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An antisense oligonucleotide targeting the αV integrin gene inhibits adhesion and induces apoptosis in breast cancer cells

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Abstract

The aim of this study was to show the anti-adhesive potential of an antisense oligodeoxynucleotide (ODN) approach when designed to suppress the cellular function of the αV integrin subunit in breast cancer cells. The αV integrins play major roles in favouring breast cancer spreading. In this study, we inhibited αV subunit synthesis in the human breast carcinoma cell line, MDA-MB231, by a partially phosphorothioated antisense oligodeoxynucleotide (5543-ODN). The αV antisense 5543-ODN reduced αV, but not actin, mRNA transcription and protein expression by 55% and 65% respectively (1 μM, 72 h). Control sense and mismatch reagents were inactive. The antisense, but not the sense and mismatch, 5543-ODN induced dose- and time-dependent inhibition of MDA-MB231 adhesion to serum, vitronectin, fibrinogen and fibronectin substrates but was inactive on adhesion to laminin. Thus, the αV integrin was located in adhesion structures, which were disrupted by treatment with the αV antisense 5543-ODN. Antisense treated cells also showed evidence of programmed cell death with the appearance of apoptotic bodies. MDA-MB231 cells express a mutant form of the pro-apoptotic factor p53; however, no changes in the expression of p53 were observed by Western blotting. Immunofluorescence did reveal an increased nuclear translocation of p53 suggesting activation of the protein, but such a translocation did not lead to significant changes in either the expression of the cyclin dependent kinase inhibitor, $p21^{WAF1/CIP1}$ the cell survival factor Bcl-2 or the pro-apoptotic factor Bax.

Keywords: Breast cancer; Bone metastasis; αV integrin; Osteolytic lesions; Osteoclasts

1. Introduction

In the UK breast cancer affects between 7 and 10% of women and globally accounts for one-fifth of female malignancies, and at least half of these patients will die from metastatic disease which involves primarily the skeleton [1,2]. The spread of cancer requires the disengaging of cells from a primary tumour site and migration to and attachment at a secondary settlement location. Many workers have shown that a number of cell adhesion molecules play a role in metastasis and that integrins are especially involved in tumorigenic spread [3].

Integrins are non-covalently associated α/β glycoprotein type I heterodimers and in mammals at least 16 α and 8 β subunits have been described and shown to combine into 22 different heterodimers, each with specific recognition and affinities for various components of the extracellular matrix (ECM) milieu or for other cell-bearing adhesion molecules [4,5]. Integrins are not only implicated in cell–cell and cell–ECM interactions but also signalling into the cell, and are thus involved in sensing the cellular microenvironment, playing key roles in cellular activities such as migration, differentiation, survival and tissue (re)modelling in both normal and pathological states [6,7].

In the case of breast cancer cell lines there is considerable evidence of altered integrin levels in the tumorigenic situation in comparison with the native, parental background. αV integrins, are highly expressed

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and could have a prominent role in breast carcinoma metastasis to bone [8]. A number of breast carcinoma cell lines are available for studying the effect of various anti-adhesive treatments, such as monoclonal antibodies (MAbs) and peptides, and these include the MDA-MB cell line series. In a study by Meyer and colleagues [8] where they assessed the expression of αV integrins in eight different breast cancer cell lines, they found that the classical, αVβ3, vitronectin receptor was only expressed in MDA-MB231 cells, whereas αVβ5 is expressed by all breast cancer cells, αVβ1 is expressed on the majority and the αVβ6 and αVβ8 are not expressed. Their work suggests that the αV subunit could be a key target for anticancer therapies, since the vitronectin receptor has been shown to be crucial in tumour progression and expression of the invasive phenotype [8,9] as shown in the melanoma cell types [10,11]. Therefore, there is considerable interest in trying to modify breast cancer integrin expression profiles with consequential effects upon primary tumour growth and spread.

The rationale of the work described herein was to use cell lines to reflect *in vivo* tumour responses when treated with exogenously added antisense ODNs directed against the αV integrin subunit. ODNs are short nucleic acid sequences that are designed to complement a target gene in a specific mRNA species. Upon sequence recognition and hybridisation, the mRNA–ODN duplex prevents sequence recognition by the translational apparatus. In many cases, however, the mRNA part of the duplex is cleaved by intracellular RNase H enzyme. The efficacy of the ODN is reflected by its ability to reduce target protein expression [12–15].

2. Materials and methods

All chemical reagents (AnalaR grade) were from the Sigma Chemical Company (Poole, Dorset, UK) or Pharmacia (St Albans, Herts, UK). Cell culture media, antibiotics, sera, lipofectamine and plasma vitronectin were from Gibco, Life Technologies (Paisley, Renfrewshire, UK). Culture dishes and all sterile plasticware were purchased from Falcon, Becton-Dickinson (Cowley, Oxford, UK) or Costar (Cambridge, MA, USA). TdT-mediated dUTP-biotin nick-end labelling (TUNEL) apoptosis/DNA fragmentation detection kit and antibodies PIB5 (α3), PID6 (α5) and PIF6 (αVβ5) were from Chemicon (Tamecula, CA, USA). The human αvβ3, 23C6, and αV, 13C2, MAbs were produced in our laboratory [16]. The human αV MAb (clone #139) and the human β3 polyclonal antibody (clone #1264) were kindly donated by J. Gailit [17] Department of Dermatology, State University of New York at Stony Brook, NY, USA. The anti $p21^{WAF1/CIP1}$ (Ab-5) and the p53 (OP09) antibodies were from Oncogene Research Calbiochem (Cambridge, MA, USA). The anti Bcl-2 (sc 492), the anti-Bax (sc 493), the anti-actin (sc 1616) and the secondary antibodies were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Antibodies 7.2 (α4) and PM6/13 (β3) were from Serotec Ltd (Oxford, UK) Antibodies B5-IVH2 (β5) and GOH3 (α6) were from Signal Transduction Laboratories (Lexington, KY, USA). Antibodies TS2/21 (α1) and TS1/18 (β2) were kindly donated by T. Springer, Department of Pathology, Center for Blood Research and Harvard Medical School, Boston, MA, USA. Antibodies P5D2 (β1) and HAS6 (α2) were kindly provided by F. Watt, ICRF Keratinocyte Laboratory, London, UK. Enhanced ChemiLuminescence (ECL) kit and Hybond nitrocellulose were from Amersham International Inc. (Little Chalfont, Bucks, UK). Bovine serum albumin (fraction V) was from Boehringer Mannheim GmbH (Mannheim, Germany). Reagents for RT-PCR were from Promega Italia (Milan, Italy).

2.1. Sequence assessment and ODN design

cDNA sequences encoding the integrin αV from a variety of species were assessed using the DNA Star and GCG molecular biology packages. Sequence alignment of the available αV cDNAs demonstrated a high start site similarity with a number of possible target sites. After a number of preliminary experiments and comparisons with other antisense sequences, one 18 bp target region, nucleotide positions 40–57 (sequence numbering as in [18]) in the human cDNA, was chosen. This site showed high sequence homology to all mammalian αV subunits known, but not to other α integrin sequences (determined using a BLAST search of the current EMBL database). The synthesis of the corresponding αV 5543-ODN sequence used was:

Antisense: 5′-G*C*GGC*GGAAAAGC*CA*T*C*G-3′ (*represents phosphorothioate linkages). Sense (5′-C*G*ATGGC*TT*TT*CCGCC*G*C-3′), and mismatch (5′-G*C*AAC*GAGAGAGC*CG*T*C*G-3′) control sequences were also designed according to the αV ODN target region. The AS-3 antisense sequence is 5′-A*AG*C*CAT*C*GC*GAAG*T*G*C-3′ and AS-4 is 5′-G*AC*TGT*C*CA*CGT*T*C*TAG*G*T-3′. AS-3 is complementary to nucleotide positions 31–48 and AS-4 to nucleotides 136–152, respectively. AS-3 and AS-4 sequences have been used previously as uniformly modified phosphorothioates to inhibit αV expression [19].

