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Effects of collagenase-cleavage of type I collagen on $\alpha_2\beta_1$ integrin-mediated cell adhesion

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SUMMARY

In this paper we show that collagenase-3 cleavage of type I collagen has a marked effect on $\alpha_2\beta_1$ integrin-mediated interactions with the collagen fragments generated. Isolated $\alpha_2\beta_1$ integrin and α_2 integrin A-domain were found to bind to both native collagen and native $\frac{3}{4}$ fragment and, to a lesser degree, native $\frac{1}{4}$ fragment. Whole integrin and integrin A-domain binding were lost after heat denaturation of the collagen fragments. At physiological temperature, cell adhesion to triple-helical $\frac{3}{4}$ fragment via $\alpha_2\beta_1$ integrin was still possible; however, no $\alpha_2\beta_1$ integrin-mediated adhesion to the $\frac{1}{4}$ fragment was observed. Unwinding of the collagen fragment triple helices by heating to physiological temperatures prior to adsorption to plastic tissue culture plates resulted in total abrogation of HT1080 cell attachment to either fragment. These results provide significant evidence in support of a role for matrix-metalloproteinase cleavage of the extracellular matrix in modifying cell-matrix interactions.

Key words: Type I collagen, Collagenase-3, $\alpha_2\beta_1$ integrin, A-domain, Cell adhesion

INTRODUCTION

In order to invade and metastasize, it is necessary for malignant tumour cells to cross the basement membrane and penetrate the connective tissue stroma. It has been postulated that this process involves tumour cell production and/or activation of proteolytic enzymes, particularly matrix metalloproteinases (MMPs), which degrade the extracellular matrix (ECM) (reviewed by Murphy et al., 1989; Stetler-Stevenson et al., 1993; Mignatti and Rifkin, 1993). MMP cleavage of ECM components may also have more subtle effects on cell-matrix interactions, possibly modulating cell attachment to and/or movement through ECM proteins.

MMPs are zinc-dependent enzymes that are active at neutral pH and cleave ECM proteins (reviewed by Murphy and Reynolds, 1993; Murphy and Knäuper, 1997). MMPs are predominantly secreted in a pro form and are activated in close proximity to the cell surface by other active MMPs (Sato et al., 1994; Atkinson et al., 1995) or by serine proteinases (Gavrilovic and Murphy, 1989; Matrisian, 1992). MMP activity is tightly regulated in vivo by tissue inhibitors of metalloproteinases (TIMPs). ECM degradation by MMPs has been implicated during both normal and pathological events; for example, upregulation of MMPs by tumours and surrounding connective tissue stroma is a common feature of many cancers, and it is postulated that MMPs are necessary for tumour cell invasion through ECM prior to tumour metastasis (Aoyama and Chen, 1990; Stetler-Stevenson et al., 1993). One member of the MMP family, collagenase-3 (CL-3), was isolated from a human breast carcinoma library (Freije et al., 1994). CL-3 is expressed by rat ovary during ovulation (Balbin et al., 1996), by human bone cells during fetal development (Johansson et al., 1997; Ståhle-Bäckdahl et al., 1997) and by the osteoarthritic human synovial membrane (Wernicke et al., 1996). CL-3 exhibits potent collagenolytic ability against fibrillar collagens (Knäuper et al., 1996) and has been used in this study to generate fragments of type I collagen, a major component of connective tissue stroma of most mammalian tissues.

Type I collagen consists of three polypeptide chains, each of molecular mass of about 95 kDa, which self-associate into a triple-helical structure. The triple helix is particularly resistant to degradation by most mammalian proteinases at physiological temperatures with the exception of collagenases, which are able to cleave all three chains of type I collagen at a single locus between residues 775 and 776, that is, between one Gly-Ile in each α1 chain and one Gly-Leu in the α2 chain (Highberger et al., 1979). The fragments generated are approximately $\frac{3}{4}$ and $\frac{1}{4}$ of the total length of the native molecule (Gross and Nagai, 1965) and melt below 37°C (Stark and Kühn, 1968).

Cell-matrix interactions are frequently mediated by integrins, a family of cell-surface glycoproteins that bind ECM

proteins. Integrins are heterodimers, composed of an α subunit and a β subunit, and these are non-covalently associated. At least 16 α subunits and 8 β subunits have been identified to date. A major cell surface receptor for native type I collagen is the $\alpha_2\beta_1$ integrin (Elices and Hemler, 1989; Kirchhofer et al., 1990). Other integrins such as $\alpha_1\beta_1$ integrin have also been reported to bind type I collagen (Gulberg et al., 1992). A region at the N terminus of the α_2 integrin subunit, named the A-domain (or I-domain), has been demonstrated to be the ligand-binding region of the molecule (Kamata and Takada, 1994; Tuckwell et al., 1995). This region is similar in sequence to the collagen binding motif found in the A-domain of von Willebrand factor (Pareti et al., 1987) and A-domain-like sequences found in type VI collagen, complement factor B and cartilage matrix protein (Colombatti et al., 1993). It has been suggested that denaturation of type I collagen causes unfolding of the collagen triple helix to reveal cryptic RGD sequences. Cell interaction with denatured type I collagen has been postulated to be mediated by $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrin rather than $\alpha_2\beta_1$ integrin, which binds to proteins via RGD motifs (Davis, 1992; Montgomery et al., 1994; Jones et al., 1997), or via a fibronectin bridge (Tuckwell et al., 1994).

The adhesion of cells expressing $\alpha_2\beta_1$ integrin to type I collagen has previously been investigated. Cell adhesion to monomeric type I collagen, type I collagen fibres, α1(I) and α2(I) chains and the fragments of these chains derived by cyanogen bromide cleavage have been extensively studied (Barnes, 1982; Fitzsimmons and Barnes, 1985; Gulberg et al., 1992; Morton et al., 1994). Type I collagen fragments have been found to be chemotactic agents for human dermal fibroblasts (Albini and Adelmann-Grill, 1985), and type I collagen peptides have been found to be chemotactic for human peripheral monocytes (Malone et al., 1991). Isolated $\frac{3}{4}$ and $\frac{1}{4}$ fragments of type I collagen have also been shown to support human platelet aggregation (Fitzsimmons et al., 1986). However, little else is known about cell interactions with degraded type I collagen. Since collagenolytic cleavage of fibrillar collagens may be an instance of in vivo generation of gelatin (denatured collagen), it is important to evaluate the effects of collagenase activity on cell-collagen interactions. This study provides a fresh insight into cell attachment to collagen fragments generated by a physiological enzyme and studied in the native, triple-helical conformation and in the denatured, heat-unwound conformation, as might be expected *in vivo*. Since collagen degradation was carried out in a controlled manner prior to use in cell and cell-surface receptor studies, it is possible to define the effects of this cleavage on cell-collagen interactions and to speculate on the possible relevance of these results to pathological processes such as tumour cell invasion.

