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Potential role of a new anti-ß3 integrin antibody in the development of intimal hyperplasia after vascular surgery: an *in vitro* smooth muscle cell model

M.J. Gimeno[1], J. González[2], M. Rodríguez[1], C. Corrales[1], J.M. Bellón[1] and J. Buján[1]

[1]Department of Morphological Sciences and Surgery, Alcala University and [2]Department of Biophysics, CSIC. Madrid, Spain

Summary. The occurrence of intimal hyperplasia after vascular surgery is an ongoing concern in current clinical practice. Among the many factors involved in the development of this pathology, platelet adhesion and myointimal proliferation play a major role. Both these processes are mediated by integrins (mainly $\alpha v\beta 3$ integrins). Over the past years, several substances have been designed to delay or inhibit the cell proliferation that leads to hyperplasia and mainly include monoclonal antibodies directed against integrins. The aim of the present study was to evaluate the effects of an antibody denoted P37 (anti ß3 integrin) on human smooth muscle cells (SMC) and its role in blocking the ß3 subunit. To this end, SMC from human umbilical artery were cultured in the presence or absence of the cell substrate vitronectin (VN) and incubated with P37. After 4 days of treatment, determination was made of cell proliferation and migration. Smooth muscle cells grown on VN showed increased proliferation and migration compared to control VN-free cultures. However, the presence of P37 in the culture medium inhibited proliferation and reduced migration. Combined treatment with VN and P37 led to improved proliferation but VN was unable to reverse the effects on migration observed in the former cultures. Results suggest that *in vitro*, P37 is capable of blocking human SMC ß3 integrins and thus impedes cell proliferation and migration These findings may have clinical implications related to modulation of the development of hyperplasia.

Key words: ß3 integrin, Migration, Proliferation, Smooth muscle cell

Introduction

The appearance of intimal hyperplasia is a phenomenon observed in clinical practice after reparative vascular surgery including prosthesis implant or balloon catheter procedures. The excessive cell proliferation that occurs in this pathology often leads to the appearance of thrombi or to restenosis and hence to the failure of the surgical procedure. It is currently known that many factors, besides platelets, muscle and endothelial cells, are also responsible for intimal progression, including molecules of the extracellular matrix such as adhesion molecules and their receptors. Consequently, it is interactions between cells and matrix components that are responsible for the development of a myointimal layer and the appearance of immune reactions, thrombosis and vessel damage (Schwartz, 1997).

To date, several theories have been developed to explain neointimal formation although it would appear that the proliferation and migration of smooth muscle cells (SMC) is most likely to contribute to the progression of hyperplasia (deRuiter et al., 1997; Majesky and Schwartz, 1997). For SMC to migrate and proliferate, it is necessary that they interact with different extracellular matrix proteins, which mainly include osteopontin and integrins.

Osteopontin is expressed at sites where there is tissue remodelling (Thager et al., 1995). This molecule has been shown to inhibit *in vitro* and *in vivo* cell migration following angioplasty using a balloon catheter (Liaw et al., 1997).

Integrins are a family of heterodimeric glycoprotein subunits designated α and ß. The ß3 subunits mediate important interactions between cells and glycoproteins in response to vascular damage (Shattil, 1995). Further, the subunits that form integrin $\alpha v\beta 3$ (the vitronectin receptor, VN) are commonly associated with SMC migration phenomena. In normal conditions, the $\alpha v\beta 3$ integrin molecule is restricted to the media. Moreover, in atherosclerotic plaques and in certain vascular lesions, it has been shown to colocate with SMC (Hoshiga et al., 1995) and has therefore been related to different vascular diseases.

In current clinical practice, monoclonal antibodies raised against integrin receptors have proved to be of great use after surgical procedures such as angioplasty or

Offprint requests to: Dra. J. Bujan, Department of Morphological Sciences and Surgery, Faculty of Medicine, Alcala University, Ctra N-II km 33.600, 28871 Alcala de Henares, Madrid, Spain. Fax: 91 885 48 85. e-mail: mjulia.bujan@uah.es

vascular repair (Choi et al., 1994; Matsuno et al., 1994; Tam et al., 1998). However, only the so-called ReoPro antibody (abciximab) has been shown to be efficient against the integrins αIIbß3 and αvβ3 (Coller et al., 1991).