2.2. ODN synthesis

All ODNs were synthesised in our laboratory. They were routinely synthesised as partial phosphorothioates, following the 'minimal' protection strategy proposed by Peyman and Uhlmann [20]. This strategy is a combination of the end-capping technique (also known as the gap

technique) and the protection of internal pyrimidine residues (the major sites of endonuclease degradation). These modifications are suggested to decrease reagent side-effects and possibly increase specificity. ODNs were synthesised using an Applied Biosystems 394 DNA synthesiser (Perkin Elmer Applied Biosystems, Inc., Foster City, USA) and standard phosphoramidite chemistry. After coupling, phosphorothioate linkages were introduced by sulphurisation using the Beaucage reagent [21], followed by capping with acetic anhydride and N-methylimidazole. After cleavage from the solid support and final deprotection by treatment with concentrated ammonia, ODNs were purified by polyacrylamide gel electrophoresis. All ODNs were analysed by negative ion electrospray mass spectroscopy (Fisons Bio-Q) which in all cases confirmed the calculated mass.

2.3. Cells

The MDA-MB231 human breast carcinoma cell line was acquired from the Imperial Cancer Research Fund (ICRF) mammalian cell culture laboratory and the European Tissue Culture Collection (ETCC). Cells were cultured in Dulbecco's Modified Minimum Essential Medium (DMEM) with Earle's salts containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 0.2 mg/ml streptomycin, 0.2% glycine at 37°C in 5% CO_2, 95% air in a humidified atmosphere.

2.4. Treatment with ODNs

ODNs were diluted in DMEM in stock solutions and stored in aliquots at −20°C until use. Treatment of MDA-MB231 cells was performed in the presence of the uptake enhancer lipofectamine (5 μg/ml). Lipofectamine was mixed with appropriate dilutions of ODNs and pre-incubated for 30 min prior to administration to the cells. Experiments were conducted with 5000 cells/100 μl medium.

2.5. Fluorescence-activated cell sorting (FACS) analysis

MDA-MB231 cells were diluted to a density of 1–5×10^5 cells per 20 μl. Cells were stained in a 96-well round-bottomed plate with 20 μl of primary antibody; phosphate-buffered serum (PBS) was used as a negative control. The MAbs used have been described previously. The cells were then stained with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody followed by three washes with NCS buffer (10% newborn calf serum in PBS containing 0.05% sodium azide), and resuspended in 100 μl of the NCS buffer for FACS analysis.

Cell suspensions, stained for the detection of surface antigen, were analysed by flow cytometry using a FACScan (Becton-Dickinson, Cowley, Oxford, UK) with FACScan software. Data acquired were represented as a histogram of 10 000 positive events and described statistically, by the software, with the median taken as the value of that particular treatment. Percentage positive cells were compared between negative (PBS) and antibody-stained cells, and between untreated and treated cell groups.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was prepared using the acid phenol technique. For RT-PCR, 1 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase and the equivalent of 0.1 μg was added to PCR reactions. These were carried out in a final volume of 50 μl buffer containing 200 μM of dNTPs, 1.5 mM $MgCl_2$, 10 pM of each primer and 1 U Tfl-DNA-polymerase. For semi-quantitative assay, a standard calibration curve was set up to determine optimum number of cycles (25–35 cycles). 33 cycles revealed to be most appropriate. PCR conditions were, therefore: 33 cycles; 94°C (30 s), 50°C (30 s), 68°C (1.5 min). The following primer pairs were used to amplify fragments of the human αV (293 bp) and β-actin (620 bp) cDNAs: αV: forward primer 5′-TGAGGATATCACCAACTCCACA-3′; reverse primer 5′-GTTGCTAATTCTAGTGGGTCA-3′; β-actin: forward primer 5′-ATGCCTCTGGTCGTACCACGGG-CATTG-3′; reverse primer 5′-TTGCTGATCCACAT-CTGCTGGAAGGTG-3′.

Ten microlitres of PCR-amplified products were analysed on a 2% agarose gel containing ethidium bromide.

2.7. Western blotting

Cell monolayers were rinsed in PBS and lysed in a 0.5% triton X-100 extraction buffer with the addition of non-reducing sample buffer (2% sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM Tris pH 6.8). Cell extracts were electrophoresed through a 7.5–15% SDS-polyacrylamide gel and then transferred to Hybond N nitrocellulose. After blocking in 5% milk, primary antibodies were incubated with the blots at a dilution of 1/200 overnight at 4°C. Species–specific horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated at 1:5000 dilution for 60 min at 37°C prior to ECL detection of the proteins, according to the manufacturer's instructions.

2.8. Densitometric analysis

Assessment of the effects of 5543-ODNs on αV levels was performed by densitometric analysis. Band areas were analysed by scanning densitometry, using the Molecular Analyst software for the Bio-Rad Laboratories (Hercules, CA, USA) model 670 scanning densitometer, and each value converted to a percentage of the

total densitometric value. Normalisation was performed using the reference gene β-actin as an internal control.

2.9. ECM proteins and preparation and coating of glass coverslips

Thirteen millimetre glass cover slips were cleaned by 70% ethanol washing. The coverslips were coated with 250 μl of protein solution (10 μg/ml in sterile PBS) in a 24-well tissue-culture plate at 4°C overnight. Residual protein binding sites were blocked by saturating with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Coverslips were then washed three times in PBS and once in DMEM. The solutions were then replaced with 60% methanol, and the wells were further incubated for 1 h at 4°C. Methanol was removed by aspiration, and the wells were incubated with 100 μl of a buffer containing 50 mM Tris–HCl (pH 7.8), 110 mM NaCl, 5 mM $CaCl_2$, 1% BSA, and 0.1 mM phenylmethylsulphonylfluoride for 30 min at room temperature. Finally, wells were washed three times with DMEM supplemented with 0.2% BSA and immediately used for the experiment.

Table 1
Integrin expression on the human breast carcinoma cell line MDA-MB231 determined by flow cytometry[a]

Antibody	Integrin	MFI	Graded level of expression[a]
No antibody	Control	4	–
TS2/21	α1	10	–
HAS6	α2	71	+ +
PIB5	α3	110	+ + +
7.2	α4	6	–
PID6	α5	13	–
GOH3	α6	20	–
P5D2	β1	210	+ + + +
TS1/18	β2	5	–
PM6/13	β3	94	+ + +
B5-IVH2	β5	81	+ + +
13C2	αV	151	+ + + +
23C6	αVβ3	214	+ + + +
PIF6	αVβ5	76	+ + +

[a] The amount of expression was determined by comparison with a negative control (omitted primary antibody, phosphate-buffered serum (PBS) control) whose mean fluorescence intensity was ⩽5 fluorescence intensity (MFI) units. The level of expression was graded as follows: ⩽25, –; 26–49, +; 50–75, + +; 76–150, + + +; ⩾151, + + + +. The data shown are from a single experiment which was representative of three separate experiments.

