Differentiation of pre-osteoblast cells on poly(ethylene terephthalate) grafted with RGD and/or BMPs mimetic peptides

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ABSTRACT

The bone morphogenetic proteins (BMPs) are cytokines of the transforming growth factor beta family. Some BMPs such as BMP-2, BMP-7 and BMP-9 play a major role in the bone and cartilage formation. The BMP peptides corresponding to residues 73–92, 89–117, and 68–87 of BMP-2, BMP-7 and BMP-9 respectively as well as adhesion peptides (GRGDSPC) were grafted onto polyethylene terephthalate (PET) surfaces. We evaluated the state of differentiation of pre-osteoblastic cells. The behavior of these cells on various functionalized surfaces highlighted the activity of the mimetic peptides immobilized on surfaces. The induced cells (observed in the case of surfaces grafted with BMP-2, 7 or 9 mimetic peptides and GRGDSPC peptides) were characterized on several levels. First of all, we focused on the evaluation of the osteoblastic markers such as the transcriptional factor Runx2, which is a critical regulator of osteoblastic differentiation. Secondly, the results obtained showed that these induced cells take a different morphology compared to the cells in a state of proliferation or in a state of extracellular matrix production. Induced cells were characterized by an increased thickness compared to non-induced cells. Thus, our studies prove a direct correlation between cell morphology and state of induction. Thereafter, we focused on characterizing the extracellular matrix formed by the cells on various surfaces. The extracellular matrix thickness was more significant in the case of surfaces grafted with mimetic peptides of the BMP-2, 7 or 9 and GRGDSPC peptides which once again proves their activity when immobilized on material surface. These results demonstrate that GRGDSPC and BMPs peptides, grafted to PET surface, act to enhance osteogenic differentiation and mineralization of pre-osteoblastic cells. These findings are potentially useful in developing engineered biomaterials for bone regeneration.

