon different storage strategies and to test the in vivo performance of the developed bioactivated films.

Materials and Methods

Preparation of Chitin and Chitosan. Chitin was isolated from shrimps (*Pleoticus mülleri*). The material was homogenized, and the product was rinsed in order to remove the organic material. It was then treated with 9% (w/w) NaOH at 65 °C for 90 min to remove proteins and demineralized by treatment with 10% (v/v) HCl at 20 °C for 15 min. After this, it was washed and finally dried. Chitosan was directly prepared by heterogeneous deacetylation of chitin at 136 °C with 50% (w/w) NaOH for 1 h, being afterward extensively washed.

Characterization of Chitosan. We characterized the chitosan using the ASTM F2103−01 standard for the different determinations performed in this study.

Degree of Acetylation. It was determined by NMR spectroscopy. ^{1}H NMR measurements were performed on a AMX500 Bruker NMR spectrometer under a static magnetic field of 500.13 MHZ at 70 °C. A 10 mg sample was introduced into a 5 mm ϕ NMR test tube, to which 0.5 mL of 2% (w/w) DCl/D_2O solution was added, and finally, the tube was kept at 70 °C to dissolve the polymer in solution.[27]

Molecular Weight (M_v). It was performed by viscosity analysis of a chitosan solution.[28] The solvent system used was 0.3 M AcOH/0.2 M AcONa. The values for the Mark−Houwink equation "*K*" and "*a*" constants were 1.28×10^{-2} and 0.85, respectively. Viscosity measurements were made on an Ubbelohde viscometer (Schott Geräte TYP 52520/II).

Water Content. It was performed using a gravimetric technique. Calibrated drying oven at 105 °C.

Ash Content. For this determination it was used a combustion temperature of 800 °C for 6 h.

The molecular characteristics of the chitosan obtained for our work were acetylation degree 16.0 ± 0.7%, water content 5.3 ± 0.50%, ash content 0.39 ± 0.10%, and viscosity average molecular weight 56 100 g/mol.

Chitosan Films. A 1% (w/v) chitosan solution (1% acetic acid), prepared allowing for the content of water and ash, was filtered through a sterile 0.22 μm filter. 200 μL aliquots were layered over each one of 36 wells of a 48 well plate tissue culture treated (1 cm^2/well cellular surface growth, Corning Incorporated Costar). The remaining 12 wells on each plate were left untreated as internal plastic control surface for each plate. For the chitosan treated wells, the solvent was allowed to evaporate in the open plate in a sterile laminar flow hood overnight, at room temperature. Before completing the cells seeding, films were treated in several ways to stabilize them. Treatment A: buffer phosphate 0.25 M pH 7.0. Treatment B: sodium hydroxide 0.5 M. 400 μL of each solution were layered over the wells and allowed to remain on it for 30 min. Afterward, medium was removed and films were extensively washed with PBS.[26]

Scanning Electron Microscopy. A JEOL JSM-35 CF scanning electron microscope was used to characterize the mechanical stability of chitosan film coated implant versus the insertion and removal processes. The samples were prepared by gold coating using a sputter coater, Pelco 91000.

Bone Morphogenetic Protein-2 (BMP-2). Recombinant human BMP-2 (rhBMP-2) was obtained through genetic engineering techniques using BL21 (DE3), an *E. coli* bacterial expression system. The protein obtained was purified and refolded as has been previously described.[29]

Film Activation. An rhBMP-2 solution was layered overnight at 4 °C over the film surface. Protein retention was calculated through the protein concentration difference before and after the solution film contact, using the Bradford method. The rhBMP-2 activated films used in this work had approximately 8−10 μg of rhBMP-2 incorporated on the 1 cm^2 well film surface.

Stability of Bioactivated Films. To check the bioactivity stability of the retained protein, some rhBMP-2 activated plates were stored under different conditions (cold room (4 °C), room temperature, freeze-dried and later stored at 4 °C) during four months previous to the cell seeding and assay of the osseoinductive ability.

Cell Culture. C2C12 cell line (muscle myoblast, mouse, CRL 1772) was obtained from the American Type Culture Collection (Manassas, Virginia). Routine passaging of the cell line was performed on 25 cm^2 flasks with DMEM high in glucose, containing 10% fetal bovine serum plus antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin sulfate). Before the experiment, cells were grown in 175 cm^2 flasks to obtain enough cell quantity for all the seeded plates.