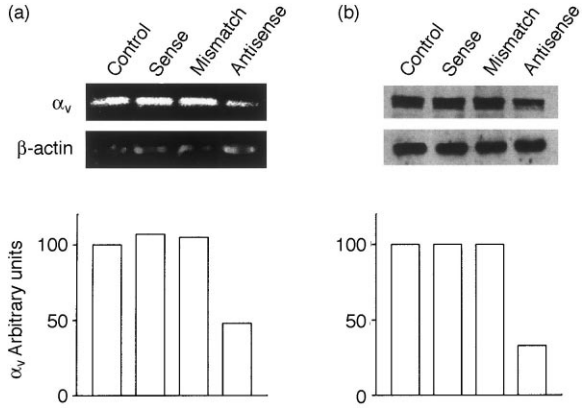

Fig. 1. Analysis of αV integrin expression in MDA-MB231 cells. 90% confluent MDA-MB231 cell monolayers were treated for 72 h with 1 μM 5543-ODNs. (a) RT-PCR. Densitometric analysis is shown below. Each value was converted as per cent of αV/β-actin ratio of 5543-ODN-treated versus control cells. The experiment was repeated three times with similar results. (b) Immunoblotting. 15 μg of protein were electrophoresed on a 7.5% SDS-PAGE gel under non-reducing conditions. Bands analysed by densitometry are shown below. Results are expressed as per cent of the αV/β-actin ratio of 5543-ODN-treated versus control cells. Similar results were observed in three independent experiments.

Table 2
The αV ODN reduces the surface protein expression of MDA-MB231 cells

	% change from IgG stained control[a]	
Antibody	αV antisense 5543 ODN	αV mismatch 5543-ODN
13C2	−35.13 (±11.2)	−2.33 (±4.33)

[a] Mean fluorescence index for IgG/no first antibody (control) on untreated MDA-MB231 cells = 3.58 (±0.39) and for 13C2 stained untreated MDA-MB231 cells = 144.1 (±2.95). $n = 3$ experiments (each the mean of 10 000 analysed cells); $P < 0.05$. Cell lines were pretreated for 24 h followed by a subsequent 24 h challenge with 2 μM of the 5543 αV ODN or mismatch control in the presence of 10% serum. Cells were trypsinised and primary stained with 13C2 (anti-αV) and secondary stained with a Fluorescein isothiocyanate FITC-rabbit anti-mouse MAb.

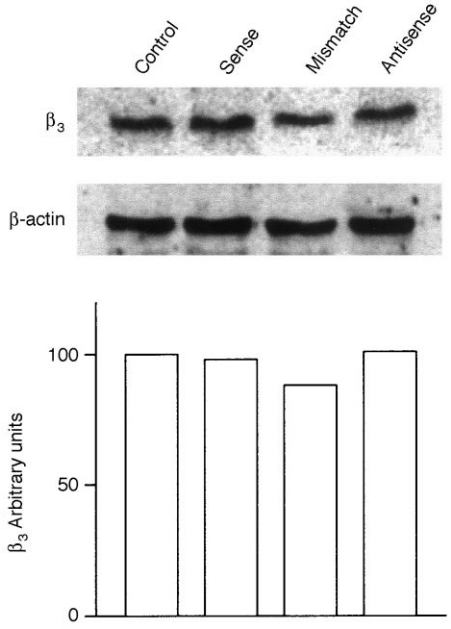

Fig. 2. Analysis of β3 integrin expression by immunoblotting in MDA-MB231 cells. 90% confluent MDA-MB231 cell monolayers were treated for 72 h with 1 μM 5543-ODNs. 15 μg of protein were electrophoresed on a 7.5% SDS-PAGE gel under non-reducing conditions. Bands analysed by densitometry are shown below. Results are expressed as per cent of the β3/β-actin ratio of 5543-ODN-treated versus control cells. Similar results were observed in three independent experiments.

2.10. Cell morphology and immunofluorescence

Cells were fixed in 3% paraformaldehyde in PBS for 15 min at 4°C, rinsed to remove non-adherent cells and observed by phase contrast microscopy. For immunofluorescence, cells were permeabilised with 0.5% Triton X-100 in PBS at 0°C for 3 min, incubated with the primary antibodies diluted 1:50 in PBS containing 1% BSA for 1 h at 37°C followed by incubation with FITC-conjugated secondary antibodies and observation by conventional epifluorescence microscopy.

2.11. Cell adhesion assay

MDA-MB231 cells were plated in 24-well multiwell plates and incubated with the ODNs in the presence of lipofectamine for different times. At the end of incubation, wells were rinsed with PBS (three times) to remove the non-adhering cells. Each well was then treated with 20% methanol (10 min), and the cells were stained with 0.5% crystal violet in 20% methanol for 5 min before rinsing with distilled water and air drying for 15 min. Crystal violet was then solubilised with 100 μl of 0.1 N sodium citrate in 50% ethanol and transferred to 96-well microtitre plates, and absorbance, which was linearly proportional to the attached cells, read spectrophotometrically at 540 nm.

2.12. Nuclear staining and apoptosis

Bisbenzimide was used for DNA staining. Cells were fixed in Carnoy's fixative (methanol-glacial acetic acid 3:1), incubated for 30 min in 0.5 μg/ml bisbenzimide, rinsed (2× in distilled water), mounted in glycerol–PBS 1:1 and observed by conventional epifluorescence microscopy.

To evaluate apoptosis, fragmented DNA was stained by the TUNEL method according to the manufacturer's instructions. Briefly, cells were fixed in 4% buffered paraformaldehyde, washed (2 min, 4× in distilled water) and incubated in terminal deoxynucleotidyl transferase (TdT) buffer for 5–10 min at room temperature. Buffer was then removed, and cells were incubated in TdT- and Biotin-deoxyuridine triphosphate (dUTP)-containing buffer for 60 min at 37°C. Cells were then washed in TdT buffer for 15 min at room temperature, washed (2 min, 4× in distilled water), blocked in blocking reagent