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1. Introduction

Tissue engineering is an emerging technology pursuing the repair or regeneration of damaged tissue with the aid of biomaterial combined with cells and appropriate stimulating factors [1]. One of the key components of many strategies in this technology is the biomaterial that is designed to provide an initial support for cell adhesion and a structural framework for cells to organize and assemble into functional tissue. To this date, numerous natural and synthetic materials have been studied and used in tissue engineering approaches. However, no single material has been considered ideal for all types of tissue, since cells grown on the artificial platform often differ in phenotype and structure as compared to those found in native conditions. The normal cells respond to various micro- and nano-scale environmental signals within the extracellular matrix (ECM) [2], which eventually alters cellular function and tissue structure. However, the presentation of these regulatory signals to cell–biomaterial is hard to achieve, and therefore, many efforts have been undertaken to control the interactions of biomaterial with cells. Previous studies have shown that it is today obvious that this microenvironment's nano-change drives a modification in cellular response [51]. In this paper, we will discuss how an engineered environment enables researchers to better understand cell–biomaterials interactions. Eventually understanding cell behavior can help us to design biomaterials assuming a definite cellular function. In the case of biomaterials for bone repair, various ways were already initiated. Two main ways are well documented: the first is chemical, and consists in chemically modifying materials by grafting proteins or mimetic peptides to support a cellular function like adhesion or cellular differentiation [3–6]. The second way is physical, and consists in modifying rigidity, nanoporosity or microporosity of materials to ensure a certain cellular response [7,8]. Until today, the first way remains a center of interest for the development of biomaterials.
International literature shows an important panel of strategies in order to functionalize bone biomaterial surfaces using different active principles and subsequently influence several intracellular pathways [9,50,56]. In this article, we propose developing biomaterials functionalized by one or several active principles in order to direct cells towards a definite cellular function such as ECM production and its mineralization. RGD sequences associated with ECM matrix proteins (collagen I, fibronectin, sialoprotein and osteopontin) interact with cells through integrin cell surface receptors to facilitate cell spreading and focal-point adhesion to the ECM [61,4]. RGD peptides have been grafted to polymers, metals, ceramics [3,24,60] to modulate cell adhesion. These studies have demonstrated that RGD peptide conjugation to materials facilitates cell adhesion, focal contact formation and promote osteogenic differentiation by increasing alkaline phosphatase (ALPase) activity, osteocalcin and osteopontin expression and calcium content. In this study, we also wanted to influence a crucial cellular function for the bone formation: the induction of osteoblasts, making them mature and able to form extracellular matrix rich in growth factors and ensuring its mineralization. The bone morphogenetic proteins (BMPs) are cytokines of the transforming growth factor beta family (TGF-β). Some BMPs such as BMP-2, BMP-7 and BMP-9 play a major role in the development of the skeleton and the maintenance of haemostasis during bone remodeling [31]. BMP-2, BMP-7 and BMP-9 are three of eighteen proteins of BMPs. BMPs are a group of growth factors known for their ability to induce bone formation [10,11,54,55]. They interact with specific cell surface receptors and activate them. The interface interaction between the BMPs and their receptors involves different types of non-covalent bonds [19,20]. The activated receptors influence the dynamics of the cytoskeleton by activating the LIM kinase-1. Then, this kinase phosphorylates the cofillin, thus suppressing its normal actin depolymerizing function [12]. Like this, the dynamics of the actin filaments is regulated to direct cell migration. Another function of the activated receptors is to regulate the expression of certain differentiation-inducing genes such as Runx2 by activating the Smad 1/5/8-signaling pathway [13,14]. Several peptides containing the knuckle epitope of BMP-2, BMP-7 or BMP-9 have therefore been used both in vitro and in vivo. Saito et al. [35] increased the efficiency of peptides derived from the knuckle epitope of BMP-2, Chen and Webster [58] have used a short peptides that the sequence containing part of the knuckle epitope of BMP-7 and Bergeron et al. [59] have used a great peptide that the sequence containing part of the knuckle epitope of BMP-9. In the same way, to study the activation of the BMP receptors, we proposed to graft adhesion peptides (using mimetic peptides of fibronectin) and BMP-2, BMP-7, BMP-9 mimetic peptides onto PET surfaces. These BMP peptides were selected after a detailed structural study of the binding interface between the BMPs and their receptors. As opposed to other studies [35,58,59], in this work, for each protein (BMP-2, 7, 9) we select the region responsible for the interaction with receptor II (Fig. 1-a, b). These selected regions have the same number of amino acids. Another surface which is bifunctionalized using at the same time adhesion (GRGDSPC) and induction peptides (using mimetic peptides of BMP-2, BMP-7 or BMP-9) in order to ensure such a involvement of the intracellular signaling pathways and have a better induction of the pre-osteoblastic cells [15,25] was synthesized. The aim of this entire study was to evaluate the rate of cell osteoinduction on various surfaces of PET modified chemically with various mimetic peptides (PET grafted with GRGDSPC peptides and PET grafted at the same time with GRGDSPC peptides and mimetic peptides of BMP-2, BMP-7 or BMP-9). The impact of these biomaterials functionalized by one or several active principles will be studied at cellular level (MC3T3-E1): we propose (i) first of all to characterize the induction mode, (ii) to evaluate the progressive evolution of cellular morphology, (iii) to measure the average thickness of the ECM formed by the induced cells and the characterization over time the osteoinduction and the ECM mineralization level.

2. Materials and methods

2.1. Materials

The PET sample used is a commercial film obtained from Goodfellow, France and is in the form of a bi-oriented film with thickness of 75 μm. Inorganic reagents (NaOH, KMnO4, H2SO4, HCl) were obtained by Sigma, France. Acetonitrile, dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-(N-morpholino)-ethanesulfonic acid (MES buffer) were obtained from Aldrich, France. Adhesion peptide (GRGDSPC) and BMP-2 mimetic peptide (RKKPVSPASSAPTQLNAISTLYF) and BMP-9 mimetic peptide (RIVKGRASSVPTRSLSPFLY) were synthesized by Genecust, France.

