Adsorption of vascular endothelial growth factor to two different apatitic materials and its release

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The aim of our study was to assess the ability of calcium phosphate powders to serve as growth factor carriers. Vascular endothelial growth factor (VEGF), in particular, is locally involved in the bone formation process throughout osteoblast differentiation. Two different apatitic substrates were tested: hydroxyapatite (HA), widely used as biomaterial, and nanocrystalline carbonated apatite (CA), which has a composition similar to bone mineral crystals. These materials have been compared for their VEGF adsorption and release properties. The adsorption of the growth factor was higher on CA than on HA probably due to differences of both the proteins and the powders involved. The specific activity of the VEGF released was also tested to determine the available activity for cells in contact with these materials. Interestingly, the bioactivity of the VEGF released from CA quantified on fetal bovine aortic endothelial cells (FBAE) by evaluating the proliferation activity, exhibited no marked difference compared to native VEGF. Qualitatively, VEGF adsorbed on CA material induced well-defined collagen type I immunostaining on osteoblast cells compared to the staining obtained after VEGF adsorption on HA.

1. Introduction

Calcium phosphate ceramics represent attractive materials to act as bone substitutes because of their analogy of composition with bone mineral. The most frequently used calcium phosphate biomaterial, hydroxyapatite (HA), differs, however, from bone mineral in many respects, notably carbonate and phosphate content, fine structure and reactivity [1]. Carbonated apatite (CA) can be synthesized [2–4] and offers a chemical composition, fine structure and morphology very close to that of bone mineral crystals and presents similar surface reactivity [5].

Binding of growth and differentiation factors which appear to control *in vitro* cellular events involved in bone physiology to these materials has been postulated to enhance bone growth and repair *in vivo*. One of them, fibroblast growth factor-2 (FGF-2) induces *in vitro* the proliferation of a wide variety of cells and in particular osteoblasts [6, 7], chondrocytes [8] and periosteal cells [9]. However, the role of FGF-2 on bone formation *in vivo*, remains unclear. FGF-2 enhances the invasion of capillaries into the metaphyseal region of the growth plate and accelerates ossification at the growth plate [10]. Vascular endothelial growth factor (VEGF) is a potent mitogenic and chemotactic factor for endothelial cells [11–13] in close association with osteoblasts and osteoclasts [14, 15]. In bone remodeling, angiogenesis has been shown to play an important role in bone healing [16]. We previously showed that VEGF is not specific for endothelial cells since it also stimulates the proliferation of non-endothelial cells such as interleukin-2-dependent lymphocytes or stromal cells cultured from neonatal hemangiomas [17] and also contributes to osteoblast differentiation [18].

The aim of this study was to examine the putative interactions of VEGF with HA and CA which could be used as carriers. Adsorption and release of the growth factor from the powders were evaluated. The bioactivity of the VEGF released was tested to determine the available activity for cells in contact with these materials. We first showed that the factor bound strongly but reversibly to HA affinity columns. Release of the growth factor from the ceramics was evaluated versus time. Fetal bovine aortic endothelial cells (FBAE) known to proliferate in response to VEGF were used as target cells. In addition we used the osteogenic sarcoma cell line SaOS-2 to demonstrate the ability of a pre-osteoblast cell line to acquire a differentiated phenotype such as type I collagen expression, by addition of VEGF released from the powders. Since the bioactivity of the released

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growth factor was conserved, this strategy provided information for the development and application of a drug-delivery system.

2. Materials and methods

2.1. Growth factors and cell-culture conditions

Human recombinant VEGF (165 amino acid isoform) was expressed in a baculovirus expression system [19]. ^{125}I-VEGF was prepared using the lodogen reagent according to [6]. Stock cultures of osteosarcoma-derived SaOS-2 cells purchased from ATCC, were cultured in α-Modified Eagle's medium (α-MEM, Gibco) supplemented with 5% fetal calf serum (FCS, Gibco) in 10% CO_2 at 37 °C. Fetal bovine aortic endothelial cells (FBAE) isolated from aortic arches were routinely grown at 37 °C with 10% CO_2 in Dubelcco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% heat inactivated fetal calf serum. The mitogenic activity of VEGF was measured on these target cells as previously described [12]. For all experiments, cells were used at passages 2 to 5. The conditioned medium of the cultured mouse pituitary tumoral cell line AtT-20 was collected as previously described [12].

