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Depletion of αV integrins from osteosarcoma cells by intracellular antibody expression induces bone differentiation marker genes and suppresses gelatinase (MMP-2) synthesis

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Abstract

Integrin heterodimers sharing the common αV subunit are receptors for adhesion glycoproteins such as vitronectin and fibronectin. They are suggested to play an essential role in cell anchoring, differentiation, and survival. Here, we describe the construction of an expression plasmid coding for an intracellular single-chain antibody against αV integrin subunit. Saos-2 osteosarcoma cells transfected with this DNA construct showed an approximately 70–100% decrease in the cell surface expression of αVβ3 and αVβ5 integrins as shown by flow cytometry. Intracellular antibody expression had no effect on the mRNA levels of αV integrin. Pulse chase experiments of metabolically labeled integrins showed that the translation of precursor αV integrin subunit was not affected. However, the maturation of αV integrins as glycoproteins was slow suggesting that the transport from endoplasmic reticulum to Golgi complex was partially prevented. Depletion of αV integrins from Saos-2 cells led to a decreased ability to spread on fibronectin and vitronectin. Furthermore, the expression of osteoblast differentiation marker genes, alkaline phosphatase and osteopontin, was induced and concomitantly the expression of matrix metalloproteinase-2 decreased. Thus, αV integrins seem to be important regulators of osteosarcoma cell phenotypes. Our data also indicate that the expression of intracellular antibodies is an effective strategy to study the significance of specific integrins for cell phenotype and differentiation.

Keywords: Osteosarcoma; Integrins; Matrix metalloproteinases; Single-chain antibody; Intracellular antibody

Abbreviations: ALP, Alkaline phosphatase; DMEM, Dulbecco's modification of Eagle's medium; EtBr, Ethidium bromide; FCS, Fetal calf serum; FN, fibronectin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HIV, human immunodeficiency virus; HON, human osteonectin; MMP, matrix metalloproteinase; OP, osteopontin; VN, vitronectin

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1. Introduction

The integrins are a large family of transmembrane glycoproteins that mediate cell–cell or cell–matrix interactions (Ruoslahti, 1991; Hynes, 1992). Their signal transduction function also connects them to the regulation of cell survival, differentiation, and proliferation. An integrin-type receptor consists of an α and β subunit which are non-covalently linked to each other. Currently, 17 different α subunits and eight β subunits have been recognized and they are known to form at least 23 αβ heterodimers.

Integrin αV functions as a vitronectin or fibronectin receptor in complex with different β subunits (β1, β3, β5, β6, and β8). Mice deficient in αV integrin subunit develop normally, except that some large vessels are fragile leading to death during or just after the birth (Bader et al., 1998). There are in situ findings that normal osteoblasts express β5 integrin in rat bone (Hultenby et al., 1993) and αV integrins in human bone (Hughes et al., 1993). The in vivo expression of integrins in osteosarcomas is not known in detail but the expression of αV integrins has been detected in several osteosarcoma-derived cell lines (Clover and Gowen, 1994; Stuiver et al., 1996). αV integrins have also been associated with various other forms of cancer, especially with human melanoma (Albelda et al., 1990; Felding-Habermann et al., 1992; Danen et al., 1995; Mitjans et al., 1995). The importance of αV integrins for cancer cell phenotype suggests that it may also play a role in the maintenance of osteosarcoma cell phenotype. The differentiation of osteoblasts in vivo is not dependent on αV integrins (Bader et al., 1998), whereas the interaction of another fibronectin receptor, α5β1 integrin, with fibronectin is essential for rat calvarial osteoblast differentiation (Moursi et al., 1996, 1997) and anti-fibronectin antibodies can decrease alkaline phosphatase mRNA levels. Promotion of osteoblast differentiation by α5β1 integrin has been suggested also in other studies (Dedhar et al., 1987).

Soluble antibody and peptide inhibitors of αV integrins have been successfully used in studies on αV integrin function. However, experimental approaches using these inhibitors may face problems in targeting the molecules to cells inside tissues. In these cases new methods are needed to specifically modify integrin expression. Intracellular single-chain antibodies have been used to prevent transportation of specific proteins to the cell surface (Beerli et al., 1994; Deshane et al., 1994; Richardson et al., 1995). They have also been used to inhibit the processing of human immunodeficiency virus (HIV-1) envelope protein (Marasco et al., 1993), to alter the function of Rev, HIV regulatory protein (Duan et al., 1994) or to reduce activity of HIV reverse transcriptase (Maciejewski et al., 1995). Here we have used intracellular single-chain antibody against αV integrin to block its expression on osteosarcoma cells and we show that the reduced expression of αV integrins leads to repression of some cellular properties typical for malignant phenotype. The data indicate that the use of constructs coding for intracellular antibodies against adhesion receptors may be considered as an effective tool in integrin research.