MATERIALS AND METHODS

Cell culture

The N-Ras transformed HT1080 human fibrosarcoma cell line (HT1080 K2P) was a gift from C. Marshall (Institute of Cancer Research, London, UK). A375M human melanoma cells were obtained from the European Tissue Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, USA) supplemented with 10% foetal calf serum (FCS; Globepharm, UK). Cells were passaged 24-48 hours prior to each experiment and had reached 80% confluency at the time of experiment.

Antibodies

Mouse monoclonal anti-human α_2 integrin antibodies were obtained from the following sources: 5E8 (Chen et al., 1991) and 6F1 (Coller et al., 1989) were kind gifts from Dr R. Bankert (Roswell Park Cancer Institute, Buffalo, NY, USA) and from Dr B. S. Coller (Mount Sinai Hospital, New York, NY, USA), respectively; P1E6 (Carter et al., 1990) was purchased from Becton Dickinson, USA. LM609, a mouse monoclonal anti-human $\alpha_v\beta_3$ integrin antibody (Wayner et al., 1991), was purchased from Chemicon, USA. Mouse IgG (Sigma, USA) was used as a negative control for these antibodies. Rat monoclonal anti-human β_1 integrin antibody (Akiyama et al., 1989) was a kind gift from Dr K. Yamada, Laboratory of Developmental Biology, National Institutes of Health, Bethesda, Maryland 20892, USA.

Generation and purification of type I collagen $\frac{3}{4}$ and $\frac{1}{4}$ fragments

Rat skin type I collagen was isolated and purified as described by Cawston and Barrett (1979). Recombinant human collagenase-3 was expressed by stable transfection of NSO mouse myeloma cells and purified from serum-free conditioned cell culture medium (Knäuper et al., 1996). Collagenase-3 was activated with *p*-amino-phenyl mercuric acetate (APMA) for 30 minutes at 37°C (Knäuper et al., 1996).

Type I collagen (5 mg) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (Tris-buffered saline, TBS) with 60 mM $CaCl_2$ was incubated with activated collagenase-3 (18 μg) for 16 hours at 25°C. Isolation of $\frac{3}{4}$ and $\frac{1}{4}$ type I collagen fragments from this cleavage mixture was performed essentially as described by Fitzsimmons et al. (1986) with minor modifications. The cleavage mixture was placed on ice at 4°C and solid ammonium sulphate added to 12% saturation to precipitate any uncleaved type I collagen. The mixture was stirred constantly for 1 hour on ice and centrifuged at 15,000 ***g*** for 1 hour at 4°C. The supernatant was removed, ammonium sulphate added to 18% saturation to precipitate the $\frac{3}{4}$ fragment, and the mixture was stirred for 1 hour on ice. The 12% pellet was washed by resuspension in TBS containing 12% ammonium sulphate and repelleted by centrifugation at 15,000 ***g*** for 1 hour at 4°C. The pellet was solubilized in 0.2 M acetic acid and dialysed for 16 hours into TBS. The precipitate produced by 18% ammonium sulphate precipitation was centrifuged as before, washed with TBS containing 18% ammonium sulphate, and solubilized and dialysed as above. The ammonium sulphate concentration of the supernatant was raised to 25% to precipitate the $\frac{1}{4}$ fragment and purified as above. The protein concentration of each solution was estimated by bicinchoninic acid (BCA) assay (Smith et al., 1985), following the manufacturer's instructions (Sigma). A known concentration of each sample was analysed with uncleaved type I collagen by 5%-15% polyacrylamide gradient gel electrophoresis and stained with silver reagent.

Adsorption of the fragments to plastic at room temperature stabilized the triple helical fragment conformation even when the fragment-coated plastic was later placed at 37°C for the duration of the assay (usually 30 minutes, as revealed by cell attachment assays performed at 37°C, see methods below). It was therefore necessary to heat denature the collagen fragments for 16 hours at 35°C prior to assay at 37°C.

The presence of CL-3 on the collagen fragments was probed by ELISA using a sheep anti-human CL-3 polyclonal antibody produced in this laboratory (L29/6; Cowell et al., 1998). No detectable level of CL-3 was found in association with fragments adsorbed to ELISA plates (Maxisorp, Nunc), although a positive control of 10 ng CL-3/well adsorbed alongside the fragments was readily detected by this method.

Purification of $\alpha_2\beta_1$ integrin from outdated human platelets

$\alpha_2\beta_1$ integrin was purified from platelet extracts as described by Kern et al. (1993) with some minor modifications. Outdated human platelet

concentrate apheresis packs of single ABO blood group were combined and centrifuged at 1,500 ***g*** for 20 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 13 mM sodium citrate, 120 mM NaCl, 30 mM glucose pH 7.0, 4°C. The suspension was centrifuged at 1,500 ***g*** for 20 minutes at 4°C and the washing procedure repeated as above. The resulting pellets were resuspended in 10 mM Tris-HCl, pH 7.4, 5 mM sodium citrate, 100 μM pepstatin A, 5 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), 2 μg/ml leupeptin and 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), and sonicated at 30% output for 3×10 seconds on ice. The sonicates were centrifuged at 100,000 ***g*** for 30 minutes at 4°C and the supernatant discarded. The platelet membrane preparations were then either stored at −80°C until needed or lysed directly by resuspending in a small volume of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM n-octyl β-D-glucopyranoside (OG), 100 μM pepstatin A, 5 μM E-64, 2 μg/ml leupeptin and 1 mM ABBSF (extraction buffer) for 30 minutes on ice. The lysate was centrifuged at 100,000 ***g*** for 30 minutes at 4°C and the supernatant collected and loaded directly onto a prepared collagen-Sepharose column.