Recent *in vivo* experiments performed in our laboratory using a new antibody, P37, directed against the 101-109 aminoacid sequence of the ß3 subunit, demonstrated its efficiency in delaying the appearance of intimal hyperplasia through the effective inhibition of platelet thrombosis (Bujan et al., 1997). With the aim of evaluating the efficiency of the action of this antibody on human SMC and its ability to block the ß3 subunit of αvβ3 integrin, we developed an *in vitro* model using a vitronectin (VN) substrate for SMC cultured in the presence of the P37 antibody.

Materials and methods

Isolation and culture of SMC

Segments of human umbilical cord artery were flushed several times with MEM under sterile conditions and then longitudinally cut open. After removal of the endothelial and adventitial layers by scraping, the medial layer was cut into small explants that were subjected to digestion in a 0.1% type I collagenase solution (Worthington) in MEM (1 h at 37 °C). Cells were subsequently cultivated in F-12 medium (GIBCO BRL) containing 2 mM L-glutamine (Gibco BRL) and supplemented with 10% bovine foetal serum (Gibco BRL), antibiotics (100,000 IU/ml penicilin/10,000 μg/ml streptomycin) (Gibco BRL) and 10 mM Hepes (Gibco BRL) in a humidified 5% CO_2 atmosphere at 37 °C.

Cells were seeded onto sterile glass slides in petri dishes to establish 4 study groups: Group I, SMC cultured in F-12 medium alone; Group II, SMC seeded on a VN substrate (5 μg/ml; SIGMA); Group III, SMC seeded on VN (5 μg/ml) and incubated with the P37 monoclonal antibody (200 nM); and Group IV, SMC incubated with P37 (200 nM). The density of seeding was $5x10^4$ cells per 3.14 cm^2 surface area in 1 ml of culture medium. Cells were left to grow for 48 h until stable and then incubated for 4 days while subjected to the different treatments.

Morphological identification

Smooth muscle cells were identified by immunocytochemistry using the smooth muscle anti-α actin (SIGMA) and total anti-actin (a gift from Dr. Gabianni) primary antibodies, both specific for SMC. Labelling was performed using cells fixed in methanol at -20 °C for 5 min. Rabbit anti-mouse IgG (Zymed Laboratories Inc., CA, USA) and goat anti-rabbit IgG (Nordic) antibodies diluted 1/20 in PBS, pH 7.6 conjugated with FITC were used as secondary antibodies. Labelled specimens were examined using a Zeiss Axiophot microscope equipped with a fluorescence lamp. Cell images were taken using Fujichrome 400 film (Fuji Photo Film Co., Ltd., Tokyo).

Cell proliferation assays

After the corresponding 4-day treatment, the cultured SMC were washed and incubated with ^{3}H-thymidine (2 μCi/ml) for a further 6 h. Cultures were then washed and subjected to autoradiography on the same day. In brief, glass slides were submerged in a (1:1) water dilution of the radiographic emulsion (NTBP-2, Eastman Kodak, Rochester, NY) at 40 °C and kept in the dark for 2 weeks at 4 °C. Glass slides were developed in D-19 (Kodak) for 5 min with agitation. Following a brief rinse in 0.5% acetate, cells were fixed with Rapid Fix (Kodak) for a further 5 min, washed for 1 h in running water and left to dry at room temperature. The SMC were stained with Carrazzi haematoxylin and mounted.

Cell migration assays

After 4 days of treatment, the edge of the cultures was traced and the dispersion area determined using software adapted for computerised image analysis (Optimas 6.5 for Windows, Data Cell Ltd., UK).

In situ DNA fragmentation

The *in vitro* identification of apoptotic cells was performed by modification of the TUNEL method (Negoescu et al., 1996). This method is based on the *in situ* detection of fragmented nucleosomal DNA characteristic of apoptosis by the specific binding of deoxynucleotides to the exposed 3-OH-ends of the fragmented chromatin. Specimens were subjected to microwave irradiation (SANYO EM-704T) for 5 min (350W) in 0.01M citrate buffer (pH 6). Fragmented DNA was determined using a commercial kit (Calbiochem, CN Biosciences Inc., USA).