2.2. HA chromatography

Since several basic proteins bind tightly to HA, we used this affinity chromatography matrix to purify VEGF. Conditioned medium from AtT-20 was concentrated by ammonium sulfate precipitation, dialysed overnight against 10 mM phosphate buffer pH 6.2 and loaded on a HA (Biorad) column (0.9 × 5 cm) previously equilibrated in the same buffer. The flow rate was 20 ml/h/cm^2, the resin was washed extensively with the equilibration buffer until the absorbance had returned to baseline, and eluted stepwise with increasing phosphate concentration. Aliquots were added every other day to low-density FBAE cells. After four days the cells were counted in a coulter counter.

2.3. Adsorption of radioiodinated VEGF onto HA and CA powder

HA powder for chromatography, obtained from Merck KGaA (Darmstadt, Germany) and CA powder was prepared as previously described [5]. Their physicochemical characterizations were reported previously [20].

Five mg of each powder was pre-equilibrated with 100 µl of tris balanced salt (TBS) (Tris-HCl 50 mM, NaCl 150 mM), pH 7.4 for 1 h at room temperature. Various concentrations (0.5–5 µg/ml) of recombinant human VEGF (rh VEGF) were added to the powders in a volume of 100 µl and incubated for 1 h at room temperature under gentle shaking. The samples were microcentrifuged for 5 min, the supernatant counted, and the plastic adsorption on the tube was also counted. It has been verified that VEGF does not precipitate in the absence of powder. The radioactivity was counted in a gamma counter.

2.4. Kinetics of the release of radioiodinated VEGF

HA and CA powders were incubated with 5 µg/ml ^{125}I-VEGF in serial tubes. After 1 h each tube was extensively washed with TBS and further incubated with 100 µl TBS. At various time intervals duplicate tubes were centrifuged, washed with TBS, and the amount of ^{125}I-VEGF released in the supernatant was counted.

As described above, release for 2 h was also quantified for various concentrations of rh VEGF incubated with the 5 mg of each powder.

2.5. Recovery of the bioactivities of the growth factors released from the powders

The same adsorption and release experiments have been realized, as previously described, with various concentrations of unlabeled VEGF. The bioactivity of the VEGF released from powders was compared to that of native recombinant VEGF using proliferation assay on FBAE cells.

FBAE cells were cultured in DMEM with 10% fetal calf serum, in 12-multiwell plates and were seeded at 5000 cells/well. VEGF released was added every other day and cells were trypsinized on day 4 and counted. All samples were measured in quadriplicate.

Bioactivities resulting from VEGF released and native VEGF, as reference from a standard curve, on FBAE cells, allows comparison of the amount of VEGF release per unity surface and concentration of native VEGF.

In control experiments the influence of powder-released buffer was assayed and exhibited neither mitogenic nor inhibitory effect on FBAE cell proliferation.

2.6. Immunostaining of type I collagen

SaOS-2 cells were seeded on glass coverslips at subconfluence and incubated for six more days in the presence or absence of 0.1 mg/ml of HA or CA. Powders have previously bound 5 µg/ml of unlabeled VEGF. The cells were then fixed for 10 min at 4 °C with 2.5% paraformaldehyde, rinsed with phosphate balanced salt (PBS) buffer pH 7.8, and permeabilized with 0.1% Triton × 100 for 10 min. Anti-human type I collagen antibody (Genzyme) was then added for 1 h in PBS. The cells were rinsed and the immunocomplexes revealed with anti-human FITC-conjugated antibody. The coverslips were rinsed and further mounted with 20% Mowiol. A Leitz epifluorescence microscope was used for the observations.

2.7. Statistical analysis

Results are expressed as the mean ± SD of four measurements. Statistical significance was determined using Student's *t* test.

3. Results

3.1. HA chromatography of VEGF

Since it had been previously shown that FGF2 bound to HA [21], we determined whether VEGF was also able to

bind to this affinity chromatography column. As shown in Fig. 1, most of the loaded material was eluted below 0.15 M phosphate concentrations and did not exhibit any mitogenic activity for FBAE cells. Elution with 0.15 M phosphate accounted for 24% of the total protein loaded and yielded one major peak of bioactivity (72% of the initial activity). This mitogenic activity was further confirmed as VEGF by radioimmunoassay (data not shown).