Type I collagen (2.25 mg/ml) was coupled to 6-aminohexanoic acid *n*-hydroxysuccinimide ester linked, cyanogen bromide-activated Sepharose 4B (Sigma) according to the manufacturer's instructions. This was washed alternately with: (1) 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl; and (2) 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, three times. The gel was washed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) and packed into a column. The column was equilibrated with two column volumes of TBS, 2 mM $MgCl_2$, 1 mM $MnCl_2$ and 25 mM OG (equilibration buffer), followed by two column volumes of extraction buffer, at a flow rate of 30 ml/hour. The platelet membrane supernatant was loaded onto the column at 10 ml/hour and the runoff reapplied to the column. One column volume of extraction buffer was then run through the column followed by at least two column volumes of equilibration buffer. Bound $\alpha_2\beta_1$ integrin was eluted off the column with TBS, 25 mM OG, 20 mM EDTA at a flow rate of 3-10 ml/hour. Proteins eluted in the presence of EDTA were monitored by spectrophotometry at 280 nm. Fractions containing protein were combined and dialysed into TBS, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02% sodium azide, and the protein concentration determined by BCA assay. The purified integrin was biotinylated using a protein biotinylation kit (Amersham) according to the manufacturer's instructions and stored at −80°C.

The purity of the $\alpha_2\beta_1$ integrin was determined by 5%-15% polyacrylamide gel electrophoresis and silver staining and identified by immunoprecipitation with P1E6 anti-α_2 integrin antibody (Carter et al., 1990) and by western blotting using mAb 13 anti-β_1 integrin antibody (Akiyama et al., 1989).

Generation and labelling of recombinant α_2 integrin A-domain

Recombinant α_2 integrin A-domain was produced as described by Tuckwell et al. (1995). Human α_2 integrin A-domain cDNA was generated by reverse transcription/PCR and cloned into pGEX-2T (Smith and Johnson, 1988). Induction of bacteria that carried the plasmid led to production of an α_2 integrin A-domain-glutathione-S-transferase (GST) fusion protein, which was purified from bacterial lysates on a glutathione-affinity column.

Recombinant α_2 integrin A-domain-GST fusion protein was biotinylated as described by Tuckwell et al. (1996), and the GST removed on a glutathione-agarose column after incubation of the fusion protein with thrombin (Sigma).

$\alpha_2\beta_1$ integrin and α_2 integrin A-domain binding assays

Assays were performed as previously described (Tuckwell et al., 1996), with minor modifications. Briefly, for experiments at room temperature (23°C), 96-well ELISA plates (Maxisorp, Nunc) were coated with native type I collagen and native type I collagen $\frac{1}{4}$ and $\frac{3}{4}$ fragments in TBS for 1 hour at room temperature. Unbound substrate was flicked off, the wells washed three times with TBS and blocked with 50 mg/ml bovine serum albumin (BSA) in TBS for 1 hour at room temperature. BSA was flicked off and wells washed three times with TBS, and biotinylated $\alpha_2\beta_1$ integrin or α_2 integrin A-domain was added at 0.5 μg/ml in TBS, 2 mM $MgCl_2$, 1 mg/ml BSA. Whole integrin assays were performed in the presence of 1 mM $MnCl_2$ in addition to $MgCl_2$. Plates were incubated for 2 hours at room temperature for whole integrin and 3 hours at room temperature for α_2 integrin A-domain. Wells were washed three times with TBS, 1 mM $MgCl_2$, and streptavidin-peroxidase (Amersham) was added at 1:1,500 dilution in TBS, 1 mM $MgCl_2$, 1 mg/ml BSA for 15 minutes at room temperature. Wells were washed three times with TBS, 1 mM $MgCl_2$, and bound A-domain was visualized using 3,3′,5,5′-tetramethylbenzidine-peroxidase substrate mixed 1:1 with H_2O_2. The colour reaction was stopped with 2.5 M H_2SO_4. The absorbance was read at 450 nm using a multiwell plate reader.

Assays at 37°C were carried out as above, but with collagen and fragments incubated at 35°C for 16 hours prior to adsorbing to 96-well ELISA plates to ensure denaturation of the fragments. Collagen and fragments were plated and blocked at 37°C to prevent renaturation of the fragments. Assays were run for 30 minutes at 37°C for both whole integrin and α_2 integrin A-domain, and developed as for assays performed at room temperature.

Cell attachment assays

Cell attachment assays were performed essentially as described by Gamble et al. (1993). Briefly, 96-well tissue culture plates (Corning) were (1) coated for 1 hour at room temperature with type I collagen, CL-3 cleaved type I collagen or type I collagen $\frac{1}{4}$ and $\frac{3}{4}$ fragments in TBS, then washed and blocked with 1% heat-denatured BSA in PBS for 1 hour at room temperature, or (2) coated for 1 hour at 37°C with collagen and fragments incubated at 35°C for 16 hours immediately prior to assay, washed and blocked for 1 hour at 37°C with 1% heat-denatured BSA in PBS. Cells were trypsinized off flasks, centrifuged in the absence of serum and resuspended in DMEM. Where indicated, cells were preincubated with antibodies at 2×10^6 cells/ml in DMEM for 30 minutes at 37°C. The block was washed off with DMEM and 6×10^4 cells/well plated in DMEM. Cells were allowed to attach for 1 hour at 37°C, as determined by time-course experiments (data not shown). Unattached cells were flicked off and washed three times with warmed DMEM. Attached cells were fixed with 4% paraformaldehyde for at least 30 minutes and stained with 1% Methylene Blue in 0.01 M borate for 30 minutes. Excess dye was washed off with water and bound cells were lysed with ethanol/0.1 M HCl (1:1 ratio). The absorbance of released dye was read at 630 nm on a multiwell-plate reader. Results are expressed as percentage of cells binding to native type I collagen, allowing convenient comparison of the two cells lines, HT1080 and A375M.

All experiments were performed at least three times, with similar results, and representative experiments are shown.

RESULTS

Platelet proteins eluted from the collagen affinity column were analysed by SDS-PAGE and silver staining, revealing the presence of two bands of approximate molecular mass 150 and 120 kDa (Fig. 1A). These proteins were identified by immunoprecipitation with an anti-α_2 integrin antibody (P1E6) (Fig. 1B). The identity of the β_1 integrin subunit was also confirmed by western blotting with an anti-β_1 integrin antibody (mAb13; results not shown). Type I collagen $\frac{3}{4}$ and $\frac{1}{4}$ fragments were subjected to SDS-PAGE and silver staining, and were found to be free from uncleaved collagen and/or each other (Fig. 1C).