Experimental cell cultures were performed on 48-well plates with the chitosan films on it that had been previously described. Trypsinized cells were seeded in triplicateat a cellular density of 10 000 cells/cm^2 for all of the different surfaces on each plate. Afterward, 400 μL of pre-warmed complete culture medium was added. 400 μL of the complete culture medium was also added to the triplicate blank wells present in the plate for each surface type.

In the case of the experiments performed in order to check the rhBMP-2 activity, the chitosan films were rhBMP-2 activated as described in Film Activation, and the complete culture medium added to the cells seeded on the control cell culture activated plastic surface was supplemented with 10 μg of rhBMP-2 (the maximum amount adsorbed on the chitosan films). Growth medium change was performed at day 3 and was also supplemented with 10 μg of rhBMP-2 for the control plastic seeded cells into the case of rhBMP-2 activity experiments. In any case, rhBMP-2 was added to the complete medium of the cells seeded on chitosan films surfaces.

Cellular Assays. Total cellular protein content was measured using the Bradford BioRad microassay. Typically, the culture medium from 48-well plate was removed and wells were carefully washed twice with phosphate buffer saline (PBS) in order to eliminate the possible interference of FBS protein. 200 μL of Bradford reagent and 800 μL of distilled water were added, mixed, and incubated at room temperature for 30 min. Colorimetric measurements were done at 590 nm in a Microplate Reader (Biotek FL-600). Blank readouts were subtracted from the measured absorbance.

Viability assays were performed in order to test two different cellular activities: unspecific esterase activity of the intact cell with Calcein AM (Molecular Probes) and mitochondrial activity through MTT assay (Sigma).

Calcein AM Assay. The medium from 48-well plate cultures was removed and added 100 μL of 2 μM Calcein AM (in PBS). Plates

Table 1. Results Obtained for the Different Assays Performed on Cultured C2C12 Cells over Distinct Surfaces without rhBMP-2 Activation[a]

assay	surface	day 1	day 2	day 3	day 4	day 5	day 6	day 7
Bradford	A	3.358 ± 0.415	4.961 ± 0.201	10.670 ± 0.526	6.165 ± 0.897	22.6587 ± 0.310	15.070 ± 1.068	19.487 ± 1.118
micro	B	2.171 ± 0.714	5.710 ± 1.378	8.035 ± 0.493	3.314 ± 1.216	11.992 ± 0.302	14.606 ± 0.552	17.138 ± 0.646
	control	3.184 ± 0.244	7.561 ± 0.518	22.268 ± 0.303	19.472 ± 0.3827	22.524 ± 0.382	18.578 ± 0.871	3.0257 ± 1.584
calcein AM	A	14609 ± 698	27446 ± 1507	50603 ± 4016	76148 ± 3245	84434 ± 4247	66700 ± 7338	57376 ± 8925
	B	10470 ± 174	18236 ± 1430	35604 ± 8107	52593 ± 6257	42813 ± 2789	31240 ± 2254	28544 ± 3831
	control	15206 ± 222	40184 ± 920	64271 ± 1707	99753 ± 2811	99101 ± 4050	63418 ± 16143	15874 ± 8435
MTT	A	0.108 ± 0.002	0.273 ± 0.017	0.398 ± 0.026	0.507 ± 0.020	0.701 ± 0.084	0.857 ± 0.078	0.695 ± 0.054
	B	0.124 ± 0.016	0.244 ± 0.017	0.326 ± 0.027	0.391 ± 0.022	0.499 ± 0.035	0.636 ± 0.039	0.675 ± 0.007
	control	0.132 ± 0.015	0.328 ± 0.032	0.533 ± 0.021	0.925 ± 0.029	0.941 ± 0.021	0.551 ± 0.008	0.216 ± 0.010

[a] Numbers stand for μg of protein/well (Bradford micro assay). Number of cells (Calcein AM assay) and (DO 570 nm − DO 690 nm) for the MTT assay.

were stored 1 h in the dark and the measurement was taken at 530 nm after exciting at 490 nm.

MTT (in Vitro Toxicology) Assay. reconstituted MTT (dilution 1/10, 40 μL MTT-400 μL medium) was added to the cell culture medium. The plates were returned to the incubator for 2.5 h. After the incubation period, 440 μL MTT solubilization solution was added. Absorbance at 570 nm and the background absorbance at 690 nm were measured in a Microplate Reader (Biotek FL-600).

Films Bioactivity Assay (rhBMP-2 Assay). The activity of the adsorbed protein was evaluated through the colorimetric alkaline phosphatase activity assay of the C2C12 cells seeded on the activated films. This activity is only present in osteoblastic cells, and it appears in this cell line due to the rhBMP-2 induced differentiation toward osseoblastic lineage.