2.2. Methods

2.2.1. Mimetic peptides design

The FATCAT [16] program was used as a tool to search the BMP-2 protein homologues. The structural alignment was performed with STAMP [17] and then optimized with VITO [18]. The interactions between the three BMPs and the receptor II were determined according to the experimental data [19,20] and also by analyzing the crystallographic structure of the BMPs in complex with their receptor (PDB code: 2H6V, 2ZCO, 3LXS). The distances between the BMPs residues and those of the receptor were calculated with Pymol (DeLano Scientific LLC, http://www.pymol.org).

2.2.2. PET surface preparation and covalent grafting of mimetic peptides

Polymer surfaces were modified according to Boxus et al. [21] with some modifications [3]. The peptide immobilization strategy was performed following the procedure described in previous works [3]. Briefly, materials were modified in order to create COOH functions on PET surfaces. Next, PET-COOH samples were immersed in a solution of EDC (0.2 μM) + NHS (0.1 μM) in MES buffer (0.1 μM in MiliiQ water) and then rinsed in MiliiQ water (50 ml during 30 min). Same protocol was applied for each surface. Finally, immobilization of mimetic peptides was achieved in a solution of mimetic peptides (GRGDSPC and/or BMPs mimetic peptides)/PBS for 16 h at room temperature. After grafting, the disks were rinsed in MiliiQ water (100 ml) for 1 week in order to remove non-grafted peptides [3]. Oxidized PET, PET grafted with GRGDSPC peptides, PET grafted with GRGDSPC and BMP-9 mimetic peptides, PET grafted with GRGDSPC and BMP-7 mimetic peptides, PET grafted with GRGDSPC and BMP-2 mimetic peptides, PET grafted with BMP-2 mimetic peptides are named as surface 1, 2, 3, 4, 5 and 6 respectively. (C/GRGDSPC = 10-4 M; C/BMP mimetic peptides = 10-3 M).

2.2.3. Surface characterization

Surfaces were characterized using fluorescent peptides using fluorescent microscopy. Mimetic peptides of adhesion (GRGDSPC peptides) were linked to TAMRA fluorochrome (RGD-TAMRA) and BMP-2 mimetic peptides were linked to FITC fluorochrome (BMP-2 mp-FITC). FITC or TAMRA fluorochromes were linked in a covalent way at their C terminal end of the different peptides. Other methods of characterization such as X-ray photoelectron spectroscopy (XPS) and high-resolu- tion μ-imager were used to characterize these materials. These results were detailed in previous publications [3, 22–24].

2.2.4. Cell culture

MC3T3-E1 (pre-osteoblast like) cells are a non-transformed cell line established from newborn mouse calvaria and exhibit an osteoblastic phenotype. Cells were grown on materials in Alpha-MEM medium supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycine. Cells were subcultured once a week using EDTA and maintained at 37 °C in humidified atmosphere of 55% CO2 in air. Cells were used for experiments at passages 4 or 5. Throughout this study, cells are seeded at a density of 50 000 cells/cm2. The cells were seeded on surfaces without FCS during 12 h that to allow the BMP mimetic peptides act on receptors without hassle of serum proteins. For evaluation of matrix mineralization, we put dexamethasone (10-8 M), ascorbic acid (50 mg/ml), and β-glycerophosphate (8 mM) in the media for 4 days [26].

2.2.5. Real-time PCR analysis of the gene expression

RNAs were analyzed by quantitative RT-PCR as described previously [27]. Briefly, total RNA was extracted using the RNeasy total RNA kit from Qiagen according to the manufacturer’s instructions. Purified total RNA was used to make cDNA by reverse transcription reaction (Gibco Brl) with random primers (invitrogen). Real-time PCR was performed with SYBR green reagents (Bio-Rad). Expression of target genes was normalized according to the HPRT gene reference by the ΔΔCt method. Data was
analyzed with iCycler IQ™ software. Results were obtained from two series of experiments performed in triplicate. Primers used for amplification are listed in Table 1.

