

## PRUEBAS DE PLANAS

# Involvement of integrins in the adhesion of osteoblastic cells to a type-I collagen matrix

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Palabras clave: células osteoblásticas; colágeno tipo-I; adhesión celular; integrinas; secuencias de reconocimiento.  
Key words: osteoblastic cells, type-I collagen, cell adhesion, integrins, recognition sequences.

**RESUMEN.** Se han desarrollado varios biomateriales con potencial aplicación en la reconstrucción de tejidos. En este sentido, existe un creciente interés en el diseño de materiales de implante óseo con máxima biocompatibilidad y adecuada adhesividad celular. El objetivo de este trabajo fue estudiar cuáles receptores integrinas participan en la adhesión de osteoblastos a una matriz de colágeno tipo-I. Se analizaron dos líneas celulares osteoblásticas: UMR106, derivada de osteosarcoma de rata; y MC3T3E1, derivada de calvaria de rata. Las células se cultivaron durante una hora sobre plástico, o sobre un gel de colágeno tipo-I, solas o co-incubadas con diferentes péptidos: (a) péptido- $\beta$ , un oligopéptido de 13 aminoácidos que corresponde a la secuencia conservada 113-125 de la subunidad  $\beta$  de los receptores integrinas; (b) RGD (Arg-Gly-Asp), que corresponde a la secuencia de reconocimiento de la subunidad  $\alpha$  de las integrinas  $\alpha_{1,5}\beta_1$ ; o (c) DGEA (Asp-Gly-Glu-Ala), la secuencia de reconocimiento de la subunidad  $\alpha$  de las integrinas  $\alpha_2\beta_1$ . La adhesión y la inducción de extensiones celulares se evaluaron microscópicamente luego de lavar, fijar y colorear las células con Giemsa. Los resultados demostraron que las células osteoblásticas se adhieren más fácilmente a un sustrato de colágeno tipo-I que al plástico ( $86 \pm 5$  células/campo vs.  $69 \pm 4$  células/campo para colágeno tipo-I vs. plástico, respectivamente,  $p < 0,02$ ). El péptido- $\beta$  inhibió la adhesión de ambas líneas celulares a una matriz de colágeno y el efecto inhibitorio mostró dependencia dosis-respuesta. Por otro lado, este péptido indujo en las células tipo osteoblastos UMR106 un aumento del agrupamiento intercelular, y una reducción en la inducción de extensiones celulares. Estos cambios morfológicos podrían estar indicando un incremento en las interacciones célula-célula y una disminución en las interacciones célula-matriz, posiblemente inducidos por el péptido- $\beta$ . De forma similar, los péptidos RGD y DGEA disminuyeron significativamente, entre un 20 y 30 %, la adhesión de las células UMR106 y MC3T3E1 a la matriz de colágeno. Las células UMR106 cultivadas sobre colágeno en presencia de DGEA mostraron un mayor estimamiento citoplasmático, con inducción de extensiones celulares en múltiples direcciones. En suma, estos resultados sugieren que las subunidades a y b de varios receptores integrinas están involucradas en la adhesión de las células tipo osteoblastos a una matriz de colágeno tipo-I. Así, el recubrimiento de ciertos biomateriales con péptidos de reconocimiento o con moléculas de colágeno intacto, podría mejorar la osteointegración de implantes para la reparación del tejido óseo.

**ABSTRACT.** Several biomaterials have been developed with applications in bone tissue engineering. In this context, there is a growing interest in developing bone implant materials with improved biocompatibility and cell adhesion properties. The aim of this study was to research which integrin receptors participate in the attachment of osteoblastic-like cells to a type-I collagen matrix. Two osteoblast-like cells, the UMR106 rat osteosarcoma cell line and the MC3T3E1 mouse calvaria-derived cells, were analyzed. Cells were plated for one hour either on plastic or a type-I collagen film, alone or co-incubated with different peptides: (a)  $\beta$ -Peptide, a 13-aminoacid oligopeptide corresponding to preserved sequence 113-125 of the  $\beta$ -subunit of integrin receptors; (b) RGD (Arg-Gly-Asp), which corresponds to the  $\alpha$ -subunit recognition sequence of  $\alpha_{1,5}\beta_1$  integrins; or (c) DGEA (Asp-Gly-Glu-Ala), the  $\alpha$ -subunit recognition sequence of  $\alpha_2\beta_1$  integrins. Cell adhesion and spreading were evaluated microscopically after fixing and staining the cells with Giemsa. The results showed that osteoblast-like cells adhere more easily to a type-I collagen substratum than to plastic ( $86 \pm 5$  cells/field vs.  $69 \pm 4$  cells/field for type-I collagen vs. plastic, respectively,  $p < 0.02$ ). The  $\beta$ -peptide inhibited the adhesion of both cell lines to the collagen matrix in dose-dependent way. Addition-