3.2. Adsorption of the growth factors

^{125}I-VEGF bound to HA in a time dependent manner and maximum binding was observed after one hour (data not shown). The VEGF content was determined in the supernatant after its release from the powder by gamma counting (specific activity 200 000 cpm/ng). The specific surface areas of the materials exhibited marked differences as shown in Table I. In spite of about a hundred fold higher specific surface area of CA compared to HA, the adsorption of the growth factor (Table II) reached about 80% of the amount incubated. The adsorption was not saturated at this range of concentrations. The adsorption increased proportionally with the specific surface area, as has been previously expressed [20]. The binding of VEGF to powders was almost linear over time with concentrations up to 5 μg/ml.

3.3. Growth factor release

Powder (5 mg) previously incubated with 5 μg/ml of iodinated VEGF was rinsed and further incubated in 100 μl of TBS under vigorous shaking. ^{125}I-VEGF in the supernatant was counted after different periods of time. Both ceramics released VEGF with the same kinetics, achieving maximal release after 2 h (Fig. 2a). The amount of ^{125}I-VEGF released from both powders was approximatly the same whatever the powder (Fig. 2b), and was proportional to the adsorbed concentration and averaged 27%.

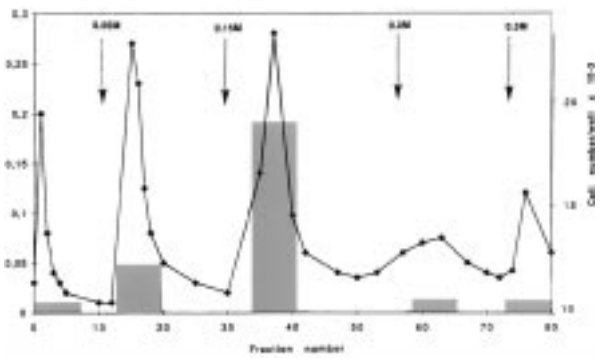

Figure 1 HA chromatography of AtT-20 conditioned medium. Conditioned medium from AtT-20 was concentrated by ammonium sulfate precipitation, dialysed overnight against 10 mM phosphate buffer pH 6.2 and loaded on a HA column previously equilibrated in the same buffer. The flow rate was 20 ml/h/cm^2. The resin was washed extensively and eluted stepwise with increasing phosphate concentrations as indicated. Aliquots were monitored for FBAE cell proliferation. OD adsorption (left, full symbols) and FBAE cell proliferation (right, bar graph).

3.4. Comparison of the biological activities of native and released growth factors

The amount of VEGF released from the powders relating to the specific surface area was evaluated for its biological activity. The bioactivity of VEGF released from ceramics in powder, was compared to a standard curve of native recombinant VEGF for its ability to induce proliferation on FBAE cells. As shown in Fig. 3, VEGF released from HA is more bioactive than VEGF released from CA comparatively to the same surface of adsorption sites.

3.5. Type 1 collagen expression in osteoblasts

It has been reported that SaOS-2 cells express a small amount of type I collagen [22] which we could not detect in culture, even after 6 days in the presence of ascorbic acid (data not shown). The cultures were incubated for 6 days in the presence of HA and CA powders previously incubated with or without VEGF (2.5 μg/ml). The addition of the two powders allowed the detection of a little immunoreactivity for type I collagen which remained diffuse and unorganized (Fig. 4). When the same amount of powder preliminarily incubated with VEGF, type I collagen expression was enhanced. This expression was qualitatively higher for CA-released VEGF than for HA-released VEGF.

4. Discussion

HA is a well-known sorbent for molecules and this property has been used as chromatographic adsorbent in protein separation. We previously showed that eye derived growth factor, the retinal FGF-2, bound strongly to a HA affinity column [21]. Similarly VEGF was retained by matrix-immobilized hydroxyapatite and eluted by 0.15 M phosphate. This chromatography showed that protein adsorption is a function of pH and protein concentration and is strongly influenced by the valency of the neutral salt present in solution.

In comparison, adsorption and release of the growth factor has been investigated with two different adsorbents in presence of a solution without phosphate ions. These ions interacted with the adsorbate by improving, for example, the adsorption of basic proteins [23].

As Misra [24] showed, the geometry of the adsorbate molecule, the location of its functional groups, its surface orientation and the nature of the solvent should have an important bearing on this type of adsorption.