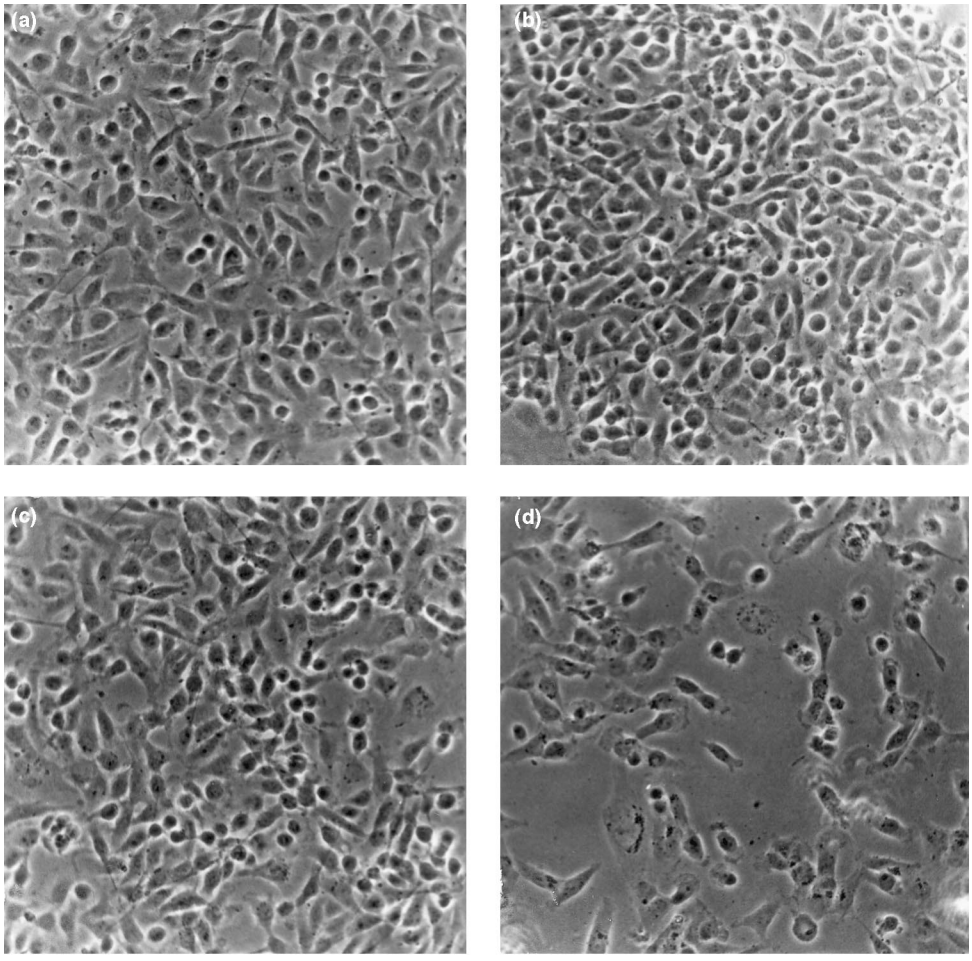

Fig. 3. MDA-MB231 cell morphology. Subconfluent MDA-MB231 cell monolayers were treated for 72 h with 1 μM 5543-ODNs and observed by phase contrast microscopy. (a) Control. (b) Sense 5543-ODN. (c) Mismatch 5543-ODN. (d) Antisense 5543-ODN. Magnification 300×.

and incubated with avidin-conjugated FITC for 30 min at 37°C. Cells were washed in PBS (5 min, 3×), counterstained with 0.5 μg/ml propidium iodide, washed in PBS (3 min), mounted and observed by conventional epifluorescence microscopy.

2.13. Statistics

Quantitative data are expressed as an average percentage of a control ±SEM (standard error of the mean). Probability data comparisons were analysed and calculated using the Mann–Whitney *U* test or the Student's *t*-test of duplicate samples in three or more replicate experiments. Statistical significance was determined by counting *P* values and considered significant if $P < 0.05$.

3. Results

3.1. Treatment with the ODNs

Incubation of MDA-MB231 cells with 5543-ODNs was performed in the presence of the uptake enhancer lipofectamine. Control experiments demonstrated that 5 μg/ml lipofectamine was most effective and that up to 72 h treatment was without toxic effect (data not shown).

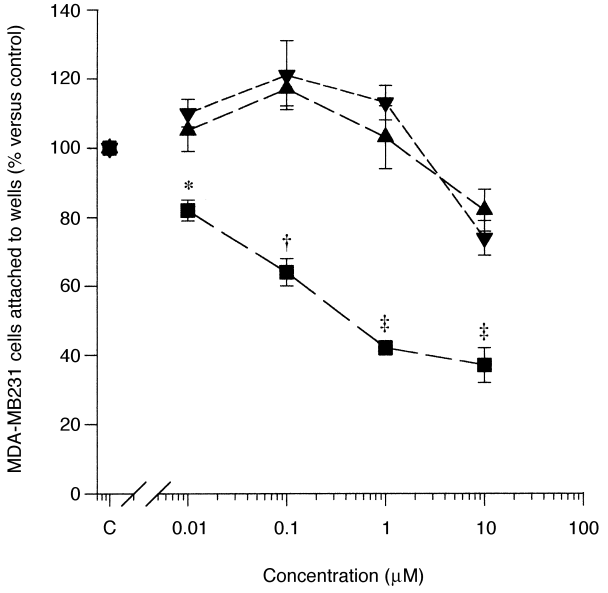

Fig. 4. Concentration-dependent inhibition of MDA-MB231 cell adhesion to substrate. MDA-MB231 cells were cultured in DMEM with the 5543-ODNs at the concentrations indicated on the abscissa and incubated for 72 h. After fixing and washing of cells, the nuclei were stained with crystal violet. This staining was assumed to be proportional to the number of adherent cells. Data are expressed as per cent versus control. Mean ± SEM $n = 3$. ■ Antisense 5543-ODN. ▼ Sense 5543-ODN. ▲ Mismatch 5543-ODN. $*P < 10^{-4}$, $\dagger P < 10^{-6}$, $\ddagger P < 5 \times 10^{-7}$ versus control.

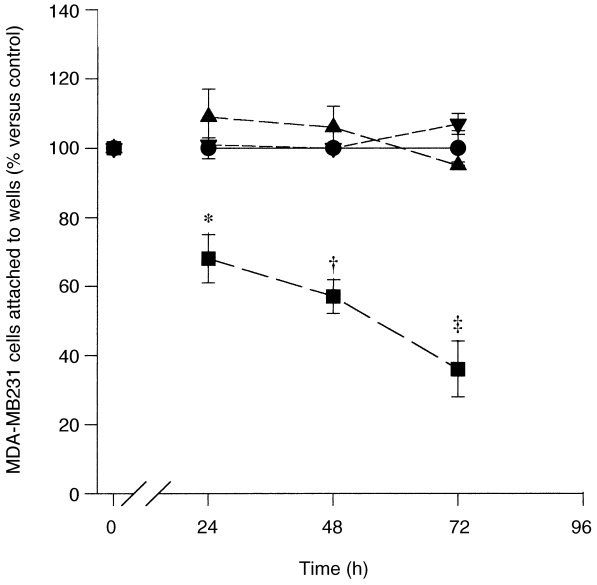

Fig. 5. Time-dependent inhibition of MDA-MB231 cell adhesion. MDA-MB 231 cells were cultured in DMEM containing 1 μM 5543-ODNs. Incubation proceeded for the times indicated on the abscissa, then cells were fixed, and adhesion was measured as described in Fig. 4. Data are the average ± SEM of per cent versus control. $n = 3$. ● Control. ■ Antisense 5543-ODN. ▼ Sense 5543-ODN. ▲ Mismatch 5543-ODN. $*P < 3 \times 10^{-4}$, $\dagger P < 10^{-6}$, $\ddagger P < 2 \times 10^{-7}$ versus control.

A summary of the integrin profile of the MDA-MB231 cells is shown in Table 1. Experiments to clarify whether the antisense 5543-ODN reduced αV expression were then carried out. RT-PCR of cDNA and immunoblotting analysis of whole cell fractions confirmed reduction of αV mRNA (53%) and protein (68%) in an

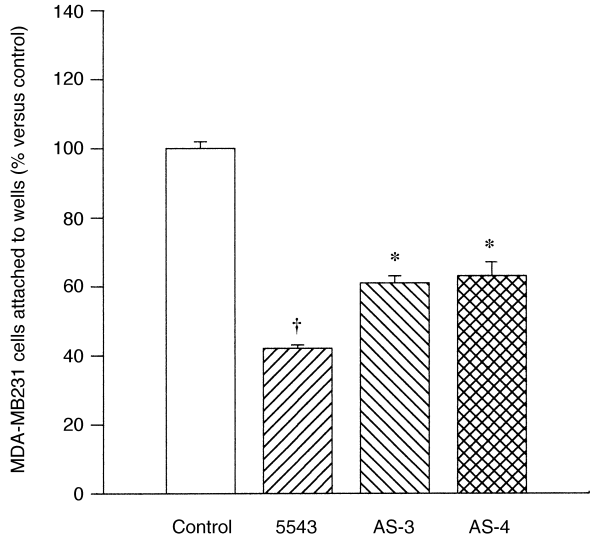

Fig. 6. Comparison of the effect of αV antisense 5543-ODN and AS-3 and AS-4. MDA-MB231 cells were incubated for 72 h with 1 μM αV antisense 5543-ODN, AS-3 or AS-4. Adhesion was measured as described in Fig. 4. Data are expressed as per cent versus control. Mean ± SEM $n = 3$. $*P < 10^{-3}$ versus 5543-ODN; $\dagger P < 10^{-4}$ versus control.

antisense-specific manner, as demonstrated by lack of effect by the sense and mismatch 5543-ODNs (Fig. 1). As reference gene, β-actin was used whose mRNA and protein expression level remained unchanged by the antisense treatment, further confirming antisense specificity. FACS analysis confirmed a significant reduction in the amount of MAb 13C2 (to αV) surface staining relative to a mismatch 5543-ODN treated culture. A summary of a number of experiments is described in Table 2.