Briefly Described. After the removal of culture medium, plate wells were washed with PBS (200 μL). Afterward, 100 μL/well of lysis buffer (50 mM Tris pH 6.8, 0.1% Triton X-100, 2 mM $MgCl_2$) was added. 10 μL samples were assayed for alkaline phosphatase activity in 96-well plates, using *p*-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol buffer as a substrate in a total volume of 100 μL; after 10 min at 37°C, the reaction was stopped with 100 μL of 0.5 M NaOH and the absorbance was measured at 450 nm on a Microplate Reader (Biotek FL-600).

For all of the assays here described, controls were performed on cells seeded at the same initial cellular densities on plastic cell culture treated wells of the same plate. In these wells, 10 μg of rhBMP-2 were added to the cell culture medium, and the rhBMP-2 doses were refreshed by changing the medium at day 3.

Activated Coated Implants. rhBMP-2 activated chitosan coated implants were fabricated upon the drying of chitosan film on Titanium implants. Coated implants were treated during 30 min with buffer phosphate 0.25 M pH 7.0 and then extensively washed with PBS as has been previously described for the chitosan films treatment. All of the operations were performed in a sterile environment. Afterward, rhBMP-2 (between 80 and 100 μg) was retained on the 1.96 cm^2 film surface.

In Vivo Study. We performed in vivo studies with New Zealand male rabbits that had a mean weight of 3 kg, using normal (as control) and activated coated implants. The rabbits were anesthetized by intramuscular injection of 2% Rompun (1 mL/10 kg, Bayer) and Imalgene 1000 (ketamine 20 mg/kg, Merial). Later, the surgical field (tibiae maesetae of both legs) was shaved and was disinfected. Defects of 3.75 mm diameter on each leg were drilled, and activated rhBMP-2 coated implants and control normal Ti implants were inserted. The rabbits were placed in individual cages with no limitation for feeding and drinking.

After a period of 5 weeks of implantation, the experimental subjects were sacrificed and the removal torque was measured with a torque measurement system (Tohnichi, Japan).

Statistical Analysis. Each experiment was done in triplicate. Results are expressed as means ± SD. Nonlinear regression fitting and ANOVA analysis were performed with Graph Pad Prism software. Upon the Anova analysis, the Bonferroni post-test was also performed, comparing the different treatments with the control (plastic).

Results and Discussion

Cellular Proliferation on Chitosan Films. The results obtained from all assays which characterize the cellular proliferation and viability, and that were performed on two different experimental chitosan surfaces (treatment A or B), were corrected for blank readout (same surface without cells) and compared with those obtained (also corrected) using commercially available multiwell plates (cell culture treated) used as surface controls.

Table 1 shows the time evolution of the total protein cellular content for C2C12 cells. It can be observed that the initial adherence is high on both experimental surfaces and very good for the one corresponding to treatment A, which has the same initial values as those of the plastic control surface.

However, the proliferation rate seems higher in plastic treated for cell culture than the one obtained in the treated chitosan films. Despite this, the final levels obtained were similar with a certain cellular proliferation onset delay compared to the controls. The decrease in the value levels on day four could be attributed to the cell culture medium exchange, which would suggest that the adherent union to the surface may be stronger on tissue culture treated plastic than over the chitosan films. The reduction of cellular proliferation levels seen at day 6−7 for the plastic control can be attributed to the presence of a totally overconfluenced monolayer that seems to be very sensible to the frictional forces of the washing step.

A more quantitative analysis can be obtained through the nonlinear regression fit of the experimental data to an exponential growth model. The results of this fitting, performed with the data obtained until culture day 3, are shown in Table 2, suggesting that the doubling time of C2C12 cell line cultured on the control surface is lower than those obtained for the different chitosan surfaces.

The influence of the washing step on the Bradford results could have caused that the values for cells cultured on chitosan films were underestimated. This can be observed on the light microscopy photographs taken directly in an inverted microscopy for the cultured cells on the different chitosan films, which show an excellent cellular proliferation (Figure 1), comparable with the one obtained on the cell culture activated plastic, which was one of the goals of this study.

The esterase activity determined by the Calcein-AM assay for the cells seeded on the developed chitosan films showed a similar response pattern to that obtained for the Bradford assay (Table 1). The fitting of the data until culture day 4 to the exponential growth model is also shown in Table 2. All of the data fit well to the model with a correlation coefficient greater than 0.98 for all of the surfaces tested. It seems that the viability of the cells, as it is described by this assay, is almost unaffected by the culture on chitosan films, being the doubling time obtained much more similar to those of plastic control.

