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In vitro and *in vivo* effects of a cyclic peptide with affinity for the αvβ3 integrin in human melanoma cells

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Abstract

Expression of the integrin αvβ3 has been shown to be associated with increasing metastatic potential in malignant melanoma. It also has a functional role on vascular endothelial cells during angiogenesis. The cyclic oligopeptide cRGDfV is known to bind with high affinity to αvβ3. We have investigated the cellular effects of cRGDfV on a panel of human melanoma cell lines *in vitro* and also on the A375 melanoma cell line growing as xenografts in nude mice. cRGDfV is a potent inhibitor of αvβ3-mediated cell adhesion, however, we have found no convincing evidence that integrin ligation by cRGDfV induces apoptosis in melanoma cell lines. However, cRGDfV when administered subcutaneously into nude mice did inhibit the growth of A375 melanoma xenografts. Histological examination of the tumours indicated that this effect was primarily one of angiogenesis inhibition. The results suggest that agents which target the αvβ3 integrin may have a useful role as anti-angiogenesis agents in clinical oncology, but that they may not exert a direct effect on αvβ3-expressing tumour cells.

Keywords: Melanoma; RGD peptides; Apoptosis; Anoikis; Xenografts

1. Introduction

A number of cellular markers have been associated with human melanoma cells, including the calcium binding protein S100, the glycoprotein MAGE, the intermediate filament HMB 45 and the gangliosides GD3 and GM2 [1]. In addition, the expression of several melanoma-associated molecules is known to increase with tumour progression (as defined by a shift towards increasing malignant behaviour), including the proteoglycan MUC-18 [2] and the integrin αvβ3 [3,4]. One common recognition site for integrin binding to substrates is the amino-acid sequence Arg–Gly–Asp (RGD) [5] and short peptides that contain this sequence will bind to integrins in competition with the native matrix protein [6]. The cyclic peptide cRGDfV binds with high affinity to αvβ3 and to laminin receptors whilst the peptide cRGDFv binds to αvβ3 with lower affinity [7,8].

The role of the αvβ3 integrin in melanoma cells is of considerable interest. Since αvβ3 and other integrins may be important determinants of melanoma cell behaviour, we have investigated the effects on human melanoma cells growing in three-dimensional Type-I collagen gels, of treatment with the peptides cRGDfV and cRGDFv. We have also investigated the effects of subcutaneous injection of cRGDfV into tumour-bearing nude mice.

2. Materials and methods

2.1. Cell lines and culture conditions

The cell lines WM115, WM239a, WM793, 1205Lu, WM164 and 451Lu, originally derived from various stages of melanoma progression were kindly supplied by M. Herlyn of The Wistar Institute, PA, USA. The cell lines WM115 and WM793 were derived from vertical growth phase (VGP) primary melanoma. Cell lines WM239a, 1205Lu, WM164 and 451Lu were derived from metastatic lesions ([9,10]; M. Herlyn, The Wistar Institute, PA, USA, data not shown). The melanoma cell line A375 was obtained from the European Collection of Animal Cell Cultures. The cell lines M21 and M21-L were kindly supplied by A. Montgomery of The Scripps Research Institute, La Jolla, CA, USA and have

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been previously described [11]. The M21-L cell line was selected as having low αv expression by negative selection using repeated fluorescence activated cell sorting (FACS). All melanoma cell lines were cultured in Dulbecco's modified Eagles medium (DMEM) (Gibco, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Normal human umbilical vein endothelial cells (HUVEC) (TCS Biologicals Ltd, Buckingham, UK) were used as a non-transformed control cell population. HUVEC were cultured in Endothelial growth Medium (TCS Biologicals Ltd) supplemented with 2% FCS. All experiments on HUVEC were performed on second passage cells.

A normal human skin fibroblast cell line (VB014) was derived in our laboratory for use as a second non-transformed biological control population. Fibroblasts were cultured in DMEM (Gibco) supplemented with 15% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

All the melanoma cell lines used overexpress the anti-apoptotic protein Bcl-2 (data not shown). All cell lines were maintained at 37°C in an atmosphere of 5% CO_2 in air.

2.2. FACS analysis of av, αvβ3, αvβ5 integrin levels

αvβ3 integrin levels were measured using the monoclonal antibody (MAb) LM609 (Chemicon, Harrow, Middlesex, UK). αvβ5 integrin levels were measured using the MAb P1F6 (Chemicon). αv integrin levels were measured using the MAb P3G8 (Chemicon). Adherent cells were detached using 0.05% EDTA in phosphate-buffered saline (PBS) for 10 min. Cells were washed in PBS and the pellet carefully resuspended in paraformaldehyde (4%). Cells were fixed for 1 h at 0°c and washed in PBS. Saturating quantities of MAb LM609, P1F6, or anti-αv (P3G8) were added to cells in PBS/0.5% bovine serum albumin (BSA), pH 7.2, and incubated at room temperature for 30 min. Cells were washed twice in PBS. Antimouse IgG–*FITC* (fluorescein isothiocyanate) was added to cells in PBS/0.5% BSA, pH 7.2, and incubated at room temperature for 20 min. Cells were washed in PBS and analysed by flow cytometry using a FACScan (Becton Dickinson, Oxford, UK). Isotype IgG immunoglobulins (Dako, Cambridge, UK) were used as controls. The results are reported as relative fluorescence, i.e. mean fluorescence of the sample minus the mean fluorescence of the corresponding isotype control for each measurement.

2.3. Cyclic peptides

cRGDfV, cRGDFv, and cRADfV were produced by Peninsula Laboratories UK Ltd (St Helens, UK) using a cyclisation procedure based on that of Gurrath and colleagues [7]. Upper-case letters denote L-amino acids and lower-case letters denote D-amino acids. Peptides were dissolved in PBS and were used as soon as possible once in solution. Where storage was required, this was at −70°C.

2.4. Demonstration of adhesion independence or anoikis

Cells were seeded into 25 cm^2 tissue flasks which were precoated with a thin layer of 2% agar in order to prevent cell attachment. Cells were sampled at 24-h intervals to assess cell morphology and DNA fragmentation.

2.5. Preparation of collagen gels

Collagen gels were prepared with acid solubilised Type-1 dermal collagen (Cellagen AC-3, ICN) dissolved in 0.1 M HCl. Isotonic collagen solutions were prepared with 2×DMEM and neutralised to pH 7.4 with 0.1 M NaOH. Melanoma cells were resuspended in cold collagen solutions before gelation at 37°C. Gels were overlaid with DMEM which was replaced at 2-day intervals, together with cyclic peptides where required. Cells were extracted from collagen gels by the addition of clostridial collagenase (Sigma) at 160 units/ml collagen and the cell number determined with a haemocytometer.

2.6. Assessment of cell morphology

Melanoma cells were seeded into 24-well tissue culture plates at 1×10^5 cells per well and were suspended in 400 μl aliquots of dermal collagen supplemented with 10% FCS. Spreading of melanoma cells within collagen gels was assessed at 24-h intervals

2.7. Analysis of DNA fragmentation

Melanoma cells were seeded into 6-well tissue culture plates at 1×10^6 cells per well and suspended in 4 ml aliquots of Type-1 collagen. Cells were extracted from the collagen gel after 5 days using clostridial collagenase (Sigma) at 640 units/well and lysed by resuspension in 400 μl lysis buffer (5 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.5% Triton X-100). Cellular debris was removed by centrifugation and the DNA in the supernatant was precipitated at −20°C by the addition of 40 μl 3.0 M sodium acetate, pH 5.2 and 800 μl absolute ethyl alcohol. The pellets were resuspended in 400 μl Tris/EDTA buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Ten microlitres RNase (2 mg/ml) was added and the mixture incubated for 1 h at 65°C. 25 μl of proteinase K (20 mg/ml) was added and the mixture incubated for an additional 2 h at 37°C. The mixture was extracted twice with an equal volume of phenol:chloroform (1:1) and twice with chloroform:isoamyl alcohol

(24:1) and the DNA was then precipitated with 40 μl 3.0 M sodium acetate pH 5.2 and 1.0 ml ethanol at −20°C overnight. The precipitated DNA was resuspended in Tris/EDTA buffer, pH 7.5, and subjected to conventional electrophoresis at 75 V for 2 h on a 1% agarose gel containing 1 μg/ml ethidium bromide and bands were visualised by ultraviolet (UV)-transillumination.