To test selectivity for the αV subunit, the effect of the 5543-ODNs on the β3 integrin was evaluated. This subunit was chosen due to the fact that our FACS data (Table 1) indicated that the αVβ3 heterodimer was the αV-receptor mostly expressed in our cell line. Fig. 2 is an immunoblotting analysis showing that the level of β3 protein was unchanged in antisense 5543-ODN treated cultures relative to controls.

3.2. Cell morphology

Morphological analysis of antisense 5543-ODN-treated MDA-MB231 cells demonstrated little change, with modest cell retraction. The most obvious modification, however, was a dramatic reduction of the number of cells attached to the culture well in the antisense ($42 \pm 2\%$, $n = 3$, $P < 0.003$) relative to control ($100 \pm 2\%$), sense ($103 \pm 9\%$) and mismatch ($113 \pm 5\%$) 5543-ODNs (Fig. 3), with a proportional increase of floating cells in the antisense ODN-treated culture supernatants only. Immunostaining and fluorescence microscopy revealed that untreated MDA-MB231 cells, as well as cells treated with the sense and mismatch 5543 ODNs, expressed high levels of αV protein, located both within the cell and at the periphery of the cells, in areas resembling focal adhesion structures. In contrast, in antisense 5543-ODN-treated cells staining intensity was

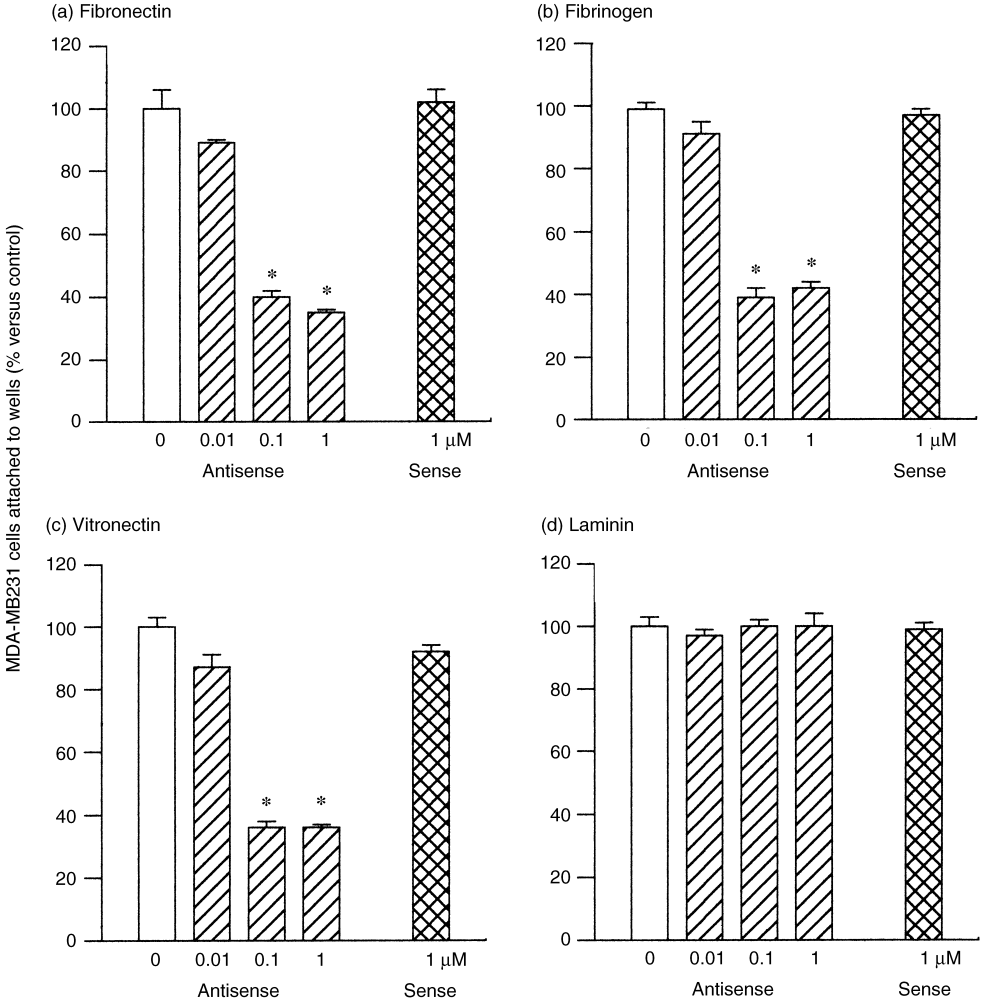

Fig. 7. Effect of αV antisense 5543-ODN on adhesion of MDA-MB231 cells to ECM substrates. MDA-MB231 cells were plated in wells coated with (a) fibronectin; (b) fibrinogen; (c) vitronectin; and (d) laminin (10 μg/ml) and incubated for 72 h in serum-free DMEM containing 0.2% BSA with 0.01, 0.1 and 1 μM antisense or 1 μM sense 5543-ODNs. Cells were then fixed and adhesion was measured as described in Fig. 4. Data are expressed as per cent versus control. Mean ± SEM $n = 3$. $^*P < 10^{-4}$.

generally reduced and focal adhesion structures were disassembled (data not shown).

3.3. Adhesion to ECM

To quantitate adhesion to ECM, MDA-MB231 cells suspended in FCS-containing growth medium, were plated onto plastic wells in the presence of the αV antisense and control 5543-ODNs where the adhesion substrate was assumed to originate from the serum proteins. A dose-dependent reduction of adherent cells in cultures treated with the antisense 5543-ODN was observed, with the plateau (58% decrease) reached at 1 μM (Fig. 4). This effect was time dependent. Fig. 5 shows maximal decrease (64%) on a 72-h incubation time. All control 5543-ODNs were without significant effect (Figs. 4 and 5). Again, non-attached, floating cells were observed in antisense ODN-treated cultures only.

In an experiment comparing the effect of our antisense 5543-ODN with that of the AS-3 and AS-4 ODNs which have been shown to be active on a melanoma cell line overexpressing the αVβ3 vitronectin receptor [19]. Fig. 6 shows that our antisense 5543-ODN was most effective, with a 58% reduction of adherent cells versus 39% for AS-3 and 37% AS-4.

The next question to be addressed was whether the effect of the antisense 5543-ODN was ECM substrate-specific. To accomplish this, MDA-MB231 cells were plated onto vitronectin-, fibrinogen-, fibronectin- and laminin-coated plastic. The number of cells attached to vitronectin, fibrinogen and fibronectin was reduced significantly in the presence of increasing (0.01–1 μM) antisense 5543-ODN concentrations (maximum decreases of 64%, 61% and 65% for fibronectin, fibrinogen and vitronectin, respectively). In contrast, the number of cells attached to laminin was unchanged. The sense 5543-ODN (1 μM) used as a control was inactive (Fig. 7).