2.2.6. Scanning electron microscopy (SEM)

SEM observations were performed as previously described, with modifications [22]. Briefly, cells were washed with PBS 1X and then fixed with parafomaldehyde in PBS (4%) during 20 min at 4 °C. Samples were dehydrated in increasing concentrations of ethanol and critical point dried. Replicas were gold-coated and observed with scanning electron microscope (SEM Hitachi S2500) at 10 kV.

2.2.7. Optical 3D profiler system (OPS)

Wyko surface profiler systems are non-contact optical profilers that use two technologies to measure a wide range of surface heights. Phase-shifting interferometry (PSI) mode allows measuring smooth surfaces and small steps, while vertical scanning interferometry (VSI) mode allows measuring rough surfaces and steps up to several micrometers high. We used PSI and VSI mode to measure the thickness of the extracellular matrix produced by the cells. To do that, we scratched with a spatula to compare the surface of the PET or PET functionalized with the extracellular matrix formed. To carry out that, we fixed samples with paraformaldehyde in PBS (4%) during 20 min at 4 °C and then metalized samples with gold or titanium.

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>sequence identity (%)</th>
<th>RMSD (Å)</th>
<th>Selected sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>-</td>
<td>-</td>
<td>KIPKACVPELTAISMLYL</td>
</tr>
<tr>
<td>BMP-7/OP-1</td>
<td>58</td>
<td>1.88</td>
<td>TVPKPSSAPQNLVISTLYF</td>
</tr>
<tr>
<td>BMP-9/GDF2</td>
<td>52</td>
<td>1.94</td>
<td>KVGAASSVPKLSPISILYK</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>-</td>
<td>-</td>
<td>RGDSPC</td>
</tr>
</tbody>
</table>

2.2.8. Immunostaining

For characterization and quantification of adhesion peptide and BMPs mimetic peptides grafted onto surfaces, we used the image analysis “Imagej” freeware (NIH, http://rsb.info.nih.gov/ji/). After smoothing, the resulting image, which appears similar to the original photomicrograph but with minimal background, was then converted to binary image by setting a threshold. Threshold values were determined empirically by selecting a setting. This gave the most accurate binary image for a subset of randomly selected photomicrographs with and without GRGDSPC-TAMRA and/or BMP-2 mp-FITC.

3. Results

3.1. BMPs mimetic peptides

BMPs have a very similar structure between themselves with identities of sequence superior to 50% and RMSD (Root Mean Square Deviation) of approximately 2 Å [Fig. 1-a, e]. These sequence and structure conservations suggest the same mechanism for all these BMPs and thus the same area of interest responsible for the interaction with their receptors [Fig. 1-a]. The structural complexes of BMP–BMP Receptors solved by X-ray approve this conception [19,28]. Indeed, the complex BMP-2 structure with BMP receptor II [19] (code PDB: 2GOO) or the BMP-7 with this same receptor [28] (code PDB: 3LX5) shows well that the area responsible for the interactions is the same one [Fig. 1-b]. We believe that the selection sequence of mimetic peptide should be a stable and common region. In our article, we focused on synthesizing BMP-2, BMP-7 and BMP-9 mimetic peptides, these BMPs being osteoinductive proteins of the immature osteoblastic cells [10,11]. Using crystallographic studies of BMP-2-receptor II interactions [35], we were able to select a region of this protein capable of interacting with its receptor [Fig. 1-c]. This region is stable enough to avoid misfolding. The goal in this kind of study was to keep a very strong interaction between mimetic peptides and their receptors; we wanted to target receptors in order to have a sufficient important action for their activation [Fig. 1-d]. The vibration of receptors finally leads to their activities that induce osteogenic markers [53].