2.8. FACS analysis of DNA fragmentation

Cells were fixed in ice-cold 70% ethanol for at least 1 h. Cells were washed once in PBS and resuspended in 1 ml PBS. Cells were allowed to stand in ice for 1 h prior to staining by the addition of 150 μl ribonuclease (1 mg/ml) and 100 μl propidium iodide (400 μg/ml). The 1-h period standing in ice allows small fragments of DNA to leak out of the cells for the subsequent detection of sub-G1 DNA contents.

2.9. Labelling of melanoma cells with chromium^{51}

Single-cell suspensions were prepared by detaching adherent cells with 0.05% EDTA in PBS for 5–10 min. Cells were washed once and resuspended in DMEM (200 μl). 100 μl of chromium^{51} (approximately 90 μCi) was added to the cells. The cells were incubated for 60 min at 37°C. Unincorporated Cr^{51} was removed by washing the cells three times using DMEM.

2.10. Cell adhesion assays

Flexible 96-well assay plates were coated with extracellular matrix proteins at 5 μg/ml in PBS for laminin, fibronectin and vitronectin (all from Sigma). Type-I collagen (Sigma) was denatured by boiling for 15 min and was coated at 50 μg/ml in PBS. After extracellular matrix (ECM) protein coating, the wells were blocked with 1 mg/ml BSA in PBS. Appropriate concentrations of RGD peptides were added simultaneously with 10^4 melanoma cells (Cr^{51}-labelled) per well. The cells were incubated for 60 min at 37°C. Unbound cells were removed by aspiration and the relative number of cells attached per well was determined using the γ-emission of Cr^{51}, measured using a gamma counter (LKB).

2.11. Electron microscopy

Cells were fixed in 1% (v/v) glutaraldehyde at 4°C overnight and resuspended in 4% agar for further processing. Cells were dehydrated in a graded series (50, 70, 90, 100%) of ethanol followed by embedding in LR White resin. Polymerisation was by the cold chemical catalytic method at 0°C for 24 h. Sections were cut on a LKB III ultramicrotome and stained with uranyl acetate and lead acetate. Samples were viewed in a Phillips EM12 electron microscope operating at 80 kV.

2.12. Measurement of intracellular calcium

Intracellular calcium was measured using the calcium-selective probe Fluo-3, loaded as the acetoxy methyl ester (Fluo-3-AM) [12]. Fluo-3 is a fluorescein derivative and was chosen as the excitation maximum corresponds to the 488 nm emission of the argon-ion laser in the FACScan.

Cells were grown in 400 μl aliquots of heat-denatured type-I collagen for 3 days prior to assay. Fluo-3-AM (Sigma) dissolved in DMSO was added directly to cells to a final concentration of 2 μM. Cells were incubated for 30 min at 37°C. Cells were washed in DMEM and resuspended in DMEM containing 0.01% $CaCl_2$. Samples were analysed by flow cytometry with on-line addition of cyclic RGD peptides. The calcium ionophore ionomycin was used at 2 μM to produce a standard intracellular calcium influx.

2.13. Flow cytometric measurement of end-labelled DNA strand breaks

In situ labelling of apoptosis-induced DNA strand breaks [13] was performed using a modification of the Boehringer *in situ* cell death detection kit (Boehringer Mannheim, East Sussex, UK) which utilises the TUNEL assay (TdT-mediated dUTP nick-end labelling). Briefly, cells were fixed in paraformaldehyde (4% in PBS, pH 7.4) for 30 min at room temperature. Cells were washed in PBS and resuspended in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C. Cells were washed with PBS and resuspended in 50 μl of TUNEL reaction mixture for 60 min at 37°C in the dark. Cells were washed in PBS and resuspended in 25 μg/ml propidium iodide in PBS. Cells were allowed to stain for 15 min prior to analysis by flow cytometry.

2.14. Assay for antitumour activity of *cRGDfV* in vivo

Male nude mice, 6–8 weeks old, were obtained from Charles River UK (Margate, UK). The animals were maintained under specific pathogen-free conditions. A375 melanoma cells (10^6 cells/mouse) in PBS were injected subcutaneously (s.c.) at day 0. Cyclic RGD containing peptides were administered s.c. when the tumour size was at least 2×2 mm. The peptide cRGDfV was administered at a dose of 50 mg/kg/day or 25 mg/kg/day by s.c. injection near the tumour site. A control peptide cRADfV was also used at a dose of 50 mg/kg/day. Tumour size was estimated daily using caliper measurements of tumour diameter in two planes. The experiment was terminated when the control population reached a humane endpoint. Antitumour effects were evaluated by tumour size and histological examination of the tumours at the end of the experiment. All experiments were carried out in accordance with the

UKCCCR guidelines for the welfare of animals in experimental neoplasia.

3. Results

3.1. αv, αvβ3 and αvβ5 integrin expression on melanoma cell lines

Flow cytometric analysis of the melanoma cell lines indicates that all of the cell lines exhibit significant expression of both αv and αvβ3 heterodimer expression (Fig. 1). The difference in expression between the lowest and highest expression of both αv and αvβ3 was approximately 2-fold for all of the cell lines except M21-L, which was negatively selected for reduced αv expression [11]. There was no apparent association between the levels of integrin expression and the nature of the clinical lesion from which the cells were derived. Only one of the cell lines (WM793) exhibited αvβ5 expression as measured by FACS analysis. The cell line M21 expressed significant levels of both αv and αvβ3 heterodimer, whereas the cell line M21-L had reduced expression of both αv and αvβ3, in accordance with previous observations [11].

FACS analysis of the HUVEC indicated that the cells expressed both αvβ3 and αvβ5. FACS analysis of the normal skin-derived fibroblast cell line indicated that it expressed high levels of αvβ5 but not αvβ3.

3.2. Inhibition of cell attachment to extracellular matrix proteins by cRGD

Cell adhesion of melanoma cells to heat denatured Type-I collagen, vitronectin, laminin and fibronectin were assayed in the presence of cRGDfV or cRGDFv. Typical adhesion assays are illustrated in Fig. 2. In all cell lines tested, inhibition of adhesion to fibronectin was incomplete, with a maximum achievable inhibition being in the region of 50%. Moreover, inhibition of adhesion to laminin was only apparent at high concentrations of peptide. IC_{50} values for inhibition of adhesion to Type-I collagen and vitronectin were in the range 100–900 nM for cRGDfV and 1.5–6 μM for cRGDFv (Table 1). Thus, substituting D-phenylalanine with L-phenylalanine in the cyclic peptide causes a reduction in activity, in agreement with the conformation/activity studies of RGD-containing peptides by Gurrath and colleagues [7].