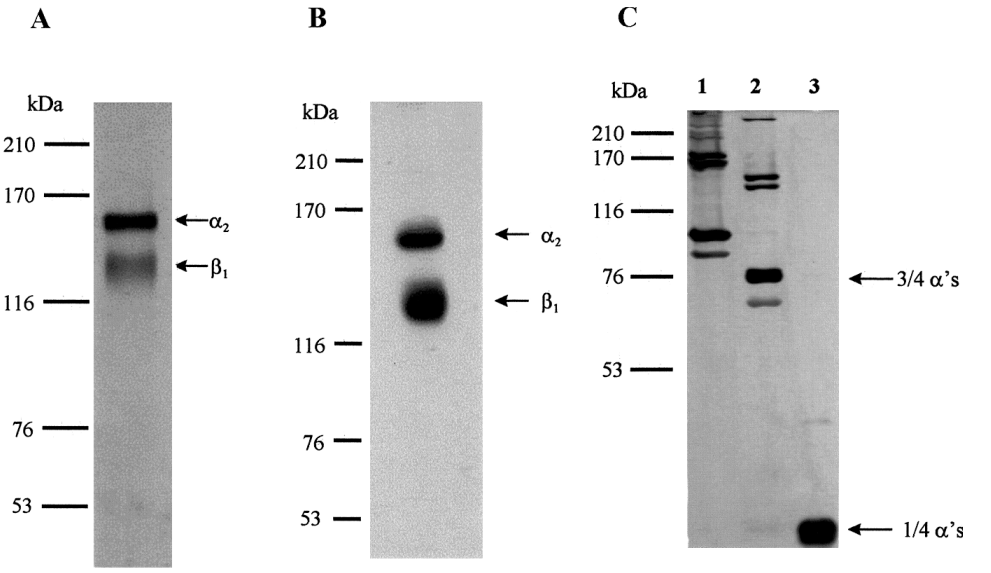

Fig. 1. (A) 6% SDS-PAGE and silver staining of purified $\alpha_2\beta_1$ integrin. (B) Immunoprecipitation of purified $\alpha_2\beta_1$ integrin using P1E6 anti-α_2 integrin antibody. Both the α and β subunits are precipitated, due to the stability of the receptor complex. (C) 6% SDS-PAGE and silver staining of: (lane 1) native type I collagen, (lane 2) isolated $\frac{3}{4}$ and (lane 3) $\frac{1}{4}$ fragments of type I collagen. The positions of molecular mass markers are shown.

$\alpha_2\beta_1$ integrin binding to type I collagen and collagen fragments

Native collagen and fragments

Purified human platelet $\alpha_2\beta_1$ integrin bound to native type I collagen and the $\frac{3}{4}$ fragment at 23°C with approximately equal apparent affinity (Fig. 2A). Some $\alpha_2\beta_1$ integrin binding to the $\frac{1}{4}$ fragment of collagen was observed at high ligand coating concentration, although this was reduced to control levels at 0.1 μg/ml coating concentration (Fig. 2A). Because a solution of $\frac{1}{4}$ fragment contains four times as many moles of ligand as a solution of native type I collagen, binding observed at high ligand coating concentration represents an overall lower level of integrin binding to $\frac{1}{4}$ fragment when compared on a molar basis relative to integrin binding to native collagen. Binding of $\alpha_2\beta_1$ integrin to the native type I collagen fragments and type I collagen was reduced to control levels by the addition of the divalent metal-ion chelator, EDTA, or anti-α_2 integrin antibodies (Fig. 2A,B).

Denatured collagen fragments

If the $\frac{1}{4}$ and $\frac{3}{4}$ fragments of type I collagen were heat-denatured at 35°C prior to coating to ELISA plates and assay at 37°C, then no binding of $\alpha_2\beta_1$ integrin to either fragment was observed (Fig. 2C). In contrast, native type I collagen was able to support $\alpha_2\beta_1$ integrin binding at 37°C.

α_2 integrin A-domain binding to type I collagen and collagen fragments

Native collagen and fragments

Isolated, recombinant α_2 integrin A-domain bound with approximately equal apparent affinity to native type I collagen and the native $\frac{3}{4}$ fragment of type I collagen at 23°C (Fig. 3A). Of interest is the fact that very little binding of A-domain was observed to the $\frac{1}{4}$ fragment, even at high ligand coating concentration (Fig. 3A). All A-domain binding to type I collagen and the $\frac{3}{4}$ fragment at 23°C was inhibited by EDTA (Fig. 3A). At 23°C, A-domain binding to native collagen and the native collagen fragments could be reduced to control levels by the addition of either EDTA or anti-α_2 integrin antibodies (Fig. 3A,B).

Denatured collagen and fragments

At 37°C, after heat denaturation of the collagen fragments at 35°C, A-domain binding to the $\frac{3}{4}$ and $\frac{1}{4}$ fragments was reduced to control levels (Fig. 3C), which was very similar to the results for whole $\alpha_2\beta_1$ integrin binding to heat-denatured fragments. A-domain binding to native type I collagen was unaffected by incubation of the collagen at 35°C, as was the case with whole integrin binding to incubated collagen.

Attachment of HT1080 and A375M cells to type I collagen and fragments

HT1080 cells attached equally well to type I collagen and the native $\frac{3}{4}$ fragment of type I collagen at high coating concentrations at 37°C; however, no cell attachment to the $\frac{1}{4}$ fragment was observed (Fig. 4A). Following heat-denaturation of the fragments, HT1080 cells were not able to attach to either fragment, although cell attachment to native type I collagen remained possible (Fig. 4B,C). Heat-denaturation of collagen to gelatin abolished the majority of HT1080 cell attachment. HT1080 cell attachment to collagen and the residual attachment to gelatin was greatly reduced by preincubating cells with a function-blocking anti-α_2 integrin antibody (6F1). A function-blocking anti-$\alpha_v\beta_3$ integrin antibody (LM609) had no effect on HT1080 cell attachment to native collagen, gelatin or the heat-denatured $\frac{3}{4}$ and $\frac{1}{4}$ fragments (Fig. 4C).

Similarly, A375M melanoma cells were able to attach to native type I collagen and this attachment could be inhibited by 6F1 anti-α_2 integrin antibody. However, in sharp contrast to HT1080 cell adhesion, gelatin and the heat-denatured collagen fragments were still able to support adhesion of A375M cells. Cell attachment to the heat-denatured substrates could be completely inhibited by LM609 anti-$\alpha_v\beta_3$ integrin antibody; however, 6F1 anti-α_2 integrin antibody had no effect on A375M cell attachment to either gelatin or the denatured fragments (Fig. 4D).