2. Results

2.1. Osteosarcoma cells expressing intracellular antibody against anti-αV integrin have reduced cell surface expression of αV integrins

Surface expression of the αV integrin subunit on human osteosarcoma Saos-2 cell clones transfected with anti-αV antibody construct was measured by flow cytometry. In all four independent single-cell clones analyzed (named a1–a4) the expression of αV integrin was decreased (70–100%) when compared to five vector-transfected control cell clones that had gone through the same selection process (named v1–v5) (Fig. 1A). In some anti-αV integrin clones the αV integrin-related fluorescence was very close to negative control level, that is cells stained with secondary antibody only (Fig. 1A). No significant changes were detected in αV integrin mRNA levels (Fig. 1B) indicating that the cell surface expression of αV integrins was suppressed at post-translational level. For immunoprecipitations cell layers were labelled with [^{35}S]methionine, detergent soluble proteins were extracted and αV integrins were precipitated with monoclonal antibody (L230) recognizing the same epitope as the intracellular anti-αV integrin single-chain antibody. In control cells L230 precipitated an approximately 150-kDa band, representing αV integrin, and a smaller broad band suggested to represent αV-associated β subunits (Fig. 1C). In cells expressing intracellular single-chain antibody against αV integrin no precipitable αV integrin was present (Fig. 1C), indicating that the intracellular production of single-chain antibody prevented the binding of L230. The expression of the anti-αV single-chain antibody was also confirmed by detection of the corresponding mRNA by RT-PCR analysis (not shown). Cell clones a1, a2, v4, and v5 were selected for further experiments.

Integrin αV can form a heterodimer with several integrin β subunits and we used flow cytometry to measure the expression of individual αV integrins (Fig. 2). The cell surface expression of αVβ3, αVβ5, and αVβ6 integrins was reduced near to negative control level. The integrin expression levels were quite