3.3. Evidence for adhesion independence of melanoma cell lines

Melanoma cells maintained in serum-containing or serum-free medium, but prevented from attaching to the surface of the culture flask by a thin layer of agar showed a rounded morphology, but the cells continued to grow and form colonies in suspension. In contrast, normal fibroblasts and normal HUVECs proceeded rapidly to apoptosis (within 24 h) following adhesion inhibition, as shown by the presence of a sub-G1 peak on DNA histograms, positive TUNEL staining and

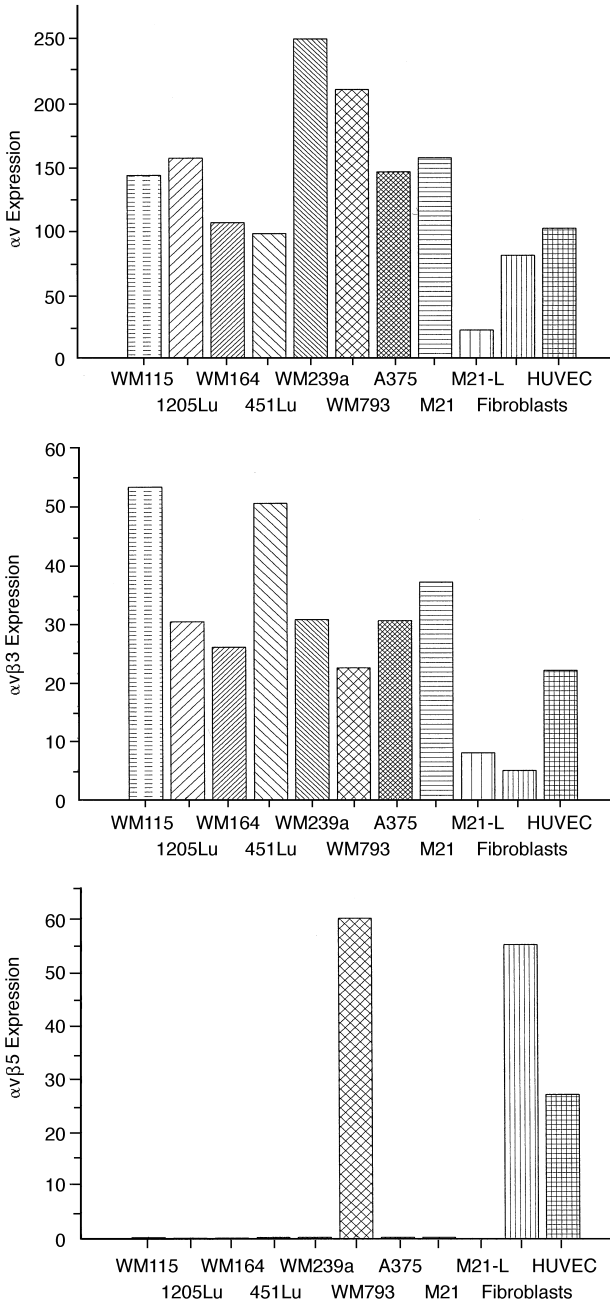

Fig. 1. Relative expression of αv, αvβ3, and αvβ5 integrins as determined by FACS analysis for the panel of melanoma cell lines, normal skin-derived fibroblasts, and HUVEC cells. Measurements were performed at least three times and typical results are shown. The results are expressed as relative fluorescence, i.e. mean fluorescence of the sample minus the mean fluorescence of the corresponding isotype control for each measurement.

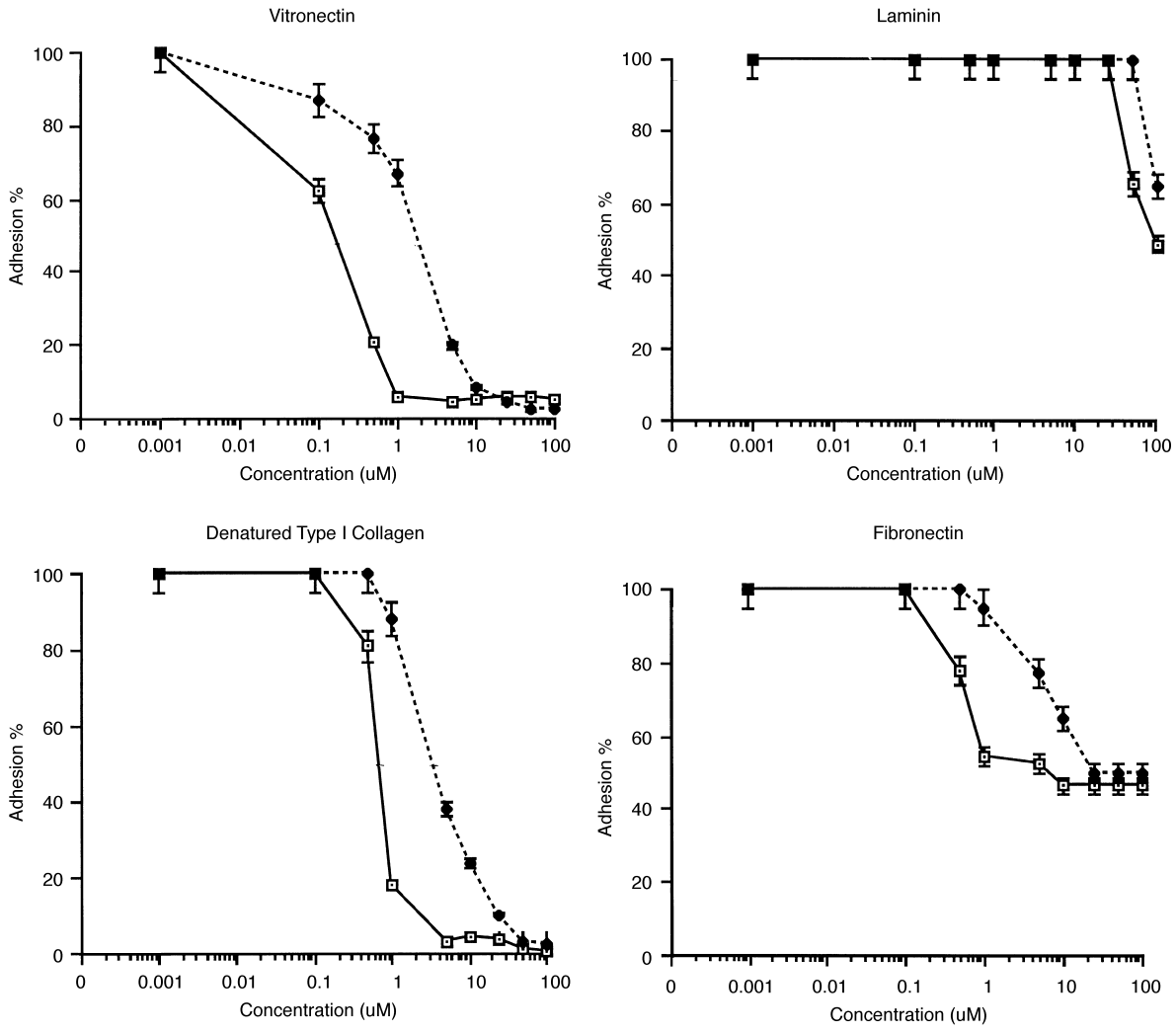

Fig. 2. Typical adhesion assay data obtained for the cell line 1205Lu treated with either cRGDfV (solid line) or cRGDFv (dotted line) in the range 0.001–100 μM. This pattern of results was broadly similar for all of the melanoma lines tested.

DNA fragmentation evidenced on agarose gels (Fig. 3). After 48 h in suspension over 98% of the cells had proceeded to fragment and disintegrate.

3.4. Integrin regulation of cell morphology in 3-dimensional collagen gels

Cells were grown in 400 μl collagen gels submerged in serum-free DMEM for 5 days. After 3 days, there was a profound difference in the morphology of control cells and cells grown in the presence of cyclic peptides (10 μM). Whilst untreated control cells remained attached and were forming colonies, treated cells were showing evidence of cell rounding and shrinkage although still apparently forming colonies, since cell numbers were similar in both peptide-treated and untreated wells. These observations were similar for both cRGDfV and cRGDFv, whereas cRADfV had no

Table 1
IC_{50} values (μM) for inhibition of adhesion to extracellular matrix proteins by cyclic RGD peptides

	WM115		WM793		WM239a		WM164		1205Lu		451Lu	
	cRGDfV	cRGDFv	cRGDfV	cRGDFv	cRGDfV	cRGDFv	cRGDfV	cRGDFv	cRGDfV	cRGDFv	cRGDfV	cRGDFv
Vitronectin	0.1	1.5	0.2	2.5	0.3	4.0	0.2	2.0	0.15	3.0	0.4	6.0
Heat denatured Type-I collagen	0.4	4.0	0.2	3.5	0.5	6.0	0.9	4.0	0.2	4.0	0.4	6.0
Laminin	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100

effect on cell morphology. Transmission electron microscopy of the RGD-treated melanoma cells did not indicate any aberrant morphological features other than a rounding up of the cells, and no evidence of chromatin margination or other features consistent with apoptosis (Fig. 4).