Table 2. Exponential Growth Model Parameters Obtained from the Fitting of the Different Assays Performed

without rhBMP-2 activation		surface A	surface B	control
total cellular protein content	start	1.486 ± 0.4342	1.694 ± 0.7934	0.9803 ± 0.1580
	K	0.6527 ± 0.1063	0.5288 ± 0.1750	1.040 ± 0.05578
	R^2	0.9815	0.9285	0.9987
	doubling time	1.062	1.311	0.6663
calcein-AM assay	start	10327 ± 1665	7517 ± 1343	13270 ± 2935
	K	0.5041 ± 0.04498	0.5038 ± 0.05238	0.5086 ± 0.06163
	R^2	0.9896	0.9859	0.9813
	doubling time	1.375	1.376	1.363
MTT assay	start	0.119 ± 0.03405	0.1190 ± 0.02778	0.09662 ± 0.01464
	K	0.3884 ± 0.08814	0.3080 ± 0.06989	0.5661 ± 0.04161
	R^2	0.9309	0.9245	0.9933
	doubling time	1.785	2.250	1.224
with rhBMP-2 activation		**surface A**	**surface B**	**control**
total cellular protein content	start	2.478 ± 0.2464	2.246 ± 0.4188	2.885 ± 0.3578
	K	0.5162 ± 0.03728	0.3435 ± 0.07363	0.5625 ± 0.04597
	R^2	0.9961	0.9594	0.9952
	doubling time	1.343	2.018	1.232
calcein-AM assay	start	12408 ± 3192	17020 ± 6035	9084 ± 2032
	K	0.4149 ± 0.07381	0.2205 ± 0.1110	0.5345 ± 0.06192
	R^2	0.9560	0.6948	0.9820
	doubling time	1.671	3.144	1.297
MTT assay	start	0.06051 ± 0.01390	0.07815 ± 0.009695	0.06197 ± 0.01758
	K	0.3518 ± 0.06748	0.2421 ± 0.03838	0.5022 ± 0.07917
	R^2	0.9400	0.9583	0.9685
	doubling time	1.970	2.862	1.380