DISCUSSION

We have shown that MMP cleavage of type I collagen has a profound effect on its interaction with the cell. The major cell

surface receptor for type I collagen and its ligand binding domain were able to adhere to collagenase-cleaved type I collagen at 23°C, but once the $\frac{3}{4}$ fragment of type I collagen had been heat denatured close to physiological temperatures, all $\alpha_2\beta_1$ integrin and α_2 integrin A-domain interactions were lost. HT1080 fibrosarcoma cells were not able to attach to the gelatinized fragments after heat denaturation at physiological temperatures; however $\alpha_2\beta_1$ integrin-mediated cell attachment to the $\frac{3}{4}$ fragment, which had not been previously denatured and therefore retained triple-helical conformation at 37°C during the assay, was possible. HT1080 cell attachment was wholly mediated by $\alpha_2\beta_1$ integrin and not by RGD-dependent integrins such as $\alpha_v\beta_3$. Futhermore, A375M melanoma cells were able to attach to gelatinized collagen and the $\frac{3}{4}$ fragment via $\alpha_v\beta_3$ integrin but not by $\alpha_2\beta_1$ integrin.

The N-terminal region of the α_2 integrin that has been shown to be involved in integrin-collagen binding has been identified as an insertion of approx. 200 amino acids, which is homologous to the von Willebrand factor A-domain (Kamata and Takada, 1994; Tuckwell et al., 1995). The data presented here is in agreement with and extends previous studies of α_2 integrin A-domain/collagen interactions. Recombinant human α_2 integrin A-domain bound strongly to native momomeric type I collagen at room temperature and at 37°C. Interestingly, although very little binding to the $\frac{1}{4}$ fragment was observed, the A-domain was able to interact at 23°C with the $\frac{3}{4}$ fragment of

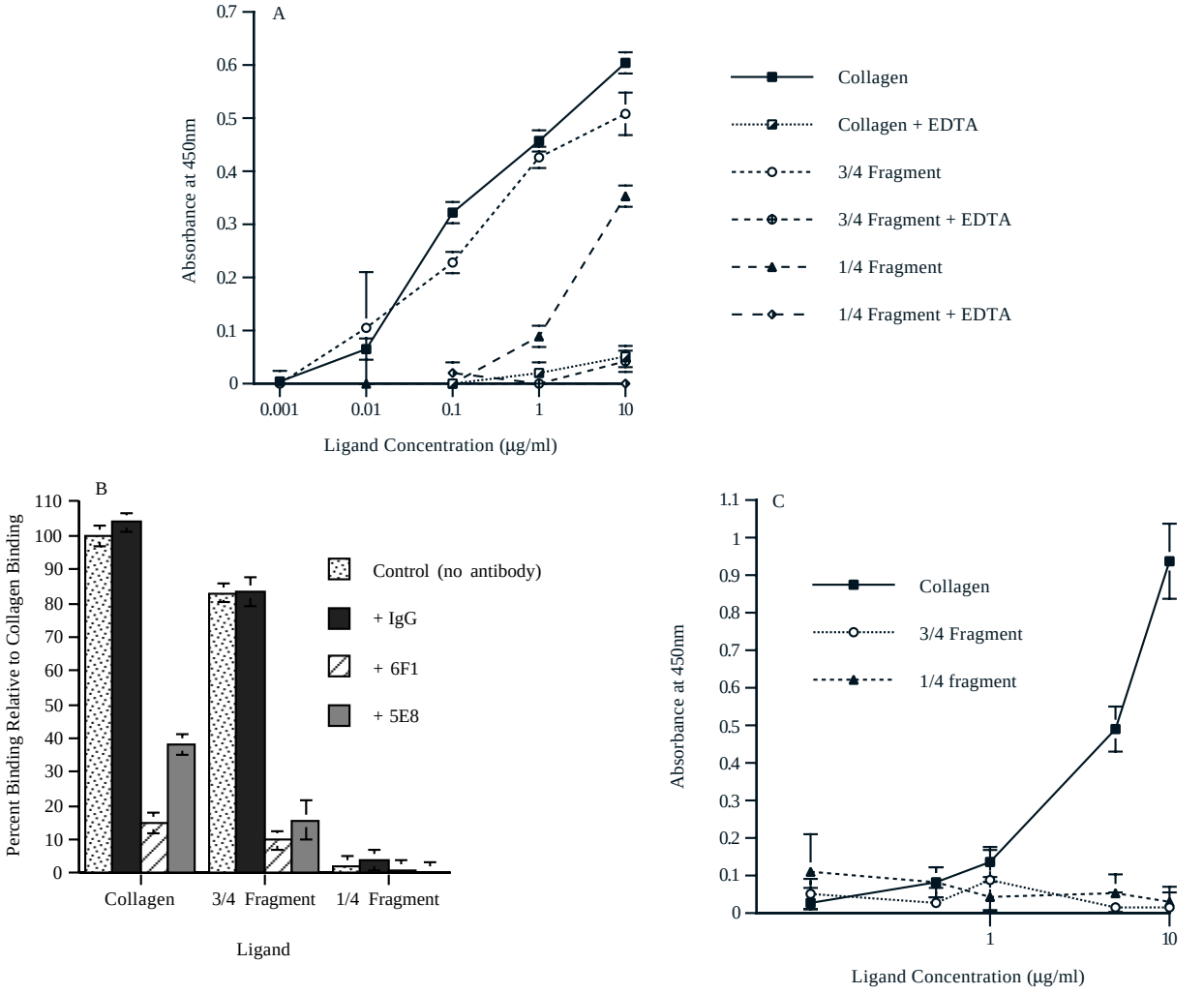

Fig. 2. (A) $\alpha_2\beta_1$ integrin (0.5 μg/ml) binding to native type I collagen and the $\frac{1}{4}$ and $\frac{3}{4}$ fragments of type I collagen coated and assayed at 23°C. $\alpha_2\beta_1$ integrin bound equally well to native collagen and the $\frac{3}{4}$ fragment of type I collagen; however $\alpha_2\beta_1$ integrin binding to the $\frac{1}{4}$ fragment was only observed at high coating concentrations of the $\frac{1}{4}$ fragment. All $\alpha_2\beta_1$ integrin binding to collagen and the $\frac{3}{4}$ and $\frac{1}{4}$ fragments could be inhibited by 10 mM EDTA. Values are the mean of triplicates ± s.d. (B) Inhibition of $\alpha_2\beta_1$ integrin (0.5 μg/ml) binding to native collagen (2 μg/ml), $\frac{3}{4}$ fragment (1.5 μg/ml) and $\frac{1}{4}$ fragment (0.5 μg/ml) coated and assayed at 23°C. Antibodies were used at 5.0 μg/ml. Data are expressed as percentage of the total binding to 10 μg/ml type I collagen. 6F1 and 5E8 anti-α_2 integrin antibodies reduced $\alpha_2\beta_1$ integrin binding to native collagen and collagen fragments down to the levels of the control. Mouse IgG was used as an antibody control but it had no effect on $\alpha_2\beta_1$ integrin binding. Values are the mean of triplicates ± s.d. (C) $\alpha_2\beta_1$ integrin binding to heat-denatured collagen fragments and native collagen plated and assayed at 37°C. Although $\alpha_2\beta_1$ integrin was still able to interact with native collagen at this temperature, no binding was observed to the denatured collagen fragments. $\alpha_2\beta_1$ integrin binding to native collagen could be inhibited by 10 mM EDTA (data not shown). Values are the mean of triplicates ± s.d.