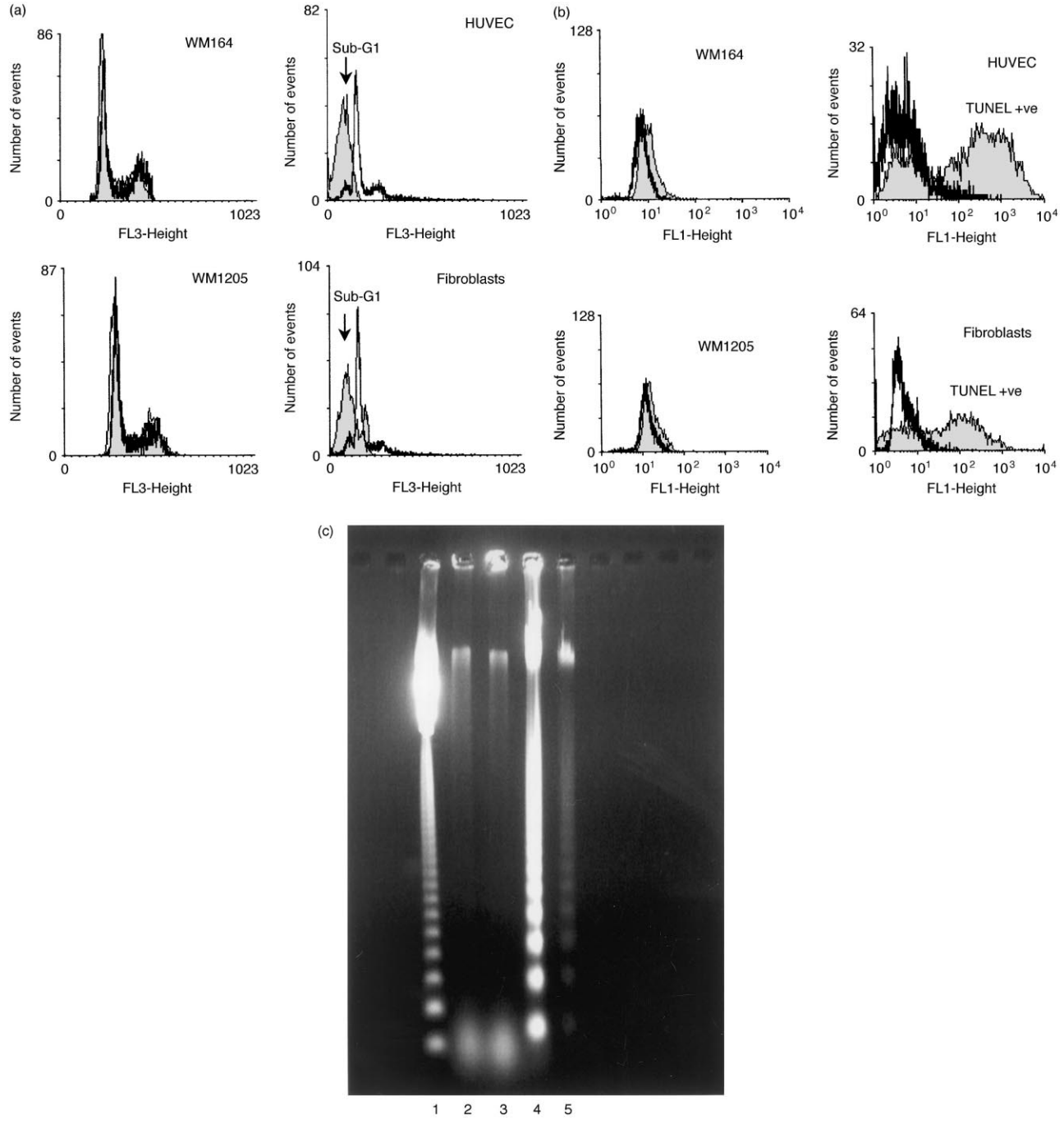

Fig. 3. (a) Evidence of apoptosis induced by adhesion inhibition (anoikis) in agar-coated flasks. The two melanoma cell lines were grown in serum-free conditions, whereas HUVEC and fibroblasts were grown in the presence of 10% fetal calf serum (FCS). The two normal cell lines (fibroblasts and HUVEC) show evidence of a sub-G1 peak by FACS analysis of DNA content, which is a typical feature of apoptosis. Shaded histograms represent cells which have been prevented from attaching to the plastic surface, compared with open histograms obtained from normally attached cells. (b) Evidence of apoptosis induced in cells by adhesion inhibition (anoikis) in agar-coated flasks. The two normal cell lines (fibroblasts and HUVEC) show positive TUNEL staining by FACS analysis, indicating DNA strand breakage, a typical feature of apoptotic cell death. Shaded histograms represent cells which have been prevented from attaching to the plastic surface, compared with open histograms obtained from normally attached cells. (c) Evidence of apoptosis induced by adhesion inhibition (anoikis) in agar-coated flasks. Lane 1, 123 BP standards; lane 2, WM164 melanoma cells; lane 3, 1205Lu melanoma cells; lane 4, normal fibroblasts; lane 5, HUVEC. The two normal cell lines show DNA fragmentation (laddering) typical of apoptosis following 18 h of adhesion inhibition.