The amount of bound VEGF increased linearly with its concentration in the solution. Proteins adsorbed mainly through electrostatic interactions. Previous studies on adsorption of a negatively charged protein onto a strongly charged apatite surface showed that the amount of incorporated ions increases when the charge differences between similarly charged adorbent and adsorbate increases [25]. One may consider that even when the total protein charge is negative in the aqueous environment, proteins are ambivalently charged, consequently, it may also interact through positively charged groups with the negatively charged surfaces and *vice versa* [26].

TABLE I Physical chemical properties as previously evaluated [20]: specific surface area, weight percentage of CO_3^{2-} ions and Ca/P ratio of the two powders at the initial state

Composition	Specific surface area ($m^2 \cdot g^{-1}$)	Weight % of CO_3^{2-} ($\pm$ 0.04)	Ca/P ($\pm$ 0.02)
HA	16 ($\pm$ 1)	0	1.66
CA	156 ($\pm$ 15)	2.54	1.55

TABLE II Adsorption of VEGF onto different powders. Each powder is a sample of 5 mg. The amount of powder (mg) is converted into its respective specific surface area (Table I)

	HA		CA	
Initial concentration of VEGF (μg/ml)	Concentration of VEGF adsorbed (μg/5 mg of powder)	Concentration of VEGF adsorbed	Concentration of VEGF adsorbed (μg/5 mg of powder)	Concentration of VEGF adsorbed ($\mu g/m^2$)
0.5	0.0006	0.0076	0.03	0.041
1	0.001	0.013	0.058	0.078
2.5	0.032	0.04	0.14	0.19
5	0.057	0.071	0.348	0.463

Another explanation for the higher adsorption on CA is that it has a much higher specific surface area than HA. However, for the same initial concentration, the amount bound per unit area CA exceed that bound per unit area HA by about 5 times. Thus, the affinity of VEGF for CA surfaces is higher than for HA surfaces. Differences of surface composition and crystal morphology may also be explained by this phenomenon. CA crystals in particular have been shown to exhibit labile phosphate and carbonate groups like bone mineral, probably located exactly at the crystal surface. In addition, crystal surfaces with many irregularities like those of bone mineral crystals could favor strong uptake.

Maximal VEGF release was linear and reached 27% of the initial amount immobilized in the device. These results are similar to those obtained with FGF-2 [20],

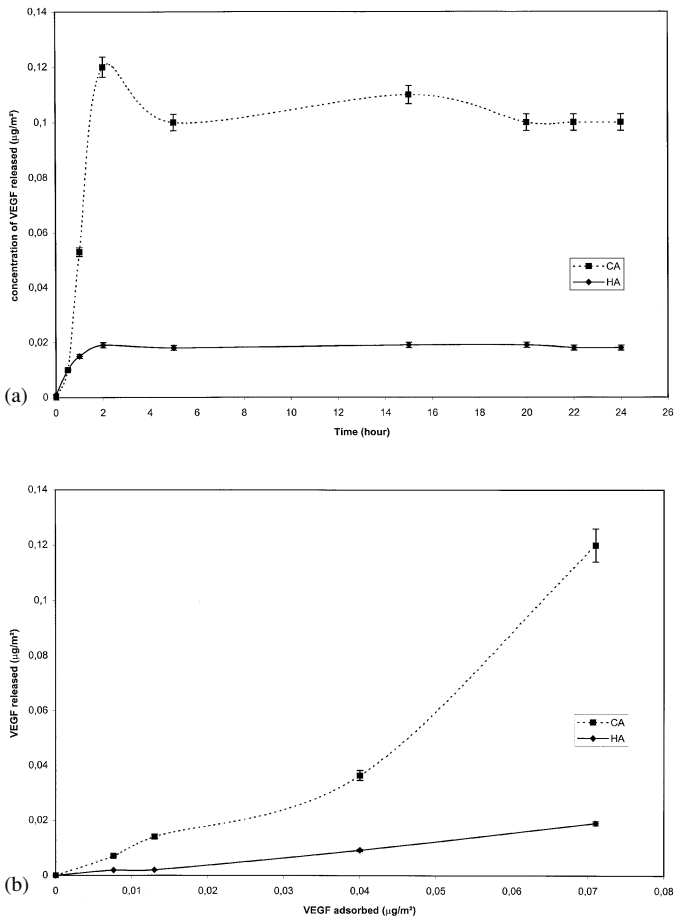

Figure 2 (a) 5 μg/ml of ^{125}I-VEGF was incubated on HA and CA powders. They were further rinsed and incubated with fresh TBS for various periods and the amount of ^{125}I-VEGF released in the buffer solution was counted. (b) Various concentrations of ^{125}I-VEGF were incubated on HA and CA powders. They were rinsed and after 2 h incubated with fresh TBS and the amount of ^{125}I-VEGF released in the buffer solution was counted. The results are the mean $\pm$ of four measurements.