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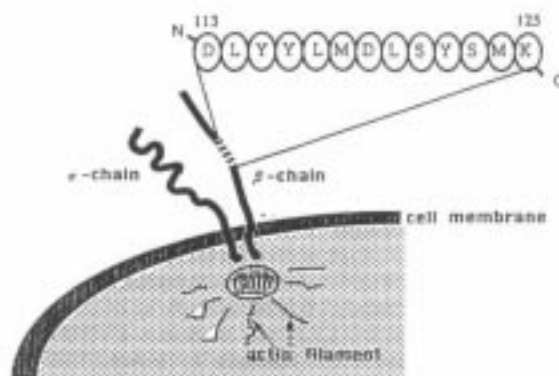
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ally, this peptide induced an increase in UMR106 cells clumping and a decrease in cells spreading. These morphological features would suggest an increase in cell-cell interactions and a decrease in cell-matrix interactions, possibly mediated by  $\beta$ -peptide. The RGD and DGEA peptides significantly decreased UMR106 and MC3T3E1 adhesion to the collagen matrix (ranging from 20 to 30 % inhibition). On the other hand, UMR106 cells grown on collagen in the presence of DGEA exhibited greater spreading with cellular extensions in multiple directions. Altogether, the obtained results suggest that both  $\alpha$  and  $\beta$  subunits of several integrin receptors are involved in the attachment of osteoblast-like cells to a type-I collagen matrix. The coating of different biomaterials with specific recognition peptides as well as with whole collagen molecules, could improve the bone integration of implants to be used in bone tissue repair.

## INTRODUCTION

Several biomaterials have been developed with possible applications in bone tissue engineering.<sup>1,2</sup> The growth and development of osteoblasts on these implants is necessary for osseointegration. However, the processes of cell adhesion and spreading (first steps in osteoblastic progression) are dependent on the substratum with which the cells interact. In this context, there is a growing interest in developing implant materials with improved biocompatibility and cell adhesion properties.

Multiple integrin receptors (integrins) have been described in osteoblasts. Integrins, by interaction of their  $\alpha$  and  $\beta$  subunits with certain consensus sequences present in extracellular matrix (ECM) proteins, can aid in osteoblasts adhesion and spreading, as well as in intracellular signalling. The consensus sequences for binding of  $\alpha$  subunits have been well characterised. Thus, the  $\alpha$  subunit of  $\alpha_2\beta_1$ , considered to be the main osteoblastic receptor for type-I collagen (which in turn constitutes > 85 % of bone ECM), recognizes the tetrapeptide sequence DGEA (Asp-Gly-Glu-Ala) present in this collagenous molecule.<sup>3</sup> On the other hand, the  $\alpha$  subunits of integrins  $\alpha_1\beta_1$  and  $\alpha_5\beta_1$  principally recognise the tripeptide sequence RGD (Arg-Gly-Asp), which is present in several ECM proteins such as type-I collagen, fibronectin, vitronectin and osteopontin.<sup>4</sup> However, the consensus sequences for binding



**Fig. 1.** Schematic structure of an integrin receptor and the  $\beta$ -peptide.

of  $\beta$  subunits to ECM have proved to be elusive to discover. Recently, an ingenious approach to this particular problem was developed by Dr. Uemura and associates. Since all integrin  $\beta$  subunits are almost identical with respect to their amino acid sequences, Uemura's team designed and synthesised the polypeptide Asp-Leu-Tyr-Tyr-Leu-Met-Asp-Leu-Ser-Tyr-Ser-Met-Lys (referred to as the  $\beta$ -peptide), which corresponds to the conserved amino acid sequence 113-125 of the  $\beta$  subunits of integrins (Fig. 1). The  $\beta$ -peptide was found to inhibit the binding of the  $\beta$  subunit of integrins to ECM proteins, presumably due to competition for the same consensus sequence(s).<sup>5</sup>

The object of this study was to characterise the integrin receptors that participate in the attachment and spreading of osteoblast-like cells to a type-I collagen matrix.

## MATERIALS AND METHODS

### Materials

Rat tail acid-soluble type I collagen, RGD peptide and DGEA peptide were purchased from Sigma (St Louis, MO, USA). The  $\beta$ -peptide was synthesised by the Peptide Institute in Osaka, Japan; the purity of this synthetic product was calculated to be 95.1 % by HPLC analysis. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco (Life Technology, Argentina). Tissue culture disposable material was from Nalge Nunc International (Roskilde, Denmark).