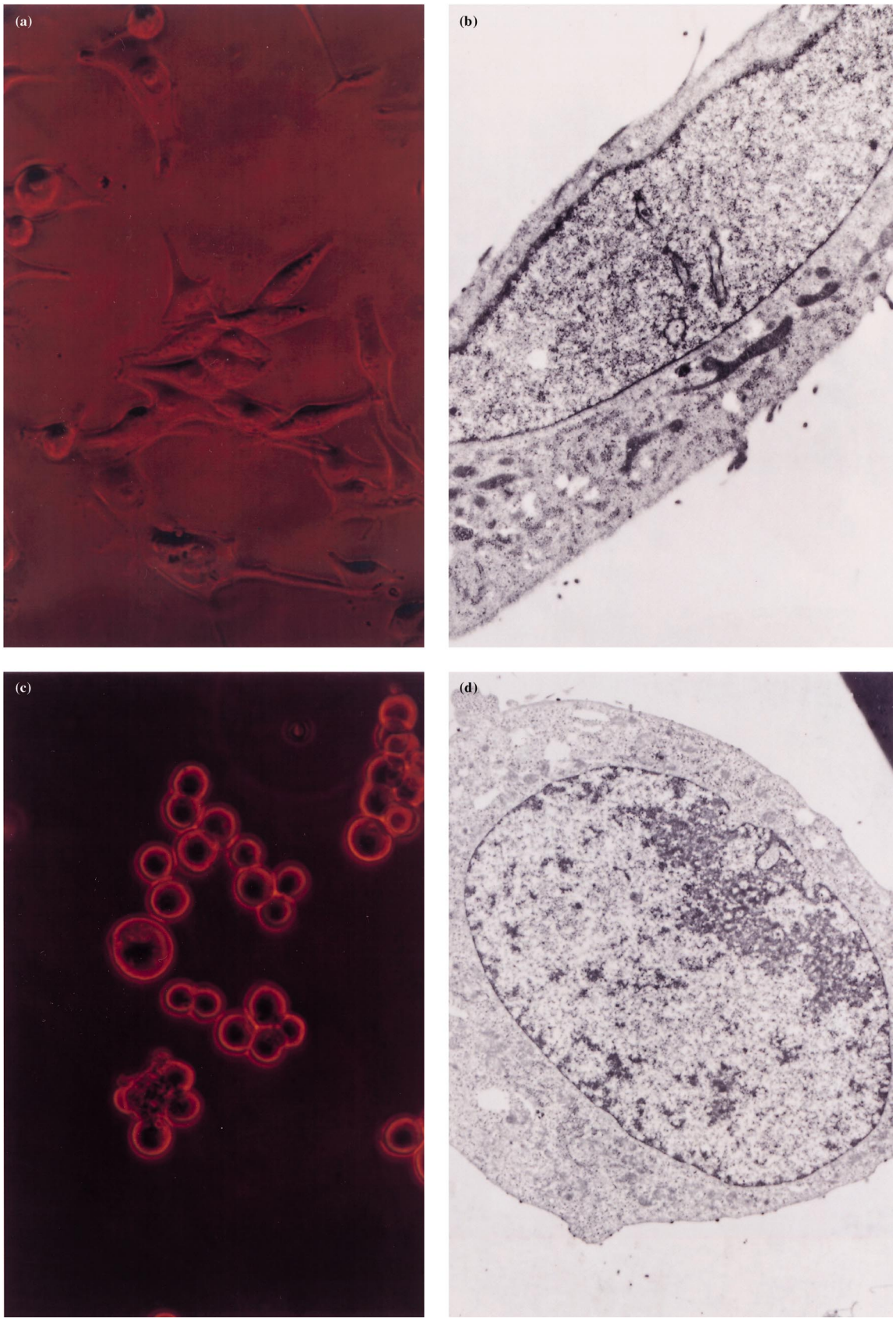

Fig. 4. Effects on cell morphology of 10 μM cRGDfV on WM115 melanoma cells embedded in three-dimensional collagen gels. (a) and (b) show control cells which exhibit a normal spread morphology and colony formation. (c) and (d) show cells treated with cRGDfV (10 μM). The cells show a rounded appearance but no obvious signs of apoptosis by transmission electron microscopy.

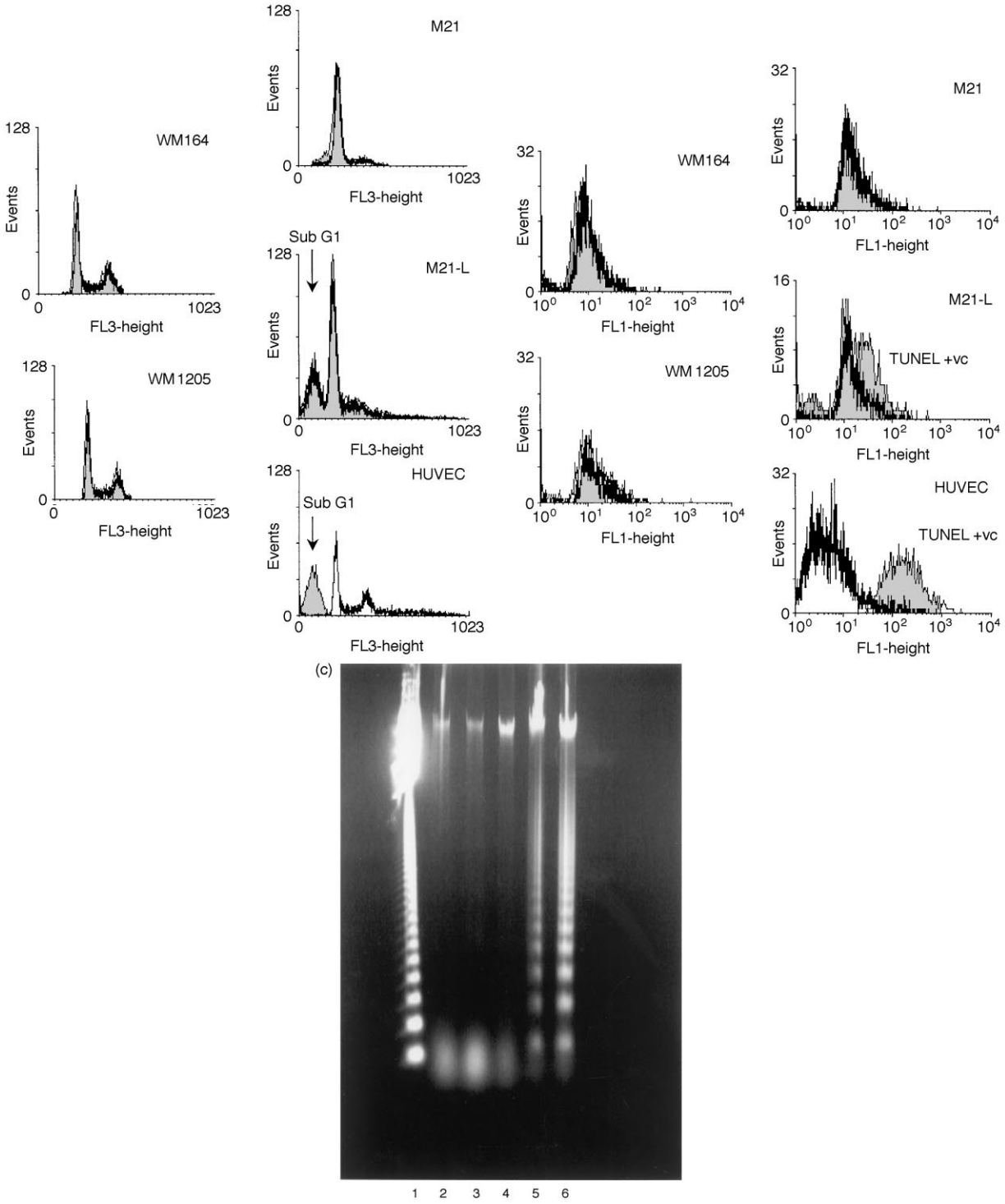

Fig. 5. (a) Evidence of apoptosis induced by treatment of cells embedded in three-dimensional collagen gels with cRGDfV (10 μM) for 5 days. Of the melanoma cell lines only M21-L shows evidence of a sub-G1 DNA peak. The data for HUVEC were obtained 15 h following addition of the peptide. All cells were grown in serum-free conditions except for HUVEC which were grown in the presence of 10% fetal calf serum (FCS). Shaded histograms represent cells which have been treated with cRGDfV compared with untreated control cells (open histograms). (b) Evidence of apoptosis induced by treatment of cells embedded in three-dimensional collagen gels with cRGDfV (10 μM) for 5 days. Of the melanoma lines only M21-L shows positive TUNEL staining. The data obtained for HUVEC were obtained 15 h following addition of the peptide. All cells were grown in serum-free conditions except for HUVEC, which were grown in the presence of 10% FCS. Shaded histograms represent cells which have been treated with cRGDfV compared with untreated control cells (open histograms). (c) Evidence of apoptosis induced by treatment of cells embedded in three-dimensional collagen gels with cRGDfV (10 μM) for 5 days. Lane 1, 123 BP standards; lane 2, WM164 melanoma cells; lane 3, 1205Lu melanoma cells; lane 4, M21 melanoma cells; lane 5, M21-L melanoma cells; lane 6, HUVEC. Of the melanoma lines only M21-L shows any evidence of DNA fragmentation. The data for HUVEC were obtained 15 h following addition of the peptide.

3.5. Evidence for cRGD-induced apoptosis in melanoma cells

Cells were grown in 400 μl collagen gels submerged in serum-free DMEM, or on surfaces coated with Type-I collagen. Melanoma cells grown in the presence of cRGDfV (10 μM) showed a lack of cell attachment and a rounded morphology but no evidence of cell death after 5 days as measured by DNA fragmentation on agarose gels, the presence of a sub-G1 peak, or positive TUNEL staining (Fig. 5). The cell line M21-L proceeded to apoptoses when grown in Type-I collagen even in the absence of cRGDfV with approximately 30–50% of the cells undergoing apoptosis in any one experiment. This value was not increased in the presence of cRGDfV or in the presence of monoclonal antibody LM609 (data not shown).

HUVEC exposed to cRGDfV (10 μM) proceeded rapidly to apoptosis (within 24 h) in 100% of cells. HUVEC were sensitive to cRGDfV even when grown on plain plastic in the absence of collagen (data not shown).