3.4. Apoptosis

The next question to be addressed was whether treatment with the antisense 5543-ODNs led to programmed cell death. To this end, DNA was stained with bisbenzimide, a reagent that binds the adenine–thymidine regions of the nucleic acid revealing its morphological appearance. Fig. 8 shows a number of fragmented DNA bodies bulging out from several nuclei in the antisense 5543-ODN-treated cells. Such fragments were very few or totally absent in control, sense and mismatch

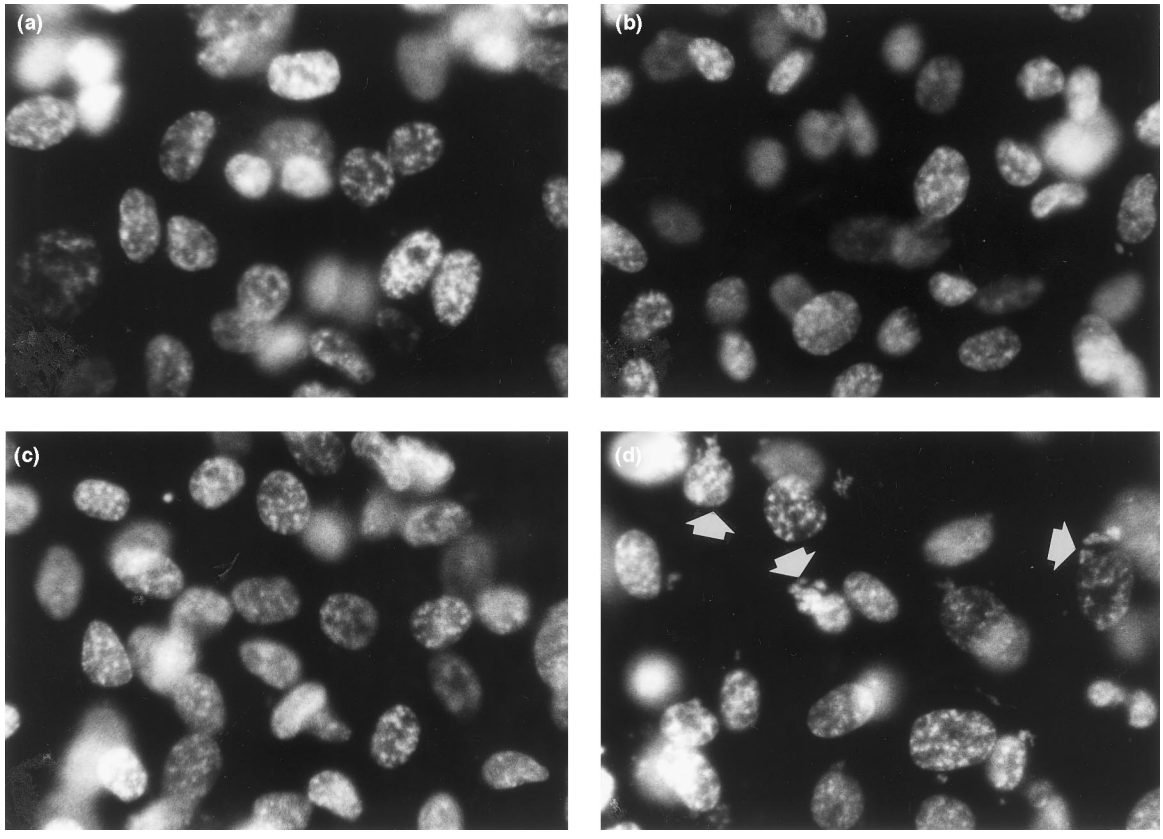

Fig. 8. Morphological evidence of apoptosis. MDA-MB231 cells were treated for 48 h as described in Fig. 3. Cells were then fixed, the DNA was stained by bisbenzimide and observed by conventional epifluorescence microscopy. (a) Control. (b) Sense 5543-ODN. (c) Mismatch 5543-ODN. (d) Antisense 5543-ODN. Magnification 2000×. Note normal nuclear morphology in (a), (b) and (c). In (d) several nuclei (arrows) are surrounded by DNA fragments, which are likely to represent apoptotic bodies.

5543-ODN-treated cultures. To confirm apoptosis with a specific method which reveals DNA fragmentation at early stage, TUNEL staining was applied. Fig. 9 shows TUNEL decoration of nuclei in the majority of antisense 5543-ODN-treated cells. In contrast, control, sense and mismatch OND-treated cultures showed staining in only a small proportion of cells, possibly revealing the 'physiological' rate of programmed cell death in this cell line in our culture conditions.

Apoptosis is known to be activated by intracellular signals which are cell type-specific and extremely heterogeneous. We, therefore, investigated the profile of a panel of genes potentially involved in the intracellular signal instigating apoptosis. The MDA-MB231 cell line is known to express a mutant form of the p53 pro-apoptotic factor [22]. Immunoblotting revealed p53 in our cultures. However, expression of the protein was unmodified following treatment with antisense and control 5543-ODNs (Fig. 10a). Therefore, the possible relocation of p53 by the antisense 5543-ODN was investigated by immunofluorescence analysis (Fig. 10b). From this experiment, in control, sense and mismatch 5543-ODN-treated cultures p53 was distributed both in the cytoplasm and nuclei of the MDA-MB231 cells (Fig. 10b — a,b,c). In contrast, in the antisense-5543-ODN treated cultures the majority of p53 protein was translocated to the nuclei (Fig. 10b–d). However, despite this, the profile of a series of genes (*Bcl-2*, *Bax*, $p21^{WAF1/CIP1}$) whose expression can be modified by activated p53, remained unchanged by 5543-ODN treatment (Fig. 11).

4. Discussion

The spread of mammary carcinomas is the most severe complication of this malignancy, and the skeleton represents a site for which this tumour has a specific competence for metastasis [1]. To date, no valid therapeutic intervention is available, though the use of bisphosphonates is currently in clinical trial [23]. Studies aimed at identifying molecules active against one of the many multistep events leading to metastasis of breast cancer could be considered of high potential impact. We developed an antisense DNA which complements the translation start site of the αV integrin gene. This antisense 5543-ODN has been tested for its anti-adhesive and pro-apoptotic effects in one of the most aggressive human breast carcinoma cell lines, the MDA-MB231, the cells being characterised by a high bone metastatic