All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

### Preparation of type-I collagen matrices and cell culture

Collagen was dissolved in sterile 0.02 N acetic acid (2.5 mg/mL) (pH 3.0), poured into plastic dishes (50  $\mu$ g/cm<sup>2</sup>) and incubated at 37 °C. Col-

lagen formed a thin film during incubation. The film was rinsed with phosphate-buffered saline (PBS) and extensively washed with DMEM, after which the cells were plated for different experiments.

UMR106 rat osteosarcoma-derived cells were grown in DMEM-10 % PBS and antibiotics. After 5-7 d, cells were sub-cultured using trypsin-EDTA and re-plated on plastic or collagen matrices to begin the experiments. MC3T3E1 osteoblastic mouse calvaria-derived cells were grown in 75 cm<sup>2</sup> plastic flasks at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere in DMEM supplemented with 10 % FBS, 100 U/mL penicillin and 100 mg/L streptomycin, passaged after 4-6 d and re-plated on plastic or on type-I collagen matrices to begin the experiments.<sup>6</sup>

### Cell adhesion and spreading: effect of various peptides

Osteoblast-like cells in DMEM-10 % PBS were plated on uncoated or collagen-coated 24-wells plates at a seeding density of  $2.5 \cdot 10^5$  cells/mL, in the presence or absence of different peptides: RGD (1 mM), DGEA (5 mM), or  $\beta$ -peptide (50 or 100  $\mu$ M). Cells were allowed to adhere for 1 h at 37 °C. Each well was then washed with PBS, fixed with methanol for 5 min and stained with Giemsa for 10 min. Cells adhered to plastic or collagen matrices were evaluated microscopically by counting several representative fields per well. A linear regression of the adhesion assay occurred between  $1 \cdot 10^5$  and  $1 \cdot 10^6$  cells/mL ( $y = 0.826 + 0.759 x$ ,  $r = 0.993$ ). Cellular spreading was also evaluated microscopically, counting the number of cells per field that presented three or more vertices.

### Statistical analysis

Three independent experiments were run for each experimental condition. Results were expressed as

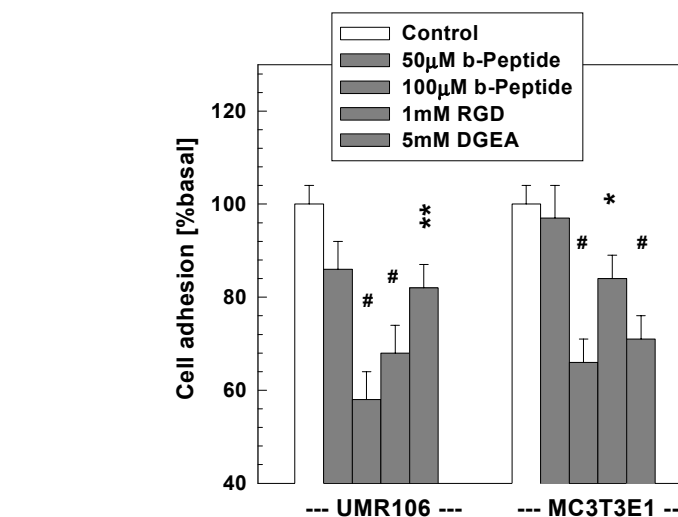
mean  $\pm$  standard error of mean. Statistical analysis of the data was performed by Student's *t* test.

### RESULTS AND DISCUSSION

Under the experimental conditions employed in this work, type-I collagen was found to be a better substratum than plastic for the adhesion of osteoblast-like cells ( $86 \pm 5$  cells/field vs.  $69 \pm 4$  cells/field for type-I collagen vs. plastic, respectively,  $p < 0.02$ ). Thus, the coating of wells with a collagenous gel increased cell attachment by 25 %.

In some experiments, the osteoblast-like cells were co-incubated the osteoblasts-like cells with the consensus sequence peptides RGD and DGEA. As expected, DGEA inhibited cell attachment to type-I collagen by 20-30 % in both cell lines (Fig. 2). Surprisingly, RGD also inhibited cell attachment by about the same amount (Fig. 2). This latter result differs to reports by other authors who found no effect of RGD on osteoblast-like cell adhesion to type-I collagen,<sup>3</sup> but is in agreement with the presence of RGD on type-I collagen. Thus, certain experimental conditions could expose this RGD sequence for interaction with  $\alpha_{1,5}\beta_1$  integrins present on osteoblastic cells, while other conditions might change type-I collagen conformation in such a way that interaction would be hindered by steric factors.