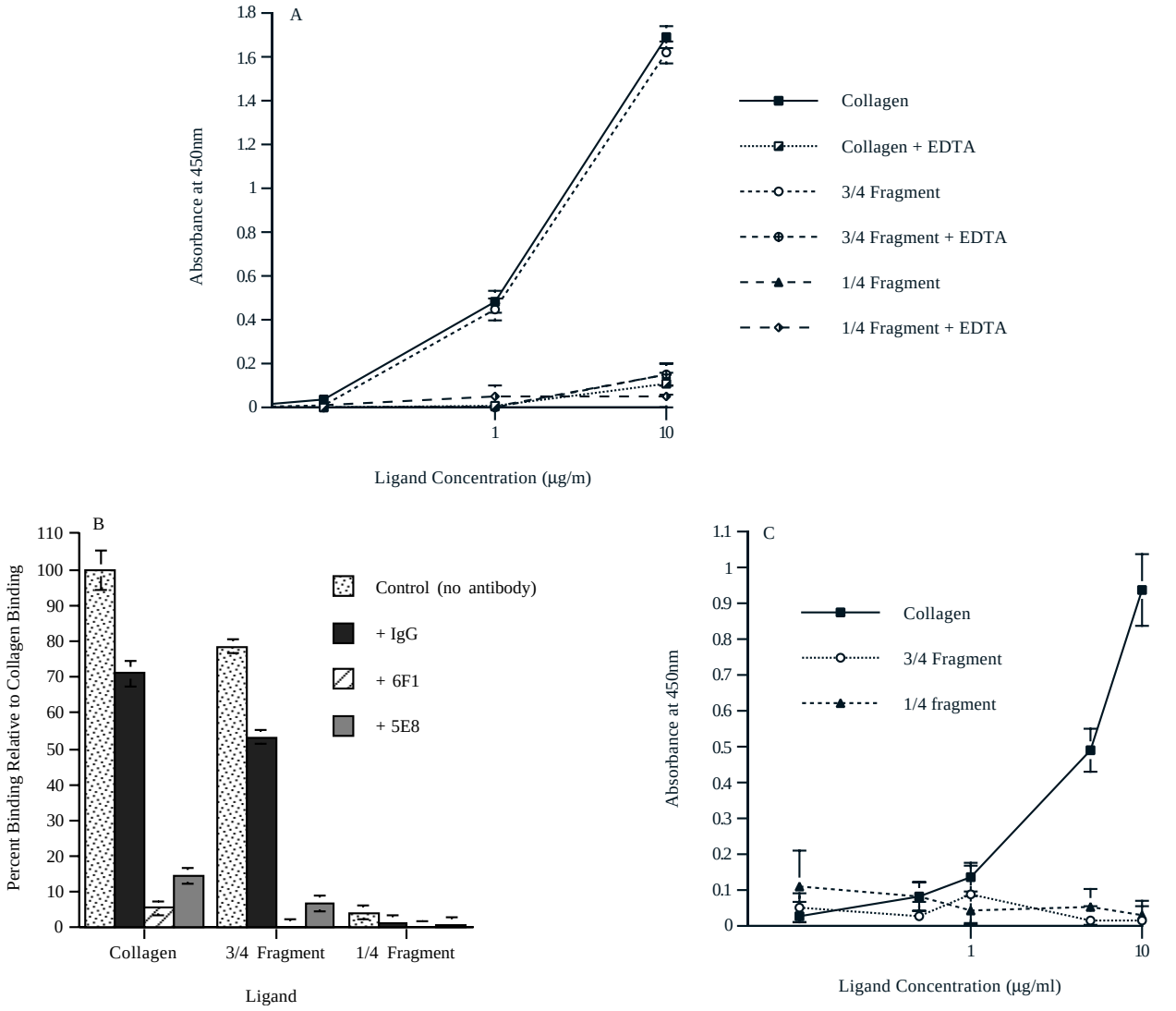

Fig. 3. (A) α_2-integrin A-domain (0.5 μg/ml) binding to type I collagen and the $\frac{1}{4}$ and $\frac{3}{4}$ fragments of type I collagen coated and assayed at 23°C. α_2-integrin A-domain bound equally well to native collagen and $\frac{3}{4}$ fragment of type I collagen. No binding to the $\frac{1}{4}$ fragment was observed, even at higher ligand coating concentrations. All binding to native collagen and native $\frac{3}{4}$ fragment could be inhibited by 10 mM EDTA. Note that the curve of A-domain binding to $\frac{1}{4}$ fragment is superimposed on that of A-domain binding to the $\frac{3}{4}$ fragment in the presence of EDTA. Values are the mean of triplicates ± s.d. (B) Inhibition of α_2-integrin A-domain (0.5 μg/ml) binding to type I collagen (2.0 μg/ml), $\frac{3}{4}$ fragment of collagen (1.5 μg/ml) and $\frac{1}{4}$ fragment of collagen (0.5 μg/ml) coated and assayed at 23°C. Antibodies were used at 5.0 μg/ml. Data is expressed as percentage of the total binding to type I collagen. 6F1 and 5E8 anti-α_2 integrin antibodies reduced A-domain binding to native collagen and collagen fragments down to control levels. Mouse IgG was used as an antibody control and reduced A-domain binding to native collagen and $\frac{3}{4}$ fragment by about 20%; however, anti-α_2 integrin antibodies caused a significantly higher degree of inhibition of A-domain binding to these ligands (P<0.05). Values are the mean of triplicates ± s.d. (C) α_2 integrin A-domain binding to heat-denatured type I collagen fragments and native type I collagen, coated and assayed at 37°C. Although A-domain was still able to bind to native type I collagen at 37°C, no binding to the denatured fragments was observed. A-domain binding to native collagen could be inhibited by 10 mM EDTA (data not shown). Values are the mean of triplicates ± s.d.

type I collagen produced by CL-3 cleavage of type I collagen. At physiological temperatures the A-domain did not bind to either collagen fragment.