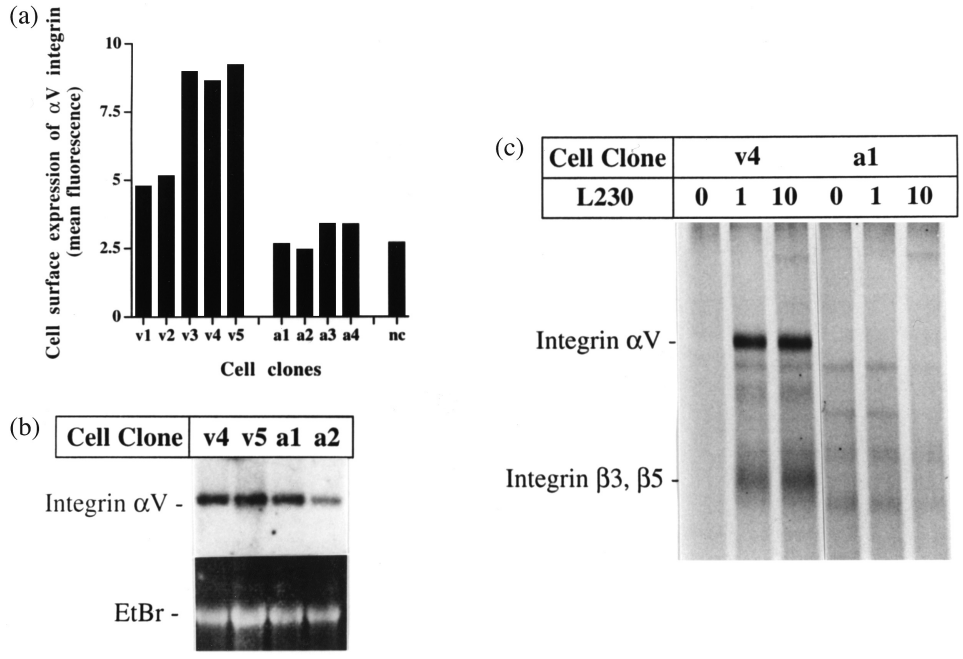

Fig. 1. Transfection of Saos-2 human osteosarcoma cells with anti-αV integrin single-chain antibody cDNA results in αV-depleted cell clones. Panel A shows flow cytometric measurement of αV integrin expression on anti-αV single-chain antibody cDNA transfected (a1–4) and vector control (v1–5) cell clones by using antibody specific to αV integrin and FITC-conjugated secondary antibody. The figures represent the median values of fluorescence pattern. Negative control (nc) was prepared by using the secondary antibody only and it showed a fluorescence level similar to a1 and a2 cell clones. Panel B shows the equal expression of αV integrin mRNA in cells expressing anti-αV single-chain antibody (cell clone a1 and a2) and in vector controls (v4 and v5) measured by Northern hybridization. Ethidium bromide (EtBr) staining of the sample RNA is shown as a control. Panel C shows immunoprecipitation of [^{35}S]methionine-labeled proteins with two different concentrations of anti-αV integrin monoclonal antibody (L230). Integrin αV subunit can be precipitated from vector control cells (v4) but not from cells (a1) expressing intracellular antibody against the same epitope as the antibody used in the immunoprecipitations. Lanes marked with 0 indicate precipitations done without primary antibody.

similar in cell clones a1 and a2. The expression of β1 integrins was also analyzed and surprisingly, there was an approximately sevenfold increase in the expression of α4 integrin. The expression of β1 and α5 integrins was slightly below the level seen on control cells. In addition to the v4 control cell clone shown (Fig. 2) the analysis of the v5 cell clone gave similar results; the expression level of αV integrins was identical but the expression level of the β1 integrins was somewhat lower than in v4 clone.

2.2. Intracellular single-chain antibody prevents the maturation of αV integrin as a glycoprotein

By using pulse chase assays and immunoprecipitations with polyclonal anti-αV integrin antiserum we showed that αV is produced as a precursor protein that has slightly faster mobility than the mature αV subunit (Fig. 3). This is in full accordance with the previous detailed report about the maturation and transport of αV integrins (Sheppard et al., 1992). The shift in the size of integrin subunits has been shown to represent the change of high-mannose type oligosaccharides to complex-type ones when integrins are removed from endoplasmic reticulum to the Golgi complex (Heino et al., 1989; Sheppard et al., 1992). In control Saos-2 cells it took approximately 2 h before 50% of αV integrins labelled during a 1-h pulse had shifted from the precursor pool to the mature pool (Fig. 3). In cell clones expressing intracellular single chain antibody the translation of αV subunit was observed (Fig. 3). However, αV subunit stayed in the precursor form substantially longer than in control cells (only 29% of radioactive αV pool maturated during the first 8 h). The fact that the maturation of αV integrins was not completely blocked was in accordance with the finding that despite the intracellular antibody expression the cells have low levels of αV integrins on their surfaces.

2.3. Reduced expression of αV integrins affects cell adhesion and spreading on both vitronectin and fibronectin

Morphological changes were seen in αV integrin-deficient cells cultured on cell culture plastic in the presence of 10% FCS. In these conditions cells use mainly serum-derived vitronectin and fibronectin as their adhesion substrate. The spreading area of anti-αV integrin-expressing cell clones remained smaller than that of the vector control cell clones (not shown).