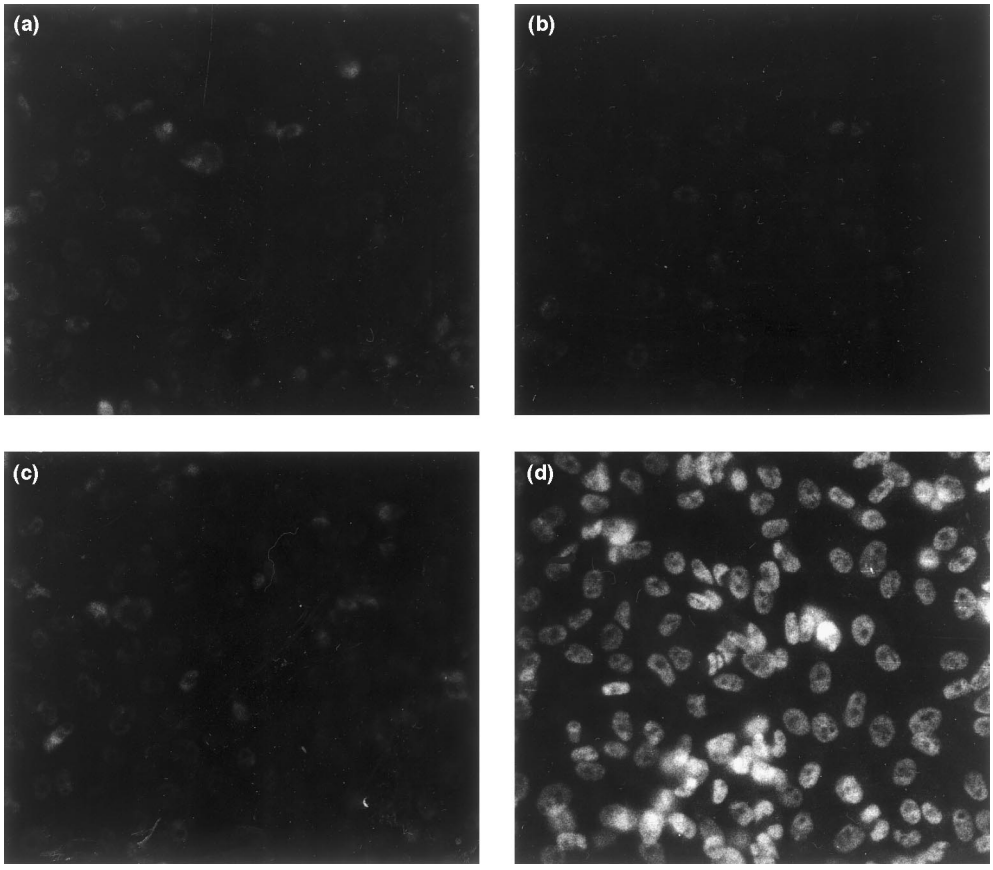

Fig. 9. Staining of fragmented DNA by TUNEL. MDA-MB231 cells were treated for 48 h as described in Fig. 3. Cells were then fixed, the fragmented DNA was stained by the TUNEL method and observed by conventional epifluorescence microscopy. (a) Control. (b) Sense 5543-ODN. (c) Mismatch 5543-ODN. (d) Antisense 5543-ODN. Magnification 800×. Note the dramatic increase of positive nuclei in (d) relative to (a), (b) and (c).

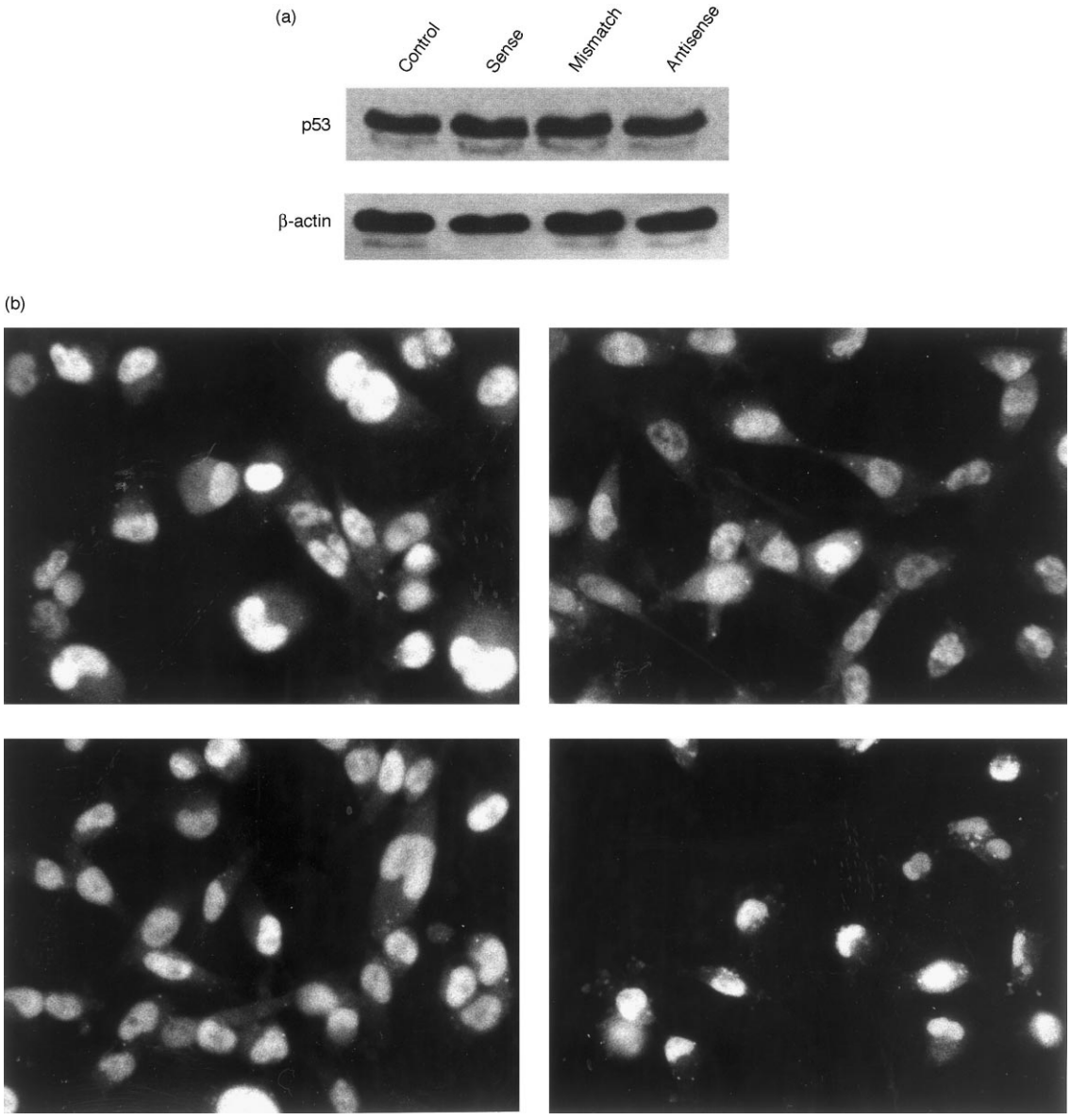

Fig. 10. Analysis of p53 expression and distribution. 90% confluent MDA-MB231 cell monolayers were treated for 72 h with 1 μM 5543-ODNs. (a) Immunoblotting. 40 μg of protein were electrophoresed on a 15% SDS-PAGE gel under non-reducing conditions. (b) Immunofluorescence. Cells were fixed, immunostained with anti-p53 antibody and observed by conventional epifluorescence microscopy. (a) Control. (b) Sense 5543-ODN. (c) Mismatch 5543-ODN. (d) Antisense 5543-ODN. Magnification 1200×. Note cytosolic and nuclear distribution of p53 in (a), (b) and (c), and nuclear staining only in (d).

potential [24,25]. Recent studies have suggested that their osteotropism is related to the high expression of the αV integrins [26], especially the αVβ3 heterodimer [27], a class of receptors recognising a number of ARG-GLY-ASP (RGD)-containing, ECM proteins, including vitronectin, fibrinogen, fibronectin and the sialoproteins of bone, osteopontin and bone sialoprotein (BSP) [26]. For example, BSP, which is abundant in bone matrix, supports breast cancer adhesion, proliferation and migration through differential usage of the αVβ3 and the αVβ5 integrins [26]. Although αV integrins are widespread in the body at a low level [28,29], only selected 'normal' cell types are known to express high levels of the αV protein, for instance, osteoclasts [28,29]. In contrast, increased levels of αV integrins have been observed in a number of pathological states, including various cancers [8–10,30]. Therefore, targeted disruption of αV-mediated adhesion could represent a valid method for preventing breast cancer metastasis to bone, without substantially altering the function of other organs.