Studies by Stark and Kühn (1968) showed that the $\frac{1}{4}$ and $\frac{3}{4}$ fragments of type I collagen lose their triple-helical structure below physiological temperatures. Thus, collagenase cleavage of type I collagen may be an instance of in vivo generation of gelatin. In this study we have shown that $\alpha_2\beta_1$ integrin and α_2 integrin A-domain are able to bind to triple-helical $\frac{3}{4}$ fragment of collagen, but not to heat-denatured $\frac{3}{4}$ fragment, which is essentially gelatin. These observations underscore the need for native, triple-helical collagen conformation as a prerequisite for $\alpha_2\beta_1$ integrin-mediated binding. Binding to the triple-helical $\frac{3}{4}$ fragment was significantly reduced by anti-α_2 integrin antibodies and completely abrogated by EDTA. Since $\alpha_2\beta_1$ integrin displayed interaction with the $\frac{1}{4}$ fragment of type I collagen only at high concentrations, at 23°C, it is possible that this peptide contains very few $\alpha_2\beta_1$ integrin binding sites. This is in agreement with data produced using cyanogen bromide-derived fragments of type I collagen (Morton et al., 1994). The

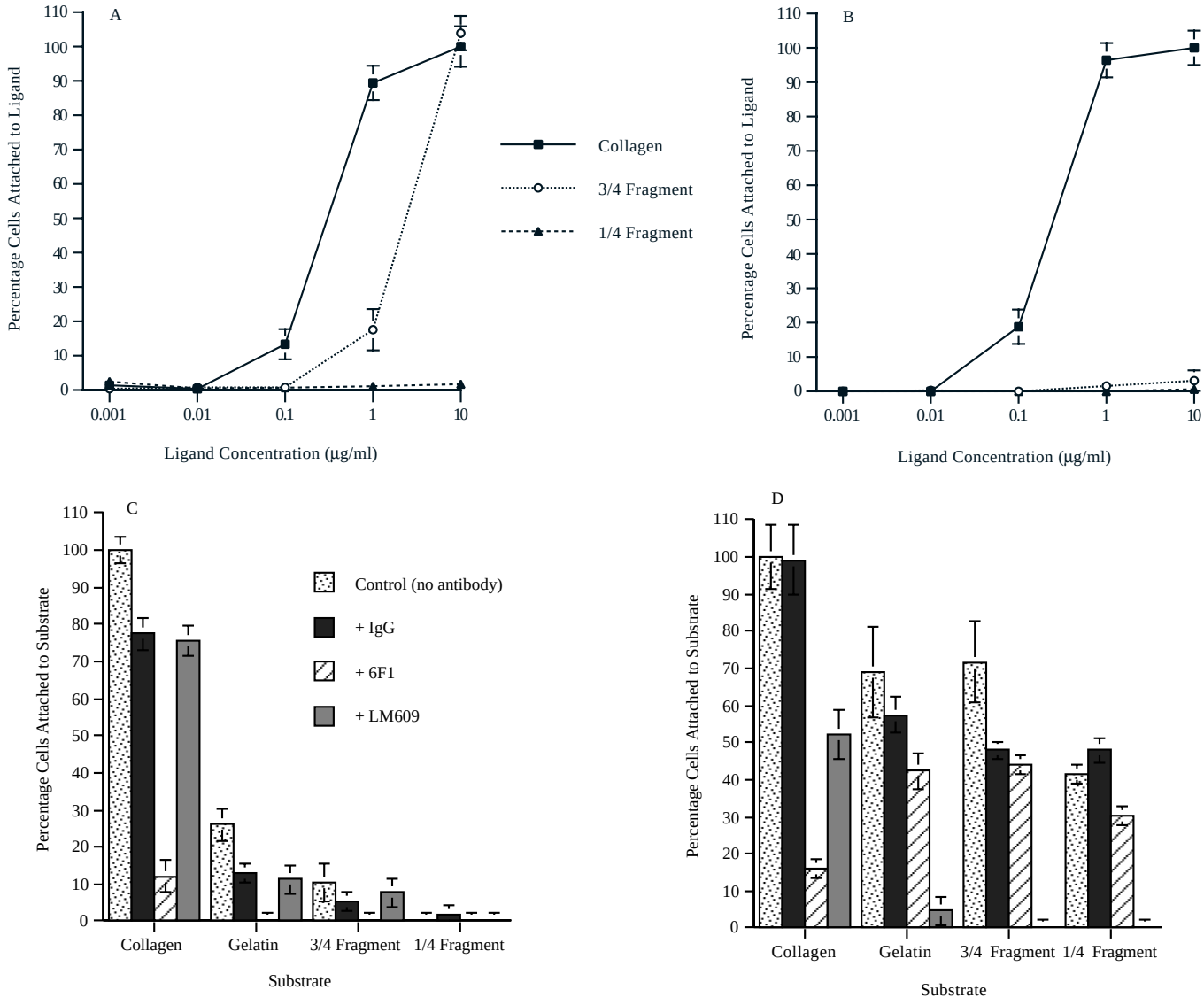

Fig. 4. (A) HT1080 cell attachment (6×10^5 cells/ml) to type I collagen and the native $\frac{1}{4}$ and $\frac{3}{4}$ fragments of type I collagen coated at 23°C. Data are expressed as percentage of cells attached to 10 μg/ml collagen. HT1080 cell attachment to native type I collagen closely parallels cell attachment to the isolated $\frac{3}{4}$ collagen fragment at high substrate concentration. Virtually no HT1080 cell attachment to the $\frac{1}{4}$ fragment of type I collagen was observed. Values are the mean of triplicates ± s.d. (B) HT1080 cell (6×10^5 cells/ml) attachment to type I collagen and the heat-denatured fragments of type I collagen, coated at 37°C. HT1080 cells are not able to attach to either denatured fragment; however, attachment to native collagen remains unchanged by coating at 37°C rather than 23°C. Values are the mean of triplicates ± s.d. (C) Inhibition of HT1080 cell attachment to native type I collagen, gelatin, denatured $\frac{3}{4}$ fragment of collagen and denatured $\frac{1}{4}$ fragment of collagen. Substrates were used at 10 μg/ml; cell concentration was 6×10^5 cells/ml; antibodies were used at 5 μg/ml. An anti-α_2 integrin antibody (+6F1) greatly reduced HT1080 cell attachment to native type I collagen and completely inhibited the low levels of attachment to gelatin and $\frac{3}{4}$ fragment of type I collagen. A control mouse IgG antibody (+IgG) and an anti-$\alpha_v\beta_3$ integrin antibody (+LM609) had no effect on cell attachment. Virtually no HT1080 cell attachment to the $\frac{1}{4}$ fragment of type I collagen was observed. Values are the mean of triplicates ± s.d. (D) Inhibition of A375M cell attachment to native type I collagen, gelatin and heat-denatured type I collagen fragments (10 μg/ml) (6×10^5 cells/ml). Antibodies were used at 5 μg/ml. A375M cell attachment to native type I collagen could be greatly reduced by preincubation with an anti-α_2 integrin antibody (+6F1); however, A375M attachment to gelatin and the $\frac{3}{4}$ fragment could be blocked with an anti-$\alpha_v\beta_3$ integrin antibody (+LM609) but not with anti-α_2 integrin antibody. A mouse IgG antibody (+IgG) had no effect on A375M attachment to any substrate. Values are the mean of triplicates ± s.d.