3.6. cRGD peptides induce a calcium transient in melanoma cells

Intracellular calcium was measured using the calcium-selective probe Fluo-3. Controlled calcium transients were induced using the calcium ionophore ionomycin. Under the experimental conditions used here, 2 μM ionomycin increases the free intracellular calcium concentration by 100% for approximately 10 min (Fig. 6). For all of the cell lines tested both cRGDfV (10 μM) and cRGDFv (10 μM) induced an increase in intracellular free calcium concentration of approximately 70% (compared with the control sample) after 3 min in contact with the peptides. The calcium levels slowly declined to initial levels over a period of approximately 12 min. The control peptide cRADfV elicited no change in the intracellular calcium concentration.

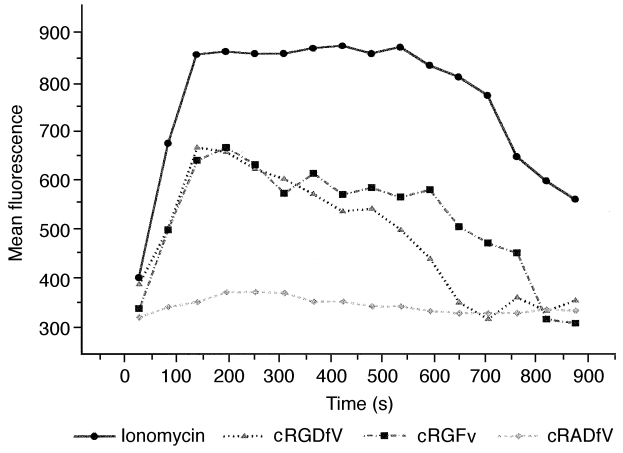

Fig. 6. Intracellular calcium was measured using the selective probe Fluo-3-AM (2 μM) and kinetic analysis of FACS data following online reagent addition. Control cells were treated with 2 μM ionomycin to induce a standard calcium flux. Cells treated with cRGDfV or cRGDFv (10 μM) produced an intracellular calcium transient within 2 min, with a duration of 7–10 min.

3.7. In vivo activity of cRGDfV

The effects of cRGDfV administered to nude mice bearing A375 melanoma xenografts was assessed daily by tumour size measurements. cRGDfV was administered s.c. near the tumour site at a dose of either 50 mg/kg/day or 25 mg/kg/day. A control peptide cRADfV was also used at 50 mg/kg/day.

Size measurements of the tumours at the end of the experimental period indicated that cRGDfV administered at a dose of 50 mg/kg/day elicited an antitumour response resulting in a mean decrease in tumour wet weight compared with control animals of approximately 50% and a mean decrease in tumour diameter of approximately 66% compared with control animals (Fig. 7). In contrast, administration of 50 mg/kg/day

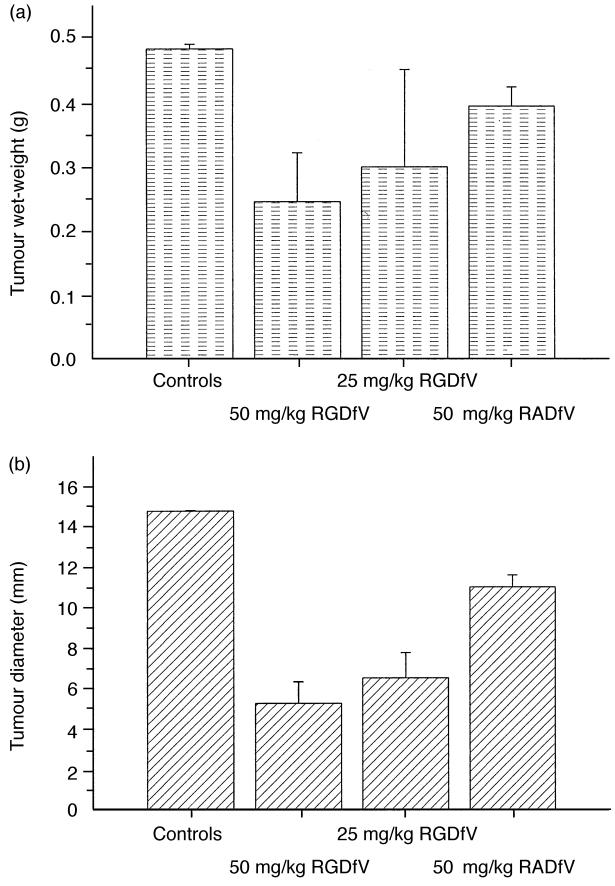

Fig. 7. Wet weight determinations and mean tumour diameters of excised tumours following a 16-day period of drug treatment. Diameters were measured in two planes and the mean value used for any one sample. The experimental groups comprised the following numbers: controls, $n=3$; 50 mg/kg cRGDfV, $n=7$, 25 mg/kg cRGDfV; $n=2$, 50 mg/kg cRADfV, $n=3$.

RADfV resulted in a corresponding disease of approximately 20% and 25%, respectively.

Examination of the mean tumour growth rates estimated from the increase in mean tumour diameter versus time (Fig. 8) indicated that the doubling times for the tumours in the different experimental groups were as follows: controls = 7.6 days; 50 mg/kg/day cRADfV = 6.9 days; 25 mg/kg/day cRGDfV = 10.1 days; 50 mg/kg/day cRGDfV = 20.2 days. Thus, the control peptide cRADfV was confirmed as having no effect on tumour growth rate, whereas the peptide cRGDfV had a marked effect in terms of reducing tumour growth rate at 50 mg/kg/day and an intermediate effect at 25 mg/kg/day.

3.8. Histological evaluation of cRGDfV-treated tumours

The histology of control tumours versus tumours treated with 50 mg/kg/day cRGDfV is compared in Fig. 9. Those tumours treated with the peptide cRGDfV do not show any evidence of an increase in the number of apoptotic tumour cells or any apparent difference in the mitotic rate of the tumour cells, however, there was a marked decrease in the number of histologically recognisable blood vessels in the capsular region of peptide-treated tumours. The blood vessels that were present in the treated tumours also mainly appeared to be mature with little evidence of new vessel formation. In order to quantify this observation, the number of histologically recognisable vessels per unit area in the peripheral capsular region (Fig. 9a and c) of the tumour were counted (Fig. 10). These measurements indicate that those tumours which responded to treatment with cRGDfV exhibited a reduction in the number of blood vessels per mm^2 of approximately 85%.

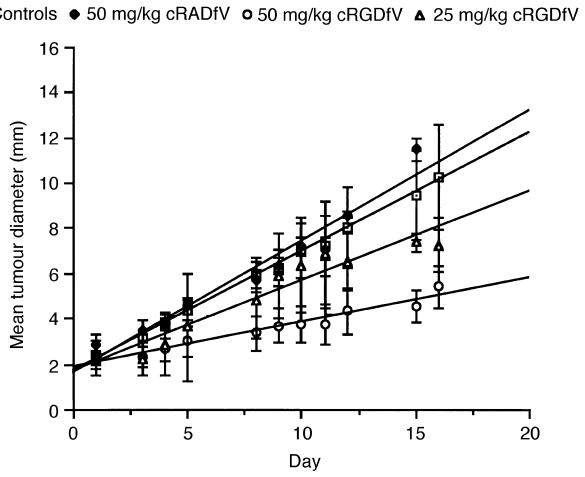

Fig. 8. Growth curves of experimental tumours. Tumour size was measured as mean tumour diameter, with the mean being obtained from caliper measurement in two different planes.