3.2. Development of biomimetic biomaterials

PET is widely used for cell culturing (as Thermanox® or Mylar®), surgical suture material, vascular grafts (as Darcon®) and anterior cruciate ligament prostheses due to its biocompatibility and its...
desirable mechanical properties, such as strength and resistance [29]. PET was chosen for this study as model material so as to accurately study cell—material interactions. The different func-
tionalized surfaces (cf Materials and Methods) were characterized by the fluorescence microscopy observation of fluorescent mimetic peptides grafted onto PET (Fig. 2). We observed the amount of peptides grafted onto PET changes depending on the peptide solution used (GRGDSPC or BMP-2 mimetic peptides or GRGDSPC and BMP-2 mimetic peptides). As expected, surfaces 2 and 6 showed only GRGDSPC-TAMRA peptides and BMP-2-FITC mimetic peptides respectively. Surface 5 showed both GRGDSPC-TAMRA peptides and BMP-2-FITC mimetic peptides. However, the amount of BMP-2 mimetic peptides was the same on surfaces 6 and 5, whereas the amount of RGD peptides was twice as low on surface 5 compared to surface 2. As shown in Fig. 2, surface 5 is actually bifunctionalized with these two types of peptides in a homogeneous way. One aim of this bifunctionalization is to firstly ensure cell adhesion via GRGDSPC peptides, and secondly to allow cell induction via BMP-2 mimetic peptides or BMP-7 and BMP-9 mimetic peptides.

3.3. Osteoinduction of cells and overexpression of osteoblast markers

The BMPs receptors thus activated act on two pathways of well-studied signalizations: (i) the first pathway does on the cytoskeleton dynamics by activating the LIM kinase1 [12] and so regulates cell migration, (ii) the second pathway regulates the expression of certain genes inducing differentiation by activating the Smad 1/5/8-signaling pathway. This Smad-dependent pathway leads to the certain genes inducing differentiation by activating the Smad 1/5/8-expression pathway. This Smad-dependent pathway leads to the certain genes inducing differentiation by activating the Smad 1/5/8-expression pathway.

3.4. Increasing of extracellular matrix thickness

The extracellular matrix represents the entire extracellular macromolecules of conjunctive tissue. It is mainly made up of glycoproteins like collagen, fibronectin and laminin, of pure proteins like elastin, as well as glycosaminoglycans and proteoglycans [31]. The aim of our study was to induce cells so that they produce natural osseous extracellular matrix. After assessing the state of inductive genes, we evaluated the thickness of extracellular matrix formed. To do this, we scratched [32] cells in order to measure the thickness ranging between the PET and the top of the extracellular matrix formed by the cells. We observed that in the case of surface 1 (oxidized PET), the cells did not form any extracellular matrix and were still in direct contact with the PET (Fig. 4-a). On the other hand, in the case of other surfaces (2, 3, 4 and 5), a formed extracellular matrix was observed (Fig. 4-b, c, g). We even measured thickness: between the PET and the top of the extracellular matrix formed. Thus, we were able to measure the thickness of the matrix formed by the cells seeded on each functionalized surface (Fig. 4-d, f). On surface 2, PET functionalized with GRGDSPC peptides, the cells produced matrix but remained in a stable condition, hence no induction (Fig. 3). On the other hand, in surfaces 3, 4 and 5, we observed a much more significant matrix. (Fig. 4-g). This is probably due to BMP-2, BMP-7 and BMP-9 mimetic peptides grafted on these surfaces. We switched from a matrix thickness of approximately 50 μm to one of approximately 200 μm after 24 h of cell culture (Fig. 4-g). This remarkable production of natural extracellular matrix by the cells shows the interest of our approach and the importance to use such chemically modified materials in therapy and tissue engineering.