Cell viability

The number of viable cells was determined by trypan blue exclusion and counted in a Neubauer chamber.

Statistical analysis

Proliferation and migration data were expressed as means ± standard deviation. The Mann-Whitney U-test was used to compare data corresponding to the different treatments. The level of significance was set at $p<0.05$.

Results

Morphological analysis

Changes in cell morphology produced during the 4

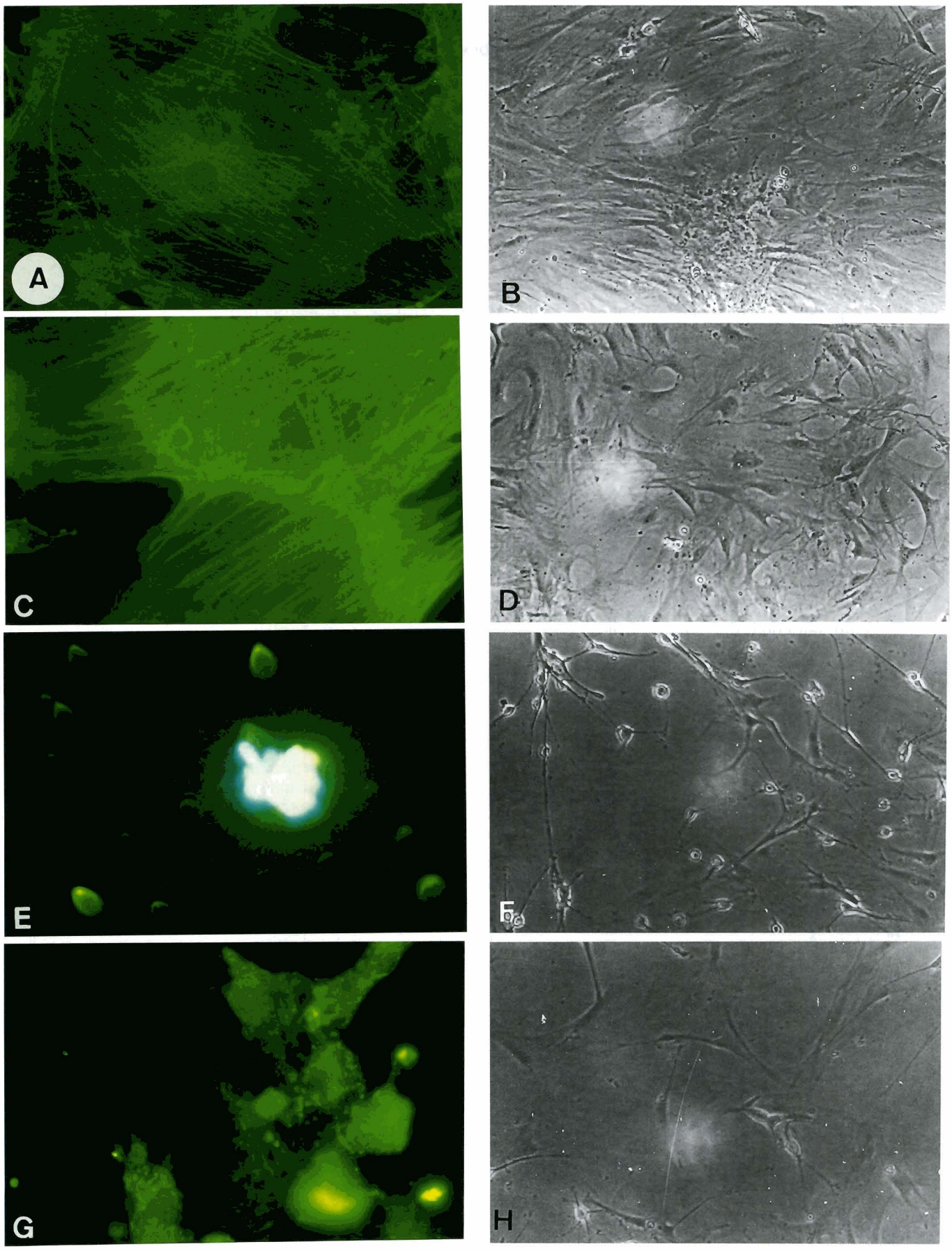


Fig. 1. Morphological findings of SMC cultures; expression of α-smooth muscle actin **(A, C, E, G)** (x 63) and cell shape appearance **(B, D, F, H)** (x 10). SMC control **(A, B)**, SMC seeded onto VN **(C, D)**, SMC incubated in the presence of P37 [200 nM] **(E, F)** and SMC seeded onto VN and incubated with P37 [200 nM] **(G, H)**.