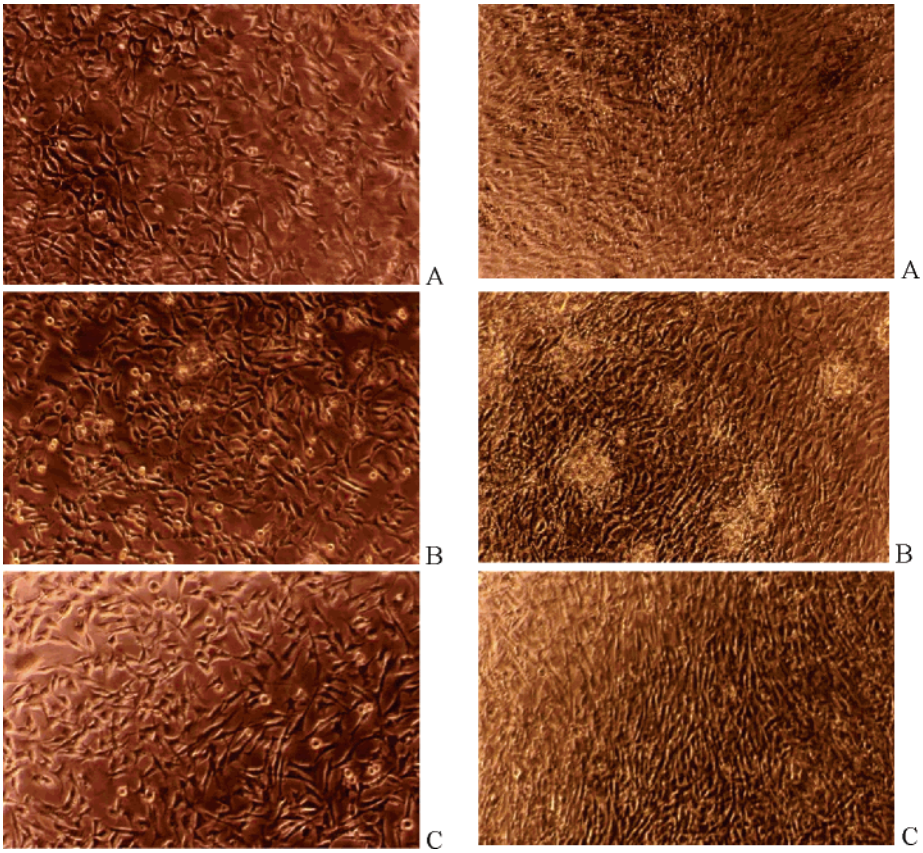

Figure 1. Light microscopy photographs (x10) taken directly over culture plates for C2C12 cell line at day 2 (left side) and 6 (right side). (A) treatment A (buffer phosphate 0.25M, pH 7.0); (B) treatment B (sodium hydroxide 0.5 M); and (C) cell culture treated plastic.

Table 1 also shows the results obtained with the MTT assay as another viability measurement. For chitosan treatment A, almost the same final values are achieved as those obtained for the plastic control with a delay of approximately 1 day.

In the methodology of this assay, it is remarkable that there is not any effect of frictional forces on the cell monolayer. Nevertheless, upon confluence of the plastic control, the mitochondrial activity decreased. The same behavior is observed

Table 3. Results Obtained for the Different Assays Performed on Cultured C2C12 Cells over Distinct Surfaces with rhBMP-2 Activation[a]

assay	surface	day 1	day 2	day 3	day 4	day 5	day 6	day 7
Bradford	A	3.939 ± 0.619	7.211 ± 0.177	11.581 ± 0.342	19.261 ± 1.557	20.259 ± 1.083	14.529 ± 1.416	18.157 ± 0.290
micro	B	3.415 ± 1.123	4.112 ± 2.571	6.419 ± 0.617	16.038 ± 1.705	15.213± 0.200	12.592 ± 0.511	11.051 ± 0.479
	control	4.722 ± 0.130	9.277 ± 0.797	15.487 ± 0.425	22.849 ± 0.287	25.236 ± 0.294	21.520 ± 0.801	0.040 ± 1.248
calcein AM	A	17092 ± 216	25288 ± 343	49472 ± 928	62870 ± 1565	56052 ± 1752	55911 ± 3255	53807 ± 3194
	B	20814 ± 774	21753 ± 2498	41294 ± 1153	37673 ± 5308	32630 ± 551	42901 ± 3360	45421 ± 4378
	control	15950 ± 358	30149 ± 1226	40371 ± 6665	78513 ± 2740	22051 ± 2223	39787 ± 5994	7865 ± 2766
MTT	A	0.094 ± 0.005	0.131 ± 0.017	0.149 ± 0.015	0.257 ± 0.031	0.465 ± 0.092	0.284 ± 0.050	0.274 ± 0.071
	B	0.090 ± 0.004	0.131 ± 0.012	0.173 ± 0.037	0.199 ± 0.032	0.253 ± 0.061	0.202 ± 0.026	0.167 ± 0.054
	control	0.088 ± 0.019	0.148 ± 0.013	0.321 ± 0.036	0.448 ± 0.022	0.666 ± 0.008	0.515 ± 0.018	0.216 ± 0.005

[a] Numbers stand for μg of protein/well (Bradford micro assay). Number of cells (Calcein AM assay) and (DO 570 nm − DO 690 nm) for the MTT assay.

for the cell growth on chitosan films with treatment A, and for the other treatment, it seems that at day 7 the cells are reaching its confluence value, lower than for the other surfaces. Table 2 shows the results of the different fits performed for each one of the cellular properties tested.

If we take into account all of these experimental results, it seems that a proper cellular adhesion is observed on both surfaces used, although the cellular densities appeared to be somehow lower than in commercially available multiwell plates used as surface controls. The levels obtained for cellular proliferation had an onset delay of 1−2 days compared to this plastic control. Among chitosan treatments used, it seems that the one with 0.25 M buffer phosphate pH 7.0 (treatment A) offers the best option for cell growth. The results obtained created the possibility of the activation of this biomaterial with some of the morphogenetic proteins to achieve a potent osseoinductor support for cell growth and differentiation toward osteoblastic lineage.

Effectiveness of rhBMP-2 Activation of Chitosan Films. The experimental approach performed in the study of the rhBMP-2 activated chitosan films has a double goal. First, to check that the rhBMP-2 activation does not affect the film's ability to allow cellular proliferation on it in a significant manner and, second, to evaluate the osseoinductive properties of these bioactivated films through the follow-up of the emergence of alkaline phosphatase activity in the C2C12 seeded cells.