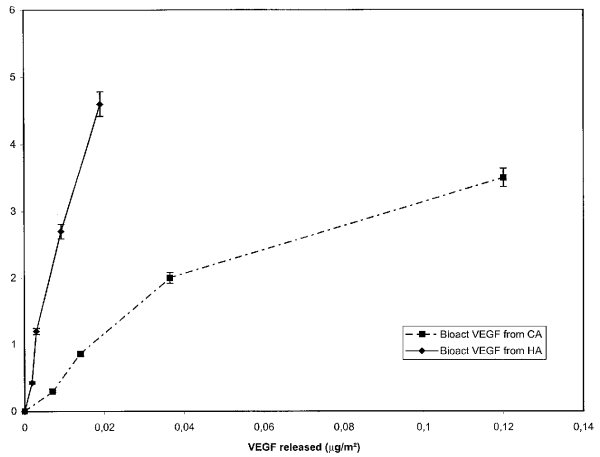

Figure 3 Unlabeled VEGF was allowed to adsorb to HA and CA powders (0.1 µg/sample) at the same concentrations as previously studied. After extensive washing, VEGF was released by vigorous shaking in TBS for 2 h, and assayed for its ability to induce FBAE proliferation as described in the text. A standard curve was used as reference. The results are the mean ± of four cultures.

another cationic growth factor promoting bone proliferation and differentiation. VEGF was steadily released from each material and a plateau was reached within two hours.

Electrostatic interactions between cationic growth factors such as FGF-2 or VEGF and acidic powders are believed to form a polyionic complex [27]. Physicalchemical changes of the proteins might result from interactions during adsorption of the growth factor with the labile environments of CA. These CO_3^{2-} and HPO_4^{2-} groups are unstable and can be exchanged rapidly, the surface modifications resulting are related to protein release [28]. For a conformation to be biologically relevant, it must satisfy a number of stringent criteria. The type of adsorption, the strength of the binding, and the actual mechanisms involved appear to be directly related to the secondary structure of the macromolecules [29]. It has been shown that some α helix structures in fibrinogen become transformed into β sheet structures during the process of adsorption/desorption denaturation [30]. This aspect could be exploited, as it has been demonstrated that alterations of the conformation of osteopontin might be required for its nucleation activity to be exerted . It is thus conceivable that growth factor molecules not yet participating in the polyionic complex with acidic ceramic may be released during the initial stage of the *in vitro* release test. Although it is not known whether the VEGF conformation does change during the adsorption/release process, the specific activity of VEGF released from each ceramic remained unchanged compared to that of native recombinant. This suggests that the association of VEGF with HA or CA does not lead to irreversible changes if any, in the conformation. However, the products resulting from the enzymatic degradation of CA may change the properties of the