4. Discussion

Recently it has been shown that mutant melanoma cells which lack the *αv* gene lose the ability to form tumours in nude mice. Transfection of these mutant cells to restore the function of the *αv* gene restores their tumorigenicity [14]. Melanoma cells growing in Type-I collagen, though they initially attach via α1β1, α2β1 and α3β1, produce degradative enzymes which expose a cryptic RGD site on the collagen molecule, and which result in high affinity binding to αvβ3 instead [15]. This may be analogous to the effects of melanoma cells growing *in vivo*, as they produce matrix-degrading enzymes, and a similar effect is seen with heat-denatured Type-I collagen [11,15]. Interference with the function of αvβ3 in melanoma cells by generating αv-deficient mutants as before not only inhibits their growth in Type-I collagen gels, but also induces apoptosis, a phenomenon also seen in fibroblasts prevented from attaching to fibronectin and recently termed anoikis [16]. Mutant cells which would otherwise undergo apoptosis in collagen gels can be 'rescued' by transfection with the missing *αv* gene. However, this phenomenon has not previously been sought in intact melanoma cells expressing αvβ3, or in melanoma cells other than the melanoma cell line M21. Antagonists of αvβ3 such as cRGDfV or the monoclonal antibody LM609 are under investigation as anti-angiogenesis agents [17–19], since proliferating endothelial cells that express αvβ3 can be induced to undergo apoptosis by injecting such agents, without an apparent effect on normal, non-proliferating endothelium. It has recently been demonstrated in lymphocytes and MCF-7 cells that induction of apoptosis by RGD peptides is by direct activation of caspase-3 [20]. There are over 20 different αβ subunit heterodimer integrins. Approximately half of these will bind the amino acid sequence RGD. RGD-containing peptides have previously been shown to inhibit tumour cell invasion, metastasis and angiogenesis [18,21–23]. It is possible to confer a degree of binding specificity to RGD-containing peptides by manipulating the conformation of the critical binding site. This can be done either by altering the amino acid sequence adjacent to the RGD sequence, or by cyclising the peptide, a process which would also render it less susceptible to enzymatic degradation if injected *in vivo* [24]. Therefore, we have investigated the activity of cyclic RGDfV, both *in vitro* and *in vivo* on human melanoma cells.

All of the melanoma cell lines we have tested express high levels of αv and αvβ3, despite their origin from different clinical stages of disease. It is noteworthy, that cRGDfV inhibits adhesion to native Type I-collagen, heat-denatured Type-I collagen, or vitronectin at 10-fold lower concentrations than cRGDFv. Inhibition of adhesion to laminin by cRGDfV was only apparent at a

high concentration and inhibition of adhesion to fibronectin was incomplete with only 50% inhibition even at high concentrations of cRGD. The observation that cRGD peptides inhibited to a much lesser extent adhesion to laminin and fibronectin than the other substrates was to be expected since fibronectin is known to bind to at least eight, and laminin to at least five integrins.

Most types of normal cells require attachment to extracellular matrix in order to proliferate and differentiate, whereas this requirement is reduced or absent in

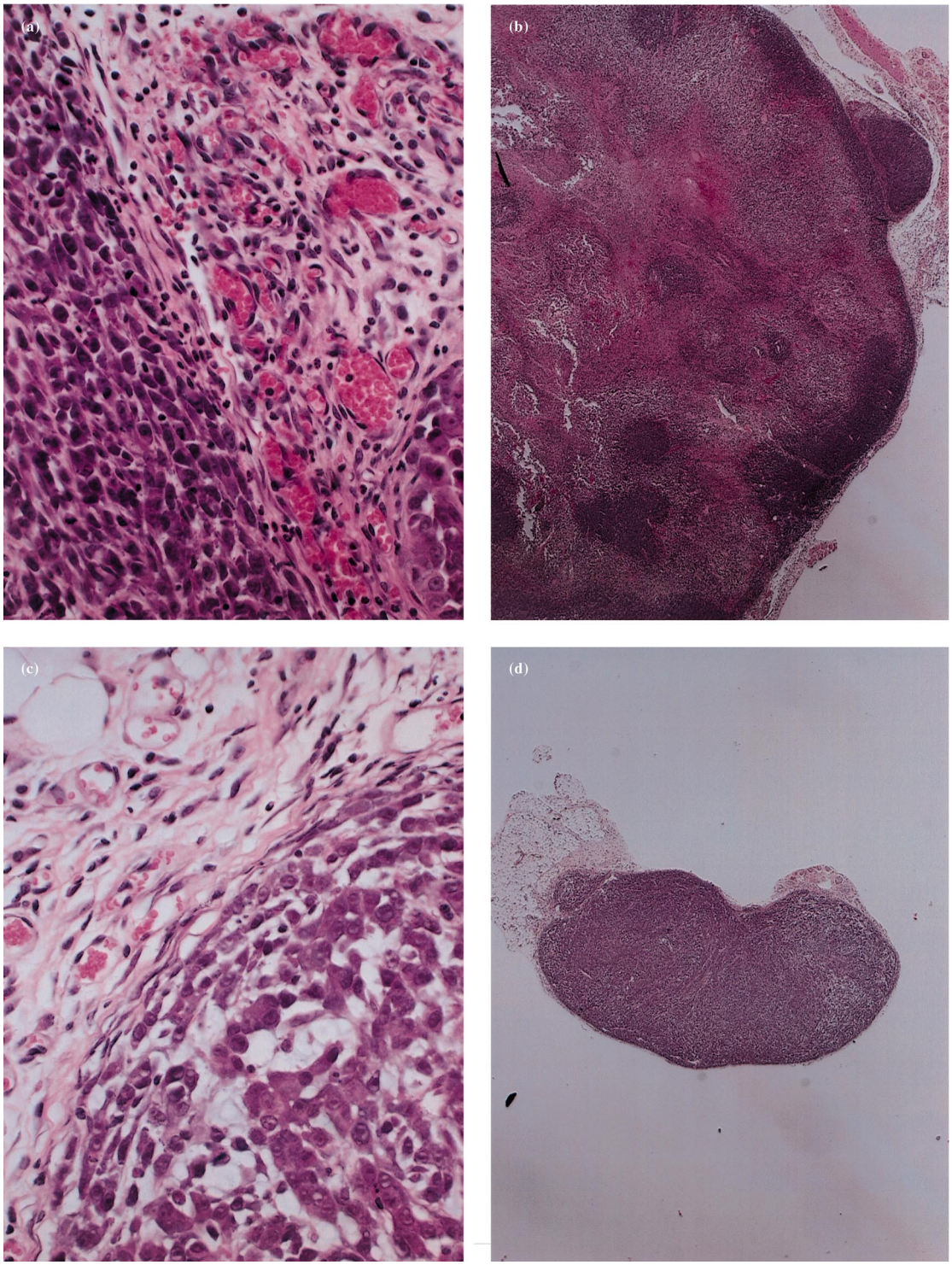

Fig. 9. A375 melanoma xenografts. (a) and (b): an untreated control tumour after 16 days of tumour growth. Tumours show extensive vascularisation. (c) and (d): a tumour treated with cRGDfV (50 mg/kg/day) after 16 days of tumour growth. The tumour shows an obvious reduction in size and decrease in the number of blood vessels infiltrating the periphery of the tumour. (a) and (c); and (b) and (d) are to the same scale.

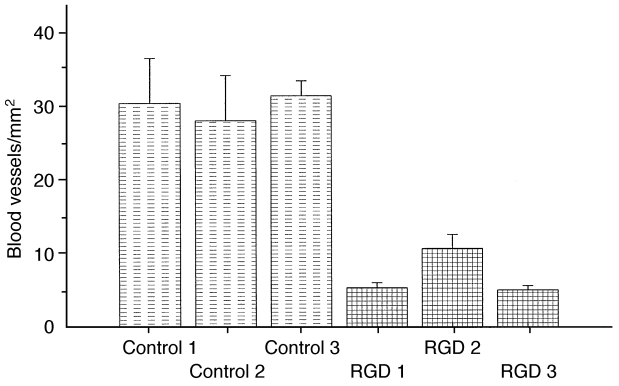

Fig. 10. Comparison of the number of blood vessels per mm^2 for three control tumours and three tumours which responded to treatment with cRGDfV. Counts were obtained by microscopic examination of three areas of the periphery of each tumour chosen at random. The mean counts are displayed.

transformed cells [25]. In addition to regulating cell growth and differentiation, the extracellular matrix is now known to be an essential cell survival factor for many cell types. Thus, matrix-adhesion inhibition has been shown to activate apoptosis in endothelial and epithelial cell types [16,26,27], and integrin-mediated events appear to be intimately involved in adhesion-inhibition of the induction of apoptosis. In contrast, anti-β1 integrin antibodies have been demonstrated to prevent detachment-induced apoptosis in epithelial cells [26]. Our observations that melanoma cells can survive and grow in an adhesion-independent manner, whereas non-transformed cells (fibroblasts and HUVEC) cannot is in agreement with previous observations. We were unable to induce apoptosis of the M21 cell line growing in three-dimensional collagen gels using cRGDfV [11]. The cell line M21-L did spontaneously proceed to apoptosis when grown in Type-I collagen in the absence of cyclic peptides, however, this was only apparent in approximately 30–50% of the population. This may reflect the fact that in M21-L cells in our culture system the αv integrin has not been completely abrogated, but rather, it expresses low levels of αv as determined by FACS analysis. We were unable to increase the amount of apoptosis using cRGDfV.