Table 3 shows the results obtained with the total protein assay, a measure of cell proliferation for the films carrying rhBMP-2. The final levels obtained were high and similar, with a certain delay on the onset of cellular proliferation, to those obtained with the controls. The observed trend behavior is very similar to that previously shown for the experiments performed without rhBMP-2 activation. Nevertheless the nonlinear fitting to an exponential growth model (Table 2), performed on the first 3 days culture data, like the one performed on non-rhBMP-2-activated chitosan indicate longer doubling time than those previously reported, also for the cells seeded on the plastic cell culture surface control. This fact could be related to the C2C12 differentiation toward an osteoblastic lineage upon the presence of rhBMP-2 (retained on the chitosan surface or present into the cell culture medium).

Photomicrographs taken directly on these cultures showed a uniform cell monolayer growth over all of the surfaces used in this study, like those previously shown for the non-rhBMP-2-activated chitosan films (data not shown).

The calcein-AM and MTT viability assays (Table 3) for the cells seeded on the developed activated chitosan films showed a similar response pattern to the one shown for the Bradford assay. The results of the fitting to the exponential growth model are also shown in Table 2. The proliferation rate obtained in these experiments is also lower than the ones attained for C2C12 proliferation on chitosan films without rhBMP-2 activation. This proliferation rate is also lower in plastic with rhBMP-2 added (positive controls) than that obtained in the Experimental Section. This fact may be reflecting, as the results obtained for total cellular protein content, a cell differentiation process where there is no proliferation.

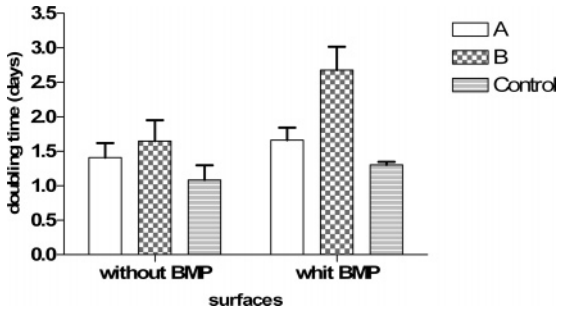

Figure 2. Doubling time for C2C12 cells as a function of the presence or absence of rhBMP-2 activation. Data are obtained from the mean of the doubling time shown in Table 2. (A) treatment A (buffer phosphate 0.25M, pH 7.0), (B) treatment B (sodium hydroxide 0.5 M). Control: cell culture treated plastic.

Table 4. Two-Way ANOVA and Bonferroni Post-Test Performed on the Doubling Time Means Obtained from Table 1

source of variation	% of total variation	*P* value	*P* value summary	significant?
interaction	9.51	0.1908	ns	no
surface	43.56	0.0045	**	yes
bmp	17.03	0.0226	*	yes

Bonferroni post-tests

control vs A

	control	*A*	difference	*t*	*P* value	summary
without BMP	1.084	1.407	0.3229	0.9738	*P* > 0.05	ns
with BMP	1.303	1.661	0.3583	1.081	*P* > 0.05	ns

control vs B

	control	*B*	difference	*t*	*P* value	summary
without BMP	1.084	1.646	0.5612	1.693	*P* > 0.05	ns
with BMP	1.303	2.675	1.372	4.137	*P* < 0.01	**

If we take the different time constants obtained from the fitting of the experimental results for the assay performed on each surface as triplicate values of the same magnitude (Figure 2), we can perform a two-way ANOVA analysis that confirms the difference between nonactivated and rhBMP-2 cells. The results obtained from such analysis support the use of treatment A for the rhBMP-2 activated chitosan films (Table 4).

The increase in the alkaline phosphatase activity of the cell line C2C12, as a consequence of the differentiation toward

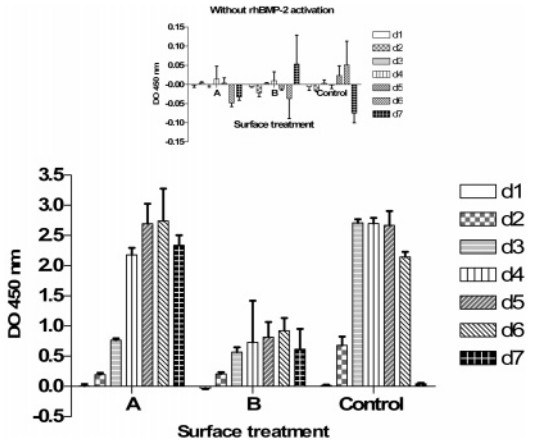

Figure 3. Time course follow up of C2C12 cell line osteoblastic differentiation (alkaline phosphatase activity) induced by rhBMP-2 activated chitosan films. (A) Treatment A (buffer phosphate 0.25M. pH 7.0); (B) treatment B (sodium hydroxide 0.5 M). Control: cell culture treated plastic. Insert graphs shows the results obtained for the same assay without rhBMP-2 activation. d1 to d7 stand for days of cell culture.