$\frac{1}{4}$ fragment produced by collagenase cleavage of type I collagen is eqivalent to a small section of α1(I)CNBr 7 and all of CNBr 6b. No human platelet adhesion to CNBr 6b via $\alpha_2\beta_1$ integrin was observed, and although CNBr 7 was found to support platelet adhesion, the collagenase-generated $\frac{1}{4}$ fragment may only contain enough of the CNBr 7 region to support α_2 integrin interactions at high concentrations. α_2 integrin A-domain did not display any interaction with the $\frac{1}{4}$ fragment, even at high concentrations. It is possible that the $\frac{1}{4}$ fragment denatured during the assays at 23°C, thus ablating any integrin-mediated interactions, although this would not be expected as the published melting temperature of this fragment (29°C;

Stark and Kühn, 1968) is several degrees higher than the assay temperature.

Human fibrosarcoma cells (HT1080) adhered to type I collagen and the $\frac{3}{4}$ fragment of collagen at 37°C. Attachment could be blocked by preincubating cells with anti-α_2 integrin antibodies. Heat-denaturation (at 35°C) of the $\frac{1}{4}$ and $\frac{3}{4}$ fragments prior to coating at 37°C completely ablated HT1080 cell attachment to either fragment. Cell attachment to native collagen was unaffected by incubating the collagen at 35°C (Fig. 4A,B). The lack of HT1080 cell attachment to the $\frac{1}{4}$ and $\frac{3}{4}$ collagen fragments was unlikely to be due to failure of the fragments to adsorb to the tissue culture plastic as the human melanoma cell line, A375M, which expresses both $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins (Marshall et al., 1991), attached equally well to gelatin and to $\frac{3}{4}$ and $\frac{1}{4}$ collagen fragments under these conditions (Fig. 4D). A375M cells express a low level of $\alpha_2\beta_1$ integrin but high levels of the $\alpha_v\beta_3$ integrin, which has been postulated as a receptor for denatured type I collagen (Davis, 1992; Montgomery et al., 1994; Jones et al., 1997). In agreement with this hypothesis, A375M cell attachment to gelatin and denatured $\frac{3}{4}$ fragment could only be perturbed by anti-$\alpha_v\beta_3$ integrin antibody, not by anti-α_2 integrin antibody. A comparison of HT1080 cell attachment to native collagen, gelatin and collagen fragments under the same conditions as the A375M cells, demonstrated that the low level of HT1080 cell attachment to gelatin could be wholly blocked with anti-α_2 integrin antibody, and that virtually no HT1080 cell attachment to either collagen fragment was observed. HT1080 cells cultured on type I collagen and both collagen fragments highly express $\alpha_2\beta_1$ integrin on the cell surface, but express very little $\alpha_v\beta_3$ integrin, as detected by immunolocalization (A. J. Messent and J. Gavrilovic, unpublished observations), therefore the lack of cell binding to potential $\alpha_v\beta_3$ integrin ligands could be explained by a deficiency in this integrin. The cell data is wholly in agreement with the data generated using the isolated binding proteins and denatured ECM components, and therefore supports the theory that $\alpha_2\beta_1$ integrin-mediated binding to type I collagen is entirely dependent on retention of the collagen triple-helical conformation (Kühn and Eble, 1994; Morton et al., 1994).

In order to remain motile, a cell must maintain a low level of adhesion to the extracellular matrix to allow traction, without attaching so firmly that it becomes immobile. Cells are able to do this by regulation of integrin activity and/or specificity (Diamond and Springer, 1994; Kirchhofer et al., 1990) and by modulation of their extracellular environment (Blasi, 1993; Friedl et al., 1997; Vernon and Sage, 1996). Our results demonstrating the inability of cells to adhere to collagenase-cleaved collagen fragments via $\alpha_2\beta_1$ integrin are very likely to be relevant to in vivo situations such as wound healing, where migrating keratinocytes degrade type I collagen as they move over the provisional wound matrix, in order to maintain motility within the wound environment, and not to become firmly attached to dermal collagen via their $\alpha_2\beta_1$ integrin (Pilcher et al., 1997). This cellular mechanism of proteinase secretion to ensure continous motility within an extracellular matrix environment, to which a cell may possess high-affinity receptors, has potential application in the process of tumour cell invasion and metastasis (Ruoslahti, 1992; Heino, 1996). High levels of MMPs have been frequently identified in the vicinity of tumour sites, although their function still remains unclear. Localized production of active, degradative enzymes such as MMPs by primary tumour cells or surrounding tissue stroma may allow a few metastatic cells sufficient freedom from tight cell-matrix bonds to facilitate movement into nearby tissues and, subsequently, the bloodstream. Initial data from studies of HT1080 cell invasion through filters coated with type I collagen fragments indicate that cells were able to move through either fragment towards a chemoattractant more readily than they could through filters coated with native type I collagen (A. J. Messent and J. Gavrilovic, unpublished observations).

This is the first demonstration of a detailed study of $\alpha_2\beta_1$ integrin interactions with type I collagen fragments generated using a specific collagenase that has been linked with pathological conditions such as tumour invasion. Such studies are vital to our understanding of how cells interact with damaged ECM, and can give valuable insight into pathological conditions where ECM degradation plays an important part in disease progression. It is hoped that further study of these interactions, particularly with reference to cell motility on and through type I collagen fragments, will contribute to a better understanding of the mechanisms by which tumour cells invade into tissue surrounding the primary tumour site and metastasize to other locations within the body.

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