days of culture were monitored in the different treatment groups. Control SMC incubated in F-12 medium alone showed the typical muscle cell morphology including extensive cytoplasm and a high density of microfilaments (Fig. 1a). The presence of these microfilaments was confirmed using the smooth muscle anti-α actin antibody. Cultures showed an even distribution on the glass slide (Fig. 1b). When cells were grown in the presence of the VN substrate (Fig. 1c), this typical morphology was maintained although punctate areas of increased cell density were observed (Fig. 1d). The addition of P37 to the culture medium in the absence of VN led to changes in cell architecture including the observation of retracted cells that had lost the ability to adhere to the substrate, loss of actin microfilaments (Fig. 1e), and changes in cell organisation giving rise to areas of low cell density (Fig. 1f). The combined use of VN and P37 induced similar cell behaviour to that observed in the presence of P37 alone, with cells lacking the morphology of control SMC (Fig. 1g) and a failure to form homogenous cultures (Fig. 1h).

Cell proliferation

The autoradiograph demonstrated the proliferative capacity of the cultures and permitted the determination of growth rates expressed as the number of proliferating cells per thousand. SMC grown in the absence (10.26±2.26) or presence (14.47±0.24) of VN showed similar progression although VN favoured growth such that at 4 days of incubation, significant differences were recorded between these groups. However, the sole presence of the antibody against the P37 ß3 subunit caused the total arrest of cell growth (0±0). In the combined presence of VN and the P37 antibody, the growth rate increased to 2.6 ±0.75.

Cell migration

The dispersion area of the SMC cultures (Fig. 2) was increased compared to that of control cultures in the presence of the VN substrate. The presence of P37 (200 nM) in the culture medium led to a reduction in the cell dispersion area that attained a difference of 50% with respect to control cultures. The combined presence of VN and P37 had no effect on the cell migration observed in the independent presence of the P37 antibody.

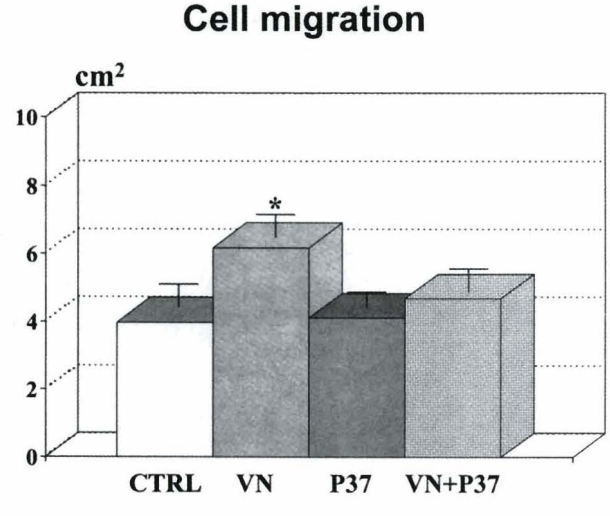
Cell damage

Owing to an insufficient number of cells in cultures grown (Fig. 3) in the presence of the P37 antibody, we were unable to detect a significant difference in the proportion of apoptotic cells between groups using the TUNEL procedure. Labelled cells were detected in all the groups but this was probably the result of the experimental conditions of culture.

Discussion

In a previous study, we were able to show that the P37 antibody is able to delay but not inhibit the appearance of intimal hyperplasia in an *in vivo* model (Buján et al., 1997). Here, we demonstrate that this antibody directed towards the αvβ3 integrin, is an inhibitor of *in vitro* SMC proliferation and migration and that the latter is dependent upon the presence of ligands such as VN that are able to bind the antibody.