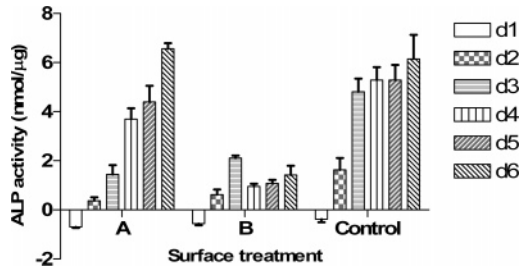

Figure 4. Alkaline phosphatase activity vs culture days for C2C12 cell line performed on rhBMP-2 activated chitosan films. (A) Treatment A (buffer phosphate 0.25M. pH 7.0); (B) treatment B (sodium hydroxide 0.5 M). Control: cell culture treated plastic. d1 to d7 stand for days of cell culture.

osteoblastic lineage due to the rhBMP-2 incorporated on the chitosan film, is an important result shown in Figure 3. This activity cannot be due to any chitosan effect by itself, as it is shown in the insert in the figure, where the results obtained with the nonrhBMP-2-activated chitosan films are plotted.

The activity levels achieved by the cells seeded on the chitosan films activated with rhBMP-2 are similar, in some cases, to those attained for C2C12 cell differentiated upon addition of the recombinant protein to the cell culture medium, used in the plastic controls.

Figure 4 shows the ratio of alkaline phosphatase activity/μg of protein on each well (obtained after dilution of the wells, to do not saturate the reader). It can be observed that this ratio is approximately the same for the plastic control and for the cells seeded on chitosan with treatment A.

The experience illustrated previously opens the possibility of using rhBMP-2 activated chitosan films as a biodegradable material with potent osseoinductive properties, being able to stimulate the cellular differentiation of mesenchymal cells toward osteoblastic lineage. This kind of material could have a possible practical utility as prosthesis or implant biodegradable cover film, enhancing the osseointegration process through its osseoinductive properties.

The rhBMP-2 protein seems to be strongly retained by the chitosan film. Figure 5 shows an indirect experimental evidence of this fact: the 1 dose column bar stands for control alkaline phosphatase levels obtained for C2C12 cells, at culture day six. In this case, rhBMP-2 (10 μg) was added to the cell culture

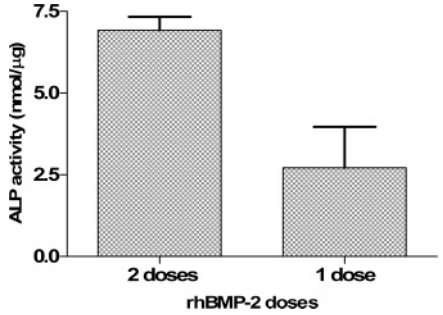

Figure 5. Alkaline phosphatase activity obtained for C2C12 cells at culture day 6 on control surface. Two-dose experiment: a 10 μg rhBMP-2 doses was added at the initial seeding and after the medium removal at day three also. One dose experiment: it was added 10 μg rhBMP-2 only at the initial seeding.

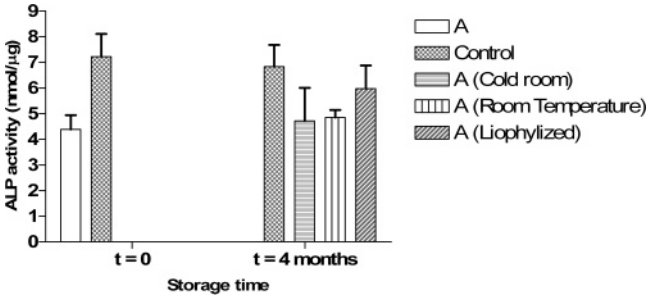

Figure 6. Different storage strategies effect on osseoinductive activity of rhBMP-2 activated chitosan films performed thought alkaline phosphatase activity of C2C12 cell line.

medium at the initial seeding, but it was omitted in the medium exchange performed at day 3. The levels attained for the alkaline phosphatase activity in this case are much lower than those obtained in the column bar corresponding to the normal (two doses) control experiment. In this one, a second 10 μg rhBMP-2 dose was added after the medium removal at day three.