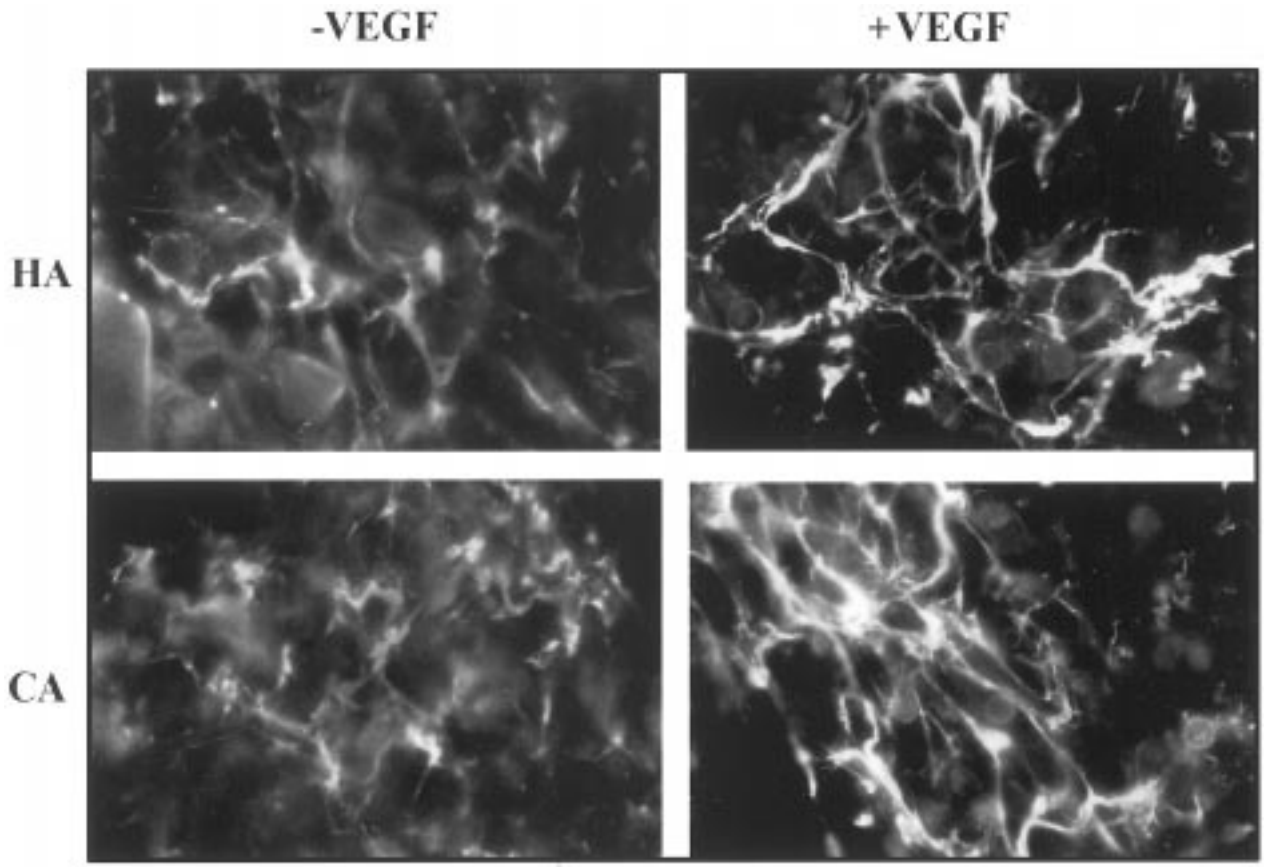

Figure 4 SaOS-2 cells were seeded on a glass coverslip at subconfluence and incubated for six more days in the presence or absence of 0.1 mg/ml of HA or CA. In some experiments, ceramic powders which had previously bound 5 µg/ml of unlabeled VEGF were used. The cells were then fixed 10 min at 4 °C with 2.5% paraformaldehyde, rinsed with PBS buffer pH 7.8, and permeabilized with 0.1% Triton X100 for 10 min. Anti-human type I collagen antibody (Genzyme) was then added for 1 h in PBS. The cells were rinsed and the immunocomplexes revealed with anti-human FITC-conjugated antibody. The coverslips were rinsed and further mounted with 20% Mowiol. A Leitz epifluorescence microscope was used for observations.

growth factor and explained the higher bioactivity of VEGF released from HA, than from CA on endothelial cell proliferation. CA, such as bone mineral, is more soluble than HA [32], the degradation particles resulting could affect, in return, the cell physiology, and therefore mitogenic activity. After a long-term exposure of cultured osteoblast cells to apatite devices, our results demonstrate that type I collagen expression was slightly more affected by CA than by HA used alone. Bone cells incubated with the same amount of VEGF-bound to the powder exhibited an increase in type I collagen expression. However, the cell monolayer only appeared to be well organized when the VEGF was released from CA samples, suggesting that in addition to a rapid effect on collagen accumulation, long-term presence of VEGF was required to induce well-defined differentiation for these cells. Thus CA behaves as a reservoir from which VEGF can be mobilized by enzymatic cleavage. Detailed consideration of the structure and the characteristics of both proteins and powders involved is necessary in order to understand adsorption processes. This *in vitro* pattern could be useful for the study of the controlled adsorption and release molecules *in vivo* and would certainly improve suitable biomaterials for bone healing.

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