Fig. 2. Fluorescence staining of mimetic peptides grafted onto PET. a, b and e represent surfaces 2, 6 and 5 respectively. c and d represent surface 5 (PET grafted at the same time with GRGDSPC and BMP-2 mimetic peptides) using GRGDSPC-TAMRA (c) and using BMP-2 mimetic peptide-FITC (d). The distribution of these two peptides is represented in e. f represents fluorescence intensity of mimetic peptides in relation to surfaces.
3.5. Osteoinduction and cell morphology

According to their function, cells adopt different morphologies [5]. Indeed, cells in migration take very different organizations from a cell in its stable state [33]. In order to understand the relation between osteoinduction and cell morphology, we focused here on the morphological observation of cells seeded on different functionalized surfaces (surfaces 1–5). On surfaces 1 and 2, we noticed cells were well spread out (Fig. 5-a, b). In surfaces 3, 4 and 5, we observed cells took different morphologies and organizations.

Fig. 3. Level of expression of Runx2, a critical regulator of osteoblast differentiation, OPN, BSP, Alk phos and Coll-α1 on different surfaces.

Fig. 4. Interference microscopy (VSI and PSI) mode to measure matrix thickness: (a) and (b) represent the behavior of the cells on surfaces 1 and 2 respectively (the numerals mean: "1" for PET, "2" for the formed ECM and "3" for cells); c: SEM micrograph of ECM formed on surface 2. d, e and f represent the various sections for measurement of ECM thickness (surface 3 was taken as an example); (g) showed evolution of ECM thickness produced on various surfaces after 24 h of culture.
We observed the cell–cell interactions decrease on surfaces 3, 4 and 5 (Fig. 5-c–e) compared to surfaces 1, 2 (Fig. 5-a, b). This can be explained by the fact that one of the characteristics of induction by the BMPs is causing the dephosphorylation of the connexins, like connexin 43 (Cx43), rendering them inactive [34]. Hence the loss of cell–cell connection and the explanation of cell behavior are characteristic of the surfaces containing BMPs mimetic peptides. We observed that the cells on surfaces 3, 4 and 5 form a filopodia to ensure various interactions with the extracellular matrix formed (Fig. 5-h to m, see arrows). These membrane prolongations show the diversity of factors that these matrices contain. Thus, we can generate a correlation between cellular morphology and organization and cellular function (induction). In the case of surface 2 where we observed a production of extracellular matrix but in a non-induced state, the morphology of these cells was specific and the cell–cell connection was conserved (Fig. 5-b and g).

3.6. Increasing of expression of growth factors and mineralization of matrix

The bone characteristic matrix mineralizes hydroxyapatite crystal deposit between collagen fibers, which confer to the bone its rupture resistance, in addition to its resistance to stretching [35]. Using the different functionalized surfaces, we proposed to study these functionalized material-extracellular matrix–cell complexes by monitoring the state of the cells over time, i.e. the evolution to a state of induction after some time (12 h, 36 h and 72 h). Then, we characterized the ECM formed and studied cell mineralization. This led us to follow the expression of a certain number of genes characterizing the induction like growth factors (BMP-2, TGF-β1 and VEGF) and BMP receptors but also mineralization markers like osteocalcin (OCN). Over time, we noticed that the cells on surfaces 1 and 2 are stable whereas the cells on surfaces 3, 4 and 5 are in continuous induction (Fig. 6). We observed a continuous increase in the expression of these growth factors as well as a remarkable increase in the OCN. These conclusions were backed up by tests of mineralization (Von Kossa), we observed a much more significant mineralization on surfaces 3, 4 and 5 than on surfaces 1 and 2 after 5 days (Fig. 7).

4. Discussion

Since their discovery in 1965 [36], BMPs have revealed a promising future in the field of tissue engineering as powerful components of biomedical products for the regeneration of body parts of human patients, namely bone and cartilage. At the present, the state-of-the-art of regenerative medicine contemplates the use of growth factors, loaded in scaffold or immobilized on materials [37,50,57].