To date, numerous molecules have been used as inhibitors of certain integrins with the sole purpose of inhibiting the phenomenon of SMC migration and thus the progression of the intima (Matsuno et al., 1994;



* $p < 0.05$

Fig. 2. The figure illustrate the cell dispersion in the presence/absence of VN as grown substrate and P37 as treatment.

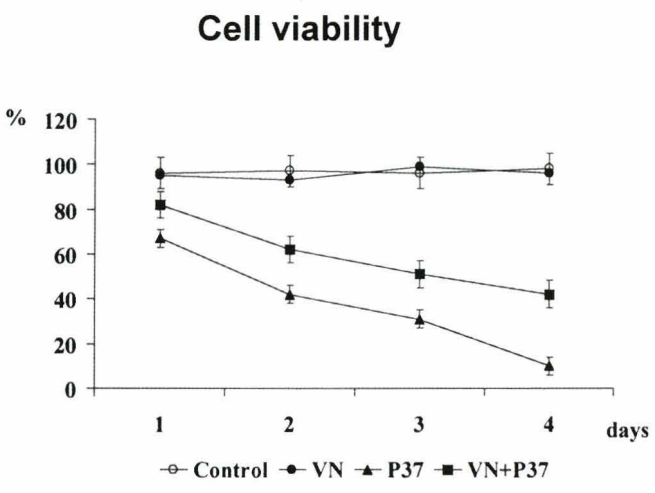


Fig. 3. Smooth muscle cell viability determined by trypan blue staining. Note the decrease in the number of cells when P37 is added to the culture medium.

Buján et al., 1997; Itoh et al., 1997). Many of these substances are antibodies against the fibrinogen receptor (αIIbß3) and are not always capable of blocking other molecules such as the vitronectin receptor (αvβ3) - with the exception of ReoPro (Coller et al., 1991) - such that their *in vivo* effects differ according to the molecules they block (Felding-Habermann and Cheresch, 1993; Clyman et al., 1994; Coller et al., 1995). Apoptotic effects on proliferating cells induced by integrin αvβ3-blocking antibodies such that they act as modulators of tumour progression (Brooks et al., 1994) have occasionally been described. We were unable to demonstrate the presence of apoptotic cells although this analysis was performed after 4 days of incubation with the different treatments. It is likely that cell damage occurs at an earlier stage, given that in experiments performed *in vivo* with this antibody, we were indeed able to detect the presence of TUNEL-positive SMC (data not shown).

In order to evaluate the capacity of these experimental antibodies to inhibit myointimal progression, several authors have analysed their effects on adhesion and migration phenomena of the SMC. The use of different growth substrates has been reported including VN as a stimulator of SMC migration regulated by ß3 integrins (Brown et al., 1994; Jones et al., 1995) and collagen for ß1 integrins (Gotwals et al., 1996). The resulting data permitted the determination of which type of integrins specifically mediated adhesion (ß1, ß3 and ß5) or migration (ß3) (Liaw et al., 1995), expanding knowledge on the role of the ß3 subunit in both these processes. The P37 antibody used in the present study is specific for this ß3 subunit, hence the evaluation of its *in vitro* effects on SMC should give us some idea of its efficiency *in vivo*.

Our finding of increased SMC proliferation in the presence of VN is consistent with the findings of Jones et al. (1996). The higher migration values reported here may be the result of different analytical criteria; we determined the area of cell migration while Jones et al. estimated the number of migrating cells.

The increase in both SMC proliferation and migration induced on the VN substrate provides support for a relevant role of αvβ3 in both these processes. Further, the capacity of the P37 antibody to inhibit cell proliferation is a clear indication of the participation of ß3 integrin in this phenomenon and a possible explanation for previous *in vivo* observations. In this situation, the antibody is able to block the receptors of platelet adhesion to the subendothelial matrix that becomes exposed after injury and thus inhibit thrombosis phenomena delaying the appearance of the neointimal layer (Bujan et al., 1997). The present findings demonstrate the participation of the P37 antibody in yet another process involved in the development of hyperplasia. We were able to show that P37 is able to block the ß3 subunit on the SMC as well as the platelet, thus inhibiting the proliferation and migration of these cells from the medial layer to the site of injury and delaying the formation of a proliferative layer that would eventually lead to vascular occlusion.

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