It becomes clear than the lack of rhBMP-2 in the cell culture medium reversed the initial C2C12 differentiation toward osteoblastic lineage, vanishing according to the alkaline phosphatase activity. In the experiments performed over rhBMP-2 activated chitosan films, rhBMP-2 was not added to the cell culture medium. The alkaline phosphatase activity levels for these cells at day 4−6 in Figures 4-5 strongly suggests that rhBMP-2 is retained on the film, even after the total cell culture medium removal performed at day three.

Storage Stability of the Osseoinductive Properties. The osseoinductive properties stability of rhBMP-2 activated chitosan films is shown in Figure 6. The alkaline phosphatase activity attained by C2C12 cells seeded on the bioactivated films, prepared and used immediately for cell seeding (time 0), is compared to the activity of the films stored in several conditions (room temperature, cold room, or freeze-dried and stored in the cold room) for four months before the cell seeding. The previously obtained results made us use only chitosan treatment A to perform this experiment.

The alkaline phosphatase levels reached are substantially the same, independently of the type of storage, suggesting a long-term stability of the bioactivated films. Now, we are extending the stability experiments performed to longer storage time. This is an important result regarding the possibility of a practical use of such a kind of rhBMP-2 activated chitosan films.

Experimental Surgery. Finally, to check the real activity in vivo of this kind of films, we performed some in vivo experiments. In these experiments, the osseointegration of implants was estimated through the measurement of the implants

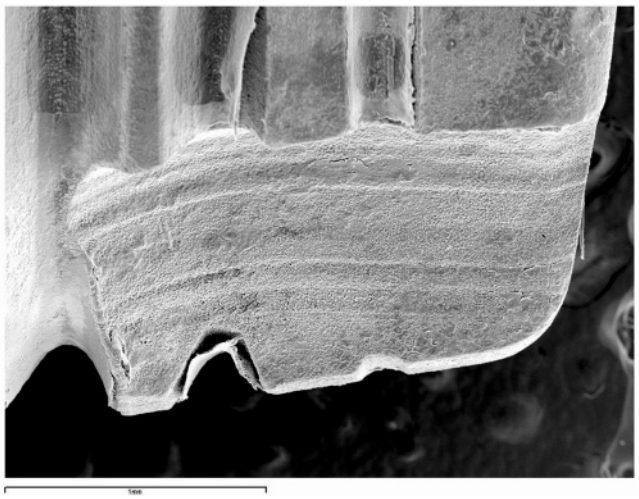

Figure 7. Electronic microscopy photograph of a coated implant after insertion and immediately removal (50X). Chitosan film although broken remain on the implant surface.

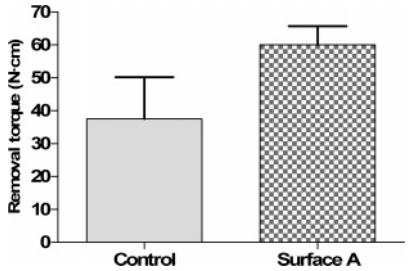

Figure 8. Comparison of removal torque (N·cm) between normal and rhBMP-2 activated coated implants.

removal torque after 5 weeks implantation, comparing the values obtained for normal (control) titanium implants and the same implants covered with the rhBMP-2 activated chitosan films. Figure 7 shows an electronic microscopy photograph of one of these covered implants after insertion and immediate removal. This experience was performed in order to check the film mechanical stability against the insertion process. Figure 8 shows the osseointegration attained through the measurement of the removal torque that has favorable results compared ($p < 0.05$) with the levels attained with the control implants after five weeks of implantation.

Conclusions

This work demonstrated that it is possible to develop chitosan film activated with rhBMP-2, in which the protein maintains its ability to induce differentiation toward osteoblastic lineage. The activity of the protein adsorbed on the film remains constant after a long time period.

The results obtained seem to make these rhBMP-2 activated chitosan films a very attractive biomaterial for the enhancement of osseointegration of surgical prostheses and implants and for tissue engineering bone regeneration.

At the moment, our ongoing work is focused on experiments in vivo using these novel activated films.

Acknowledgment. This work was supported by the Spanish Science and Technology Commission (CICYT, MAT2001-1376), Consejería de Educación de la CAM (GR/SAL 581-04) (Spain), Ministerio de Sanidad (PIO-4108) (Spain) and MMA Biomedical Research Foundation (Spain).

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BM050859E