Apoptosis of epithelial cells in suspension can be prevented by transformation with V-Ha-ras or V-Src [27], or by Bcl-2 overexpression [27,28]. It is noteworthy that all of the melanoma cell lines used in our study over-express Bcl-2 (data not shown) and this may be another reason for our inability to reliably induce apoptosis by integrin blockade. It has previously been shown that soluble fibronectin binds to active α5β1 via an RGD binding site to upregulate Bcl-2 expression [28], although it is not clear at present to what extent the cell lines used in our study express α5β1 as expression in the FACS analysis was, with the exception of WM793, uniformly low.

In non-transformed cells αvβ3 ligation suppresses p53 and the p53-induced $P21^{WAF1/CIP1}$ cell cycle inhibitor and increases the Bcl/Bax ratio. Inhibitors of αvβ3 cause the reverse effect and thus induce apoptosis (in non-transformed cells) [29]. From our results, it would appear that blockade of the αvβ3 integrin is not adequate for inducing apoptosis in malignant melanoma cells which continuously express high levels of Bcl-2.

Our results do suggest that cRGDfV will act *in vivo* to prevent tumour growth and histological examination of the excised tumours were compatible with an anti-angiogenesis mechanism.

It has been shown previously that αvβ3 blockade induces apoptosis in newly formed blood vessels and causes regression of tumours *in vivo*, but does not affect pre-existing quiescent blood vessels [17], an observation that was also observed in ocular neovascular disease [30].

In conclusion, we suggest that αvβ3 is a good target for anti-angiogenesis therapy and for reducing tumour cell invasion and metastasis, but not for inducing apoptosis in melanoma cells which express αv at high levels. We also show that cyclic RGD-containing peptides have potent antitumour activity *in vivo*, primarily by the mechanism of angiogenesis inhibition. Further work is required to determine the precise mechanism of this inhibition and also to determine the optimum drug dose and delivery route to optimise the antitumour effect. Overall, the results are in agreement with previous observations suggesting that such approaches are likely to be of benefit in the clinical setting.

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References

1. Carrel S, Rimoldi D. Melanoma associated antigens. *Eur J Cancer* 1993, **29A**, 1903–1907.
2. Johnson JD, Rothbacher U, Sers C. The progression associated antigen MUC18: a unique member of the immunoglobulin supergene family. *Melanoma Res* 1993, **3**, 337–340.

3. Hart IR, Birch M, Marshall JF. Cell adhesion receptor expression during melanoma progression and metastasis. *Cancer Met Rev* 1991, **10**, 115–128.
4. Gehlsen KR, Davis GE, Sriramarao P. Integrin expression in human melanoma cells with differing invasive and metastatic properties. *Clin Exp Metastasis* 1992, **10**, 111–120.
5. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 1984, **309**, 30–33.
6. Hautanen ETA, Gailit J, Mann DN, Ruoslahti E. Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. *J Biol Chem* 1989, **264**, 1437–1442.
7. Gurrath M, Muller G, Kessler H, Aumailley M, Timpl R. Conformation/activity studies of rationally designed potent anti-adhesive RGD peptides. *Eur J Biochem* 1992, **210**, 911–921.
8. Pfaff M, Tangemann K, Muller B, *et al*. Selective recognition of cyclic RGD peptides of NMR defined conformation by αIIbβ3, αvβ3 and α5β1 integrins. *J Biol Chem* 1994, **269**, 20233–20238.
9. Herlyn MH, Balaban G, Bennicelli J, *et al*. Primary melanoma cells of the vertical growth phase: similarities to metastatic cells. *J Natl Cancer Inst* 1985, **74**, 283–289.
10. Herlyn M, Thurin J, Balaban G, *et al*. Characteristics of cultured human melanocytes isolated from different stages of tumour progression. *Cancer Res* 1985, **45**, 5670–5676.
11. Montgomery AMP, Reisfeld RA, Cheresh DA. Integrin αvβ3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc Natl Acad Sci USA* 1994, **91**, 8856–8860.
12. Rijkers GT, Justement LB, Griffioen AW, Cambier JC. Improved method for measuring intracellular Ca^{++} with Fluo-3. *Cytometry* 1990, **11**, 923–927.
13. Gorczca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells in the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993, **53**, 1945–1951.
14. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of the αV gene in human melanoma tumorigenicity. *J Clin Invest* 1992, **89**, 2018–2022.
15. Davis GE. Affinity of integrins for damaged extracellular matrix: αvβ3 binds to denatured collagen type 1 through RGD sites. *Biochem Biophys Res Commun* 1992, **182**, 1025–1031.
16. Ruoslahti E, Reed J. Anchorage dependence, integrins and apoptosis. *Cell* 1994, **77**, 477–478.
17. Brooks PC, Montgomery AMP, Rosenfeld M, *et al*. Integrin αvβ3 antagonists promote tumour regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994, **79**, 1157–1164.
18. Brooks PC, Stromblad S, Klemke R, Visscher D, Sarker FH, Cheresh DA. Anti-integrin αvβ3 blocks human breast cancer growth and angiogenesis in human skin. *J Clin Invest* 1995, **96**, 1815–1822.
19. Hammes HP, Brownlee M, Joncyk A, Sutter A, Preissner KT. Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularisation. *Nature Med* 1996, **2**, 529–533.
20. Buckley CD, Pilling D, Henriquez NV, *et al*. RGD peptides induce apoptosis by direct caspase-3 activation. *Nature* 1999, **397**, 534–539.
21. Kumagai H, Tajima M, Ueno Y, Giga-hama Y, Ohba M. Effect of cyclic RGD peptide on cell adhesion and tumour metastasis. *Biochem Biophys Res Commun* 1991, **177**, 74–82.
22. Kawahara E, Imai K, Kumagai S, Yamamoto E, Nakanishi I. Inhibitory effects of adhesion oligopeptides on the invasion of squamous carcinoma cells with special reference to implication of alpha v integrins. *J Cancer Res Clin Oncol* 1995, **121**, 133–140.
23. Park RH, Yun I. Role of VLA-integrin receptor in invasion and metastasis of human fibrosarcoma cells. *Cancer Letts* 1996, **106**, 227–233.
24. Foster MR, Hornly EJ, Brown S, Kitchin J, Hann M, Ward P. Improved potency and specificity of Arg–Gly–Asp (RGD) containing peptides as fibrinogen receptor blocking drugs. *Thrombosis Res* 1993, **72**, 231–245.
25. Folkman J, Moscona A. Role of cell shape in growth control. *Nature* 1978, **273**, 345–349.
26. Meredith JE, Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 1993, **4**, 953–961.
27. Frisch SM, Francis H. Disruption of epithelial cell-matrix interaction induces apoptosis. *J Cell Biol* 1994, **24**, 619–626.
28. Zhang Z, Vuori K, Reed JC, Ruoslahti E. The α5β1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci USA* 1995, **92**, 6161–6165.
29. Stromblad S, Becker JC, Yebra M, Brooks PC, Cheresh DA. Suppression of p53 activity and $p21^{WAF1/CIP1}$ expression by vascular cell integrin αvβ3 during angiogenesis. *J Clin Invest* 1996, **98**, 426–433.
30. Friedlander M, Theesfeld CL, Sugita M, *et al*. Involvement of integrins αvβ3 and αvβ5 in ocular neovascular disease. *Proc Natl Acad Sci USA* 1996, **93**, 9764–9769.