Literature shows some research groups focused on functionalizing surfaces directly with entire growth factors [37]. The problem with this approach is the immobilization of these growth factors. Indeed, the grafting of the BMP-2 on its N terminal side does not systematically expose the area responsible for the interaction with the receptor [38,39]. C. Picart’s group wanted to solve these problems by changing the nature of material and developed polyelectrolyte films where they quantitatively controlled BMP-2 delivery, BMP-2 being not as much immobilized, but rather imprisoned in these films [40]. This system permitted to show the activity of these films and their role in osteoblastic induction.

Fig. 5. SEM micrographs of MC3T3-E1 cells cultured for 24 h on different surfaces (a: surface 1; b: surface 2; c: surface 3; d: surface 4 and e: surface 5). (Scale bar, 50 μm). f, g, h, i and j are SEM views of single cell. k, l and m are the details of cell interaction with ECM on surfaces 3, 4 and 5 respectively.
nevertheless the cell state must be studied precisely in relation to time (ECM formation and mineralization). We think that in the future the development of biomaterials will go more and more towards biomaterials grafted by small molecules, which provide a specific function in contact with a membrane receptor. It is all the more crucial to control the functionality of our small molecules compared to the entire protein functionality, which has the capacity to form dimers with other proteins such as for example BMP-2/BMP-7, or to be modified post-translationally by either a phosphorylation, a acetylation or a palmitoylation that modify its functionality [41,42]. In every case, we obtained an overexpression of Runx2, which among other consequences led to cell induction (Fig. 3).

Probably, the simplest method is to supply growth factors directly to the site of regeneration for cell differentiation and proliferation. However, direct injection of growth factor in solution into the regeneration site is usually rather ineffective, as the injected growth factor is rapidly diffused out from the site, and exerts no effect on bone induction or even in wound healing [43,44]. Therefore, it demands for either extremely higher doses and/or frequent injections. For clinical beneficial outcome, these growth factors require a delivery system to guide tissue regeneration and prevent the rapid dispersal of the factors from the site [45,46].

Another important issue is the short biological half-life of growth factors, usually in an order of minutes [47]. Growth factors have poor in vivo stability and consequently the biological effects are often unpredictable, unless these factors are administrated via a controlled delivery system [43]. The time required for a response in tissues is usually long compared to the half-life of the growth factor; so traditional routines of administration are not appropriate [43,48].

Peptides are emerging as a new class of biomaterials due to their unique chemical, physical, and biological properties. A certain number of studies were carried out to design BMP-2 mimetic peptides and in the majority of these studies, there were problems of reproducibility for the activity of their peptide [35,49]. All already-established peptides have areas of amino acids, which give peptides a great flexibility and thus a significant probability to unfold or fold up so as to hide the amino acids responsible for the interaction with the receptors. Actually, several teams unsuccessfully tried to better understand the role of BMP-2 mimetic peptides [50]. They put certain peptides aside and did not succeed in understanding the problem [51]. After a structural study of our BMPs mimetic peptides (BMP-2, BMP-7 and BMP-9 mimetic peptides), we could not set aside the probability of misfolding, except if we carried out a final restriction on the N terminal on our peptides. Actually, this restriction limited the degree of freedom of its dynamics and stabilized it, which is why our results are reproducible. The originality after these mimetic peptides’ restriction on the N terminal side is the biological study, i.e. the cells follow-up from their state of adhesion to the mineralization of their extracellular matrix. This represented an integral study, which enabled us to accurately conclude our surfaces 3, 4 and 5 are osteoinductive.

The purpose of the study was to select mimetic peptides of BMPs starting from the structural study of BMPs and their receptors (Fig. 1) and functionalizes surfaces with these mimetic peptides and finally to evaluate the expression of osteogenic markers and ECM synthesized. Cells status were also characterized on various surfaces, we highlighted a remarkable correlation between osteoblastic cell induction and cell morphology. Finally, we showed that BMPs mimetic peptides have an significant impact on ECM formation as well as on its mineralization.