In other experiments, osteoblast-like cells were co-incubated in presence of increasing concentrations of b-peptide. It was found that this peptide inhibited cell adhesion to type-I collagen in a dose-dependant way, in a greater extension than RGD and DGEA (Fig. 2). This result is espe-



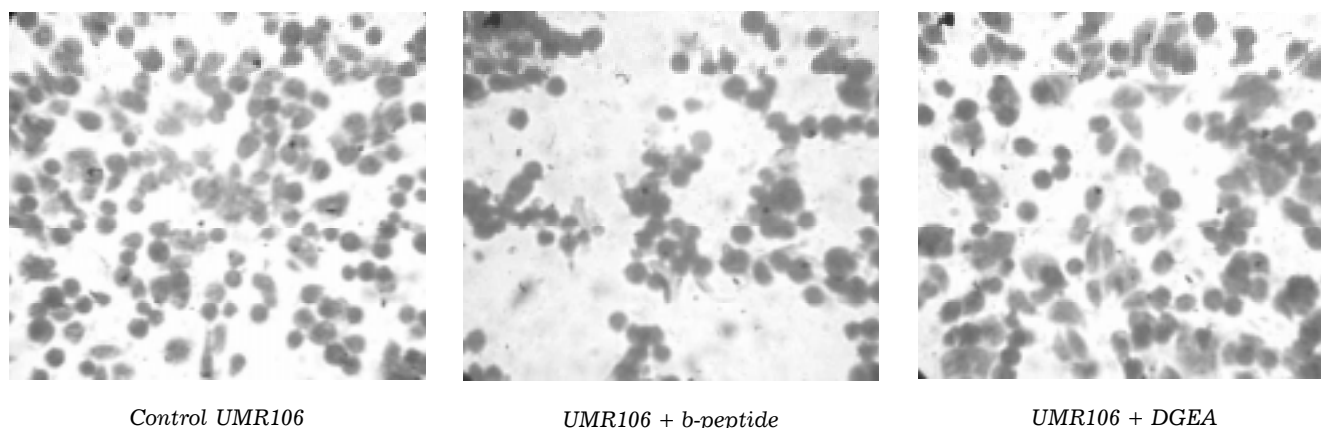
**Fig. 2.** Osteoblast-like cells adhesion to a type-I collagen matrix. Differences vs. control are: \*  $p < 0.02$ ; \*\*  $p < 0.01$ ; #  $p < 0.001$ .

cially interesting since the inhibition took place at a molar concentration of  $\beta$ -peptide tenfold lower than RGD, and fifty fold lower than DGEA. Thus, if bone implant materials were coated with peptides derived from b subunit consensus sequences, they could be just as effective as the better-known fragments with affinity for a subunits in enhancing cell recruitment and bone-forming potential.

Next, the possible effects of co-incubation with these peptides on cell spreading were evaluated. In the case of the MC3T3E1 non-transformed osteoblasts incubated with RGD, DGEA or  $\beta$ -peptide, no differences in cell spreading with regard to control (no peptide, cells plated on type-I collagen, data not shown) were observed. However, the osteosarcoma-derived UMR106 cells showed an increase in cell clumping, and a decrease in cellular spreading

when co-incubated with  $\beta$ -peptide (Fig. 3). On the other hand, UMR106 cells grown on collagen in the presence of DGEA exhibited greater spreading with cellular extensions in multiple directions (Fig. 3). These morphological features would suggest that transformed osteoblastic cells might be more susceptible to changes in cell-cell and cell-matrix interactions than non-transformed osteoblasts, when in the presence of molecules which compete with integrin binding to ECM proteins.

Altogether, the present results suggest that both  $\alpha$  and  $\beta$  subunits of integrin receptors are involved in the attachment and spreading of osteoblastic cells to a type-I collagen matrix. The coating of different biomaterials with specific recognition peptides as well as with whole collagen molecules, could improve the osseointegration of implants to be used in bone tissues.



**Fig. 3.** Effect of DGEA and  $\beta$ -peptide on the morphological features of UMR106 osteosarcoma-derived cells, compared to control (no peptide, cells plated on type-I collagen).

## CONCLUSIONS

The present results suggest that both  $\alpha$  and  $\beta$  subunits of several integrin receptors are involved in the attachment of osteoblast-like cells to a type-I collagen matrix. The coating of different biomaterials with specific recognition peptides as well as with whole collagen molecules, could improve the osteointegration of implants to be used in bone tissues.

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