The antisense 5543-ODN that was used in this study proved to be highly selective in reducing the αV mRNA and protein levels in MDA-MB231 cells. This finding, together with the lack of effect of the sense and mismatch 5543-ODNs, suggests a specific antisense mechanism of action of the reagent. In addition, we

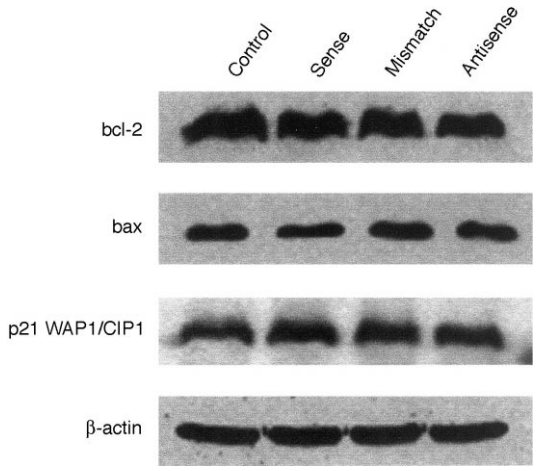

Fig. 11. Immunoblotting analysis of genes potentially involved in apoptosis. 90% confluent MDA-MB231 cell monolayers were treated for 72 h with 1 μM 5543-ODNs. 80 μg of protein were electrophoresed on a 15% SDS-PAGE gel under non-reducing conditions. The filters were then probed with anti-Bcl2, -Bax, -p21 WAP1/CIP1, and β-actin (internal control) antibodies.

have demonstrated no effect of the αV antisense 5543-ODN on β3 protein expression, thus further confirming high selectivity for αV. Prominent reduction of the number of cells attached to substrates recognised by the αV receptors (vitronectin, fibrinogen and fibronectin), but not to laminin, further confirms selectivity of the antisense 5543-ODN. The reduction in cell number could also be due to a combined effect on adhesion and on other events such as proliferation and cell death (see below). Nevertheless, it is interesting to note that no effects on cell viability and growth were observed on laminin.

Our antisense 5543-ODN was more active relative to the AS-3 and AS-4 sequences. The latter two antisense ODNs have been used previously as all-phosphorothioates to suppress adhesion to substrate of a melanoma cell line overexpressing the αVβ3 receptor [19]. In our study, all ODNs were used as partially phosphorothioated modifications in order to try to reduce non-antisense effects which have been reported for uniformly phosphorothioate modified antisense ODNs [31]. Both the 5543-ODN and AS-3 were directed against the translational start codon, although 5543-ODN is shifted downstream by nine nucleotides. AS-4 is directed against the coding region. Our study clearly shows that the selection of the mRNA target sequence is crucial with respect to the inhibitory effect.

The antisense 5543-ODN induced an increased apoptosis rate, and TUNEL staining showed that most cells undergo this process. It is known that inhibition of anchorage to substrate activates intracellular signals initiating apoptosis, and that the αV receptors play a pivotal role in preventing this event [32–35]. Apoptosis is an active process requiring transcriptional events prior to nuclear fragmentation and packaging of DNA into 'apoptotic bodies' [36]. There are several cellular 'switches' activating cellular death, one of which is the p53 protein, a gene product that senses DNA damage and arrests cells in the $G_0 \rightarrow G_1$ phase of the cell cycle through activation of $p21^{WAF1/CIP1}$, an inhibitor of the cyclin dependent kinase complexes [34,37]. Other signals to apoptosis are represented by reciprocal changes of the Bcl-2 and Bax gene products. When the Bcl-2/Bax ratio is reduced, the pro-apoptotic performance of the Bax product is favoured [38]. In our cell model, the expression of these genes was unaltered by the antisense 5543-ODN. However, the p53 protein was translocated from the cytoplasm to the nucleus, an event which occurs as a consequence of interruption of αV-mediated adhesion in other cellular models [34]. p53 expressed by the MDA-MB231 cell line is a mutant form [22], and its signalling pathway is not fully understood. In our experimental conditions, activation of this mutant p53 could stimulate alternative signals to apoptosis, which, at present, remain uncharacterised. Alternatively, it cannot be excluded that mutant p53 does not induce programmed cell death in our cellular model and the apoptosis observed is, therefore, being activated by other, p53-independent, pathways.

Breast cancer metastases to bone are destructive, or osteolytic, due to activation of osteoclastic bone resorption [1,25,39]. Osteoclasts also express αV at high levels, and αV receptors have been shown to be critical for bone resorption [28,29]. We have previously reported that the antisense 5543-ODN used in this study inhibits osteoclast adhesion and bone resorption [35]. The presence of αV integrins in both tumours and resorptive cells may be of great relevance in the pathogenesis of bone osteolytic metastasis as the development of such lesions requires the reciprocal stimulation of tumour and bone cells. Tumour cells have been shown to mediate destruction of the bone matrix via activation of osteoclastogenesis and stimulation of osteoclast resorptive function. There is little evidence for direct osteolytic activity by tumour cells themselves [1,25]. However, activated osteoclasts are thought to release paracrine factors, which, in turn, are able to stimulate tumour cell growth within the bone microenvironment [1,25]. Therefore, a reciprocal stimulation is likely to account for both local tumour expansion and osteoclastic osteolysis. Bone resorption inhibiting agents such as bisphosphonates are currently used as palliative therapy to reduce the devastating symptoms of bone malignancies; they also seem to have some inhibitory effects upon the incidence of bone metastasis [40,41].

The αV integrin receptors proved to be relevant for both osteoclast physiology and breast cancer dissemination to bone [28,29]. Other means have already been used for disrupting osteoclast adhesion to substrate. These include neutralising antibodies, the snake

venom disintegrin echistatin and RGD peptides [28,29]. Other workers have recently shown the advantage of using a non-peptide small molecule mimetic (SC-68448) to antagonise the αVβ3 integrin [42]. Compared with RGD peptides, our antisense 5543-ODN is highly specific for αV and, at variance with the antibodies and non-peptide mimetics, it blocks all the αV receptors, regardless of the associated β subunit. Osteoclasts and breast carcinoma cells express the αVβ1, the αVβ3 and the αVβ5 [8,28,29] receptors. Therefore, our antisense 5543-ODN targeted to the αV gene offers the advantage of blocking a mechanism shared by both cell types involved in the metastatic lesions, with high efficiency and specificity for all the αV receptors.

Despite the noticeable similarity of effect on adhesion, the mechanism underlying the apoptotic events activated by the αV antisense 5543-ODN in osteoclasts and MDA-MB231 cells seem to differ in some specific aspects. Osteoclasts showed an increase of $p21^{WAF1/CIP1}$ expression and a reduction of the Bcl-2/Bax ratio [35], whereas none of these genes were modified in MDA-MB231 cells. This indicates that the intracellular signals to apoptosis regulated by the αV gene are heterogeneous and may be cell type specific. Further work is necessary to better elucidate this phenomenon.

In conclusion, we have demonstrated that a valid strategy aimed at reducing the capability of MDA-MB231 cells to recognise and attach to the ECM substrates such as might also be present in bone is the use of a specific antisense 5543-ODN targeted to the translational start site of the αV integrin gene. This 5543-ODN also induces apoptosis with a mechanism probably involving p53, but independent of $p21^{WAF1/CIP1}$ Bcl-2 and Bax. The efficacy of the 5543-ODN in interrupting the contact with ECM of tumour cells and osteoclasts suggests that this compound may block metastatic bone diseases. *In vivo* studies in animal models will be required to assess if antisense 5543-ODNs could have an application as an alternative intervention for preventing or curing breast carcinoma bone metastasis.

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