In previous studies, we proved on various cellular types that the density of RGD peptide grafted onto biomaterial influences the maturity of the focal contacts [3]. This maturity informs in a quantitative way on cell adhesion. Moreover, we showed there that this maturity was directly related to the production of ECM (see effect of surface 2 on the cells, (Fig. 4)). This ECM production is independent from the process of osteoinduction via Runx2 (Fig. 3). During this state of “production”, cells took a rather particular form compared to the cells that are at “rest”. Indeed, their morphology changed and these cells connected between themselves are organized in networks (Fig. 5). On the other hand, it was obvious that this organization in networks was lost on surfaces 3, 4 and 5. On these surfaces, cells are osteo-induced by the mimetic peptides of BMPs. This process implies other laws controlling the cellular behavior by an indication going until the inhibition of the cell—cell contacts. This inhibition of contacts is characteristic of induction by BMPs [34]. This proves the activity of our BMPs mimetic peptides grafted onto PET. The production of the extracellular matrix is now more significant on the quantitative level but also on the qualitative level in terms of diversity of growth factors (Fig. 6). The state of induction
on surfaces 3, 4 and 5 acts on cell morphology. In this paper, we highlighted a correlation between osteoblastic cell induction and their morphology studied with SEM. Now, after proving we can design osteoinductive surfaces, the real question is: how can such a system be used in regenerating medicine or in tissue repair or even in tissue engineering? The answer to this question makes sense when observing the extra-cellular matrix formation. Indeed, in the case of bone repair we can imagine the functionalized material implantation in the osseous defect. Besides, osteoblasts will colonize implants, start induction and thus create a mineralized extracellular matrix rich in growth factors. These implants can undoubtedly be integrated in bone tissue. Future prospects will be to evaluate in vivo response behaviors of these materials. As already presented in an innovating strategy in vascular engineering [52], we can propose in the future to develop osseous entities in vitro via cell induction onto a material, then get these matrices back with the cells, pile them up then place them into a bioreactor before seeding. These two options seem like a direct prospect for this research program.

In addition, if we look closely at the mimetic peptide responsible for the induction, we can establish and design other mimetic peptides of other BMPs able to provide a precise cellular function. This system of osteoinduction can be a good option for other cellular types by grafting mimetic peptides of other proteins implied in various cellular processes. In the case of bone, we can generate surfaces multi-functionalized with mimetic peptides of different BMPs. This system would be a good model for example for following the differentiation of mesenchymal stem cells and studying their morphology.

5. Conclusion
In our study we proposed to mimic the function of certain BMPs by using small peptides. We covalently functionalized onto PET various mimetic peptides, one of adhesion and/or one of osteoinduction, respectively mimeting fibronectin and BMP-2, BMP-7 and BMP-9 proteins. This biomimetic modification of PET surface showed the activity of mimetic peptides and their capacity of cell induction by the increase in Runx2 expression and a spectacular production of extracellular matrix. The study of the cell morphology on various surfaces highlighted the correlation between cell morphology and cell function. Our results highlighted the fact that the BMP-2, BMP-7 and BMP-9 area determined to select our BMP-2, 7 and 9 mimetic peptide are actually implicated in the interaction with type-II receptors, which allowed inducing cells.

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Appendix

Figures with essential color discrimination. Figs. 4 and 7 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.07.042.

References

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O.F. Zouani et al. / Biomaterials 31 (2010) 8245–8253


Kloesch B, Hoering B, Dopler D, Banerjee A, van Griensven M, Redl H. Synthetic peptides derived from the knuckle epitope of BMP-2 were evaluated for inducing osteogenic differentiation in vitro and in vivo. Eur Cells Mater 2007;14(1). ISSN: 1473-2262:70.