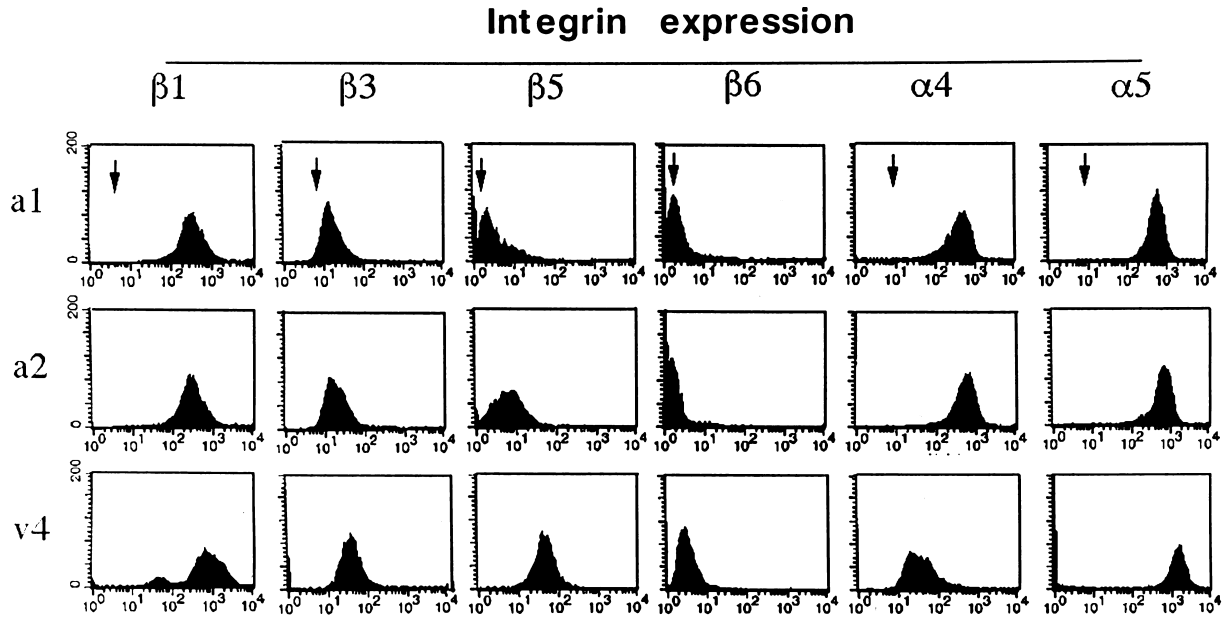

Fig. 2. Flow cytometric measurement of β1 and αV integrins on Saos-2 cell surface. Antibodies against β1, β3, β5, β6, α4, and α5 were used. In the upper panels arrows indicate the mean levels of fluorescence in negative control samples. a1 and a2 are Saos-2 cell clones expressing intracellular antibody against αV integrin. v4 is a vector control cell clone.

To investigate the influence of altered integrin cell surface expression on cell adhesion to specific matrix proteins, a1, a2, v4 and v5 cell clones were allowed to attach on fibronectin or vitronectin. All cell clones achieved their maximum adhesion on the same matrix concentration (5 μg/ml of vitronectin or 1 μg/ml of fibronectin). On vitronectin the adhesion of a1 and a2 cell clones was clearly slower (on average 43% less adherent cells after 80 min) compared to vector control cell clones (Fig. 4A) and on fibronectin a similar although smaller difference was seen (33% less adherent cells than in controls) (Fig. 4B).

A cell spreading assay was used to get more information about the role of αV integrins in cell attachment. In agreement with the cell adhesion experiments, cell spreading of anti-αV integrin expressing a1 and a2 cell clones was remarkably decreased on vitronectin (Fig. 4C). However, in the presence of the function blocking monoclonal antibody specific for the αV integrin subunit, the cell spreading of all clones was completely inhibited on vitronectin. On fibronectin cells still spread after addition of the antibody. Thus we concluded that the spreading of Saos-2 cells on vitronectin is entirely mediated by αV integrins, whereas other receptors, probably β1 integrins, are also involved in spreading on fibronectin. Furthermore, the changes in cell adhesion and spreading on vitronectin and fibronectin as a result of anti-αV integrin expression appear to be mainly due to the reduced expression of αV integrins. Finally, the data again indicates that some αV integrins remain on cell surfaces in spite of the expression of intracellular antibody.

Two independent assays were performed to analyze the growth rates of individual cell clones. In general the growth rates were very similar, although small differences were detected between anti-αV integrin transfected clones and vector controls (Fig. 5). In both experiments the cell numbers of a1 and a2 clones were slightly reduced (2% and 12% in Exp. 1; 5% and 4% in Exp. 2) during the first 26 h. In contrast, cell numbers of v4 and v5 clones slightly increased during the same period (3% and 3% in Exp.1; 17% and 10% in Exp. 2). After an incubation of 48 h the cell numbers of a1 and a2 clones started to increase and their growth rate thereafter was somewhat faster than the growth rate of v4 and v5 cell clones.

2.4. Reduced expression of αV integrins is accompanied by induction of alkaline phosphatase and osteopontin

Alkaline phosphatase, osteopontin, and osteonectin mRNA levels were measured to study the role of αV integrins in the regulation of cell phenotype. Cell clones were plated in the presence of serum in a density leading to cell confluence soon after cell attachment. The most dramatic effect was the induction of osteopontin expression in cells expressing intracellular anti-αV antibody. Fig. 6 shows the osteopontin mRNA levels in post-confluent cells. Northern blots were also quantitated by image analyzer and the mRNA levels of osteogenic marker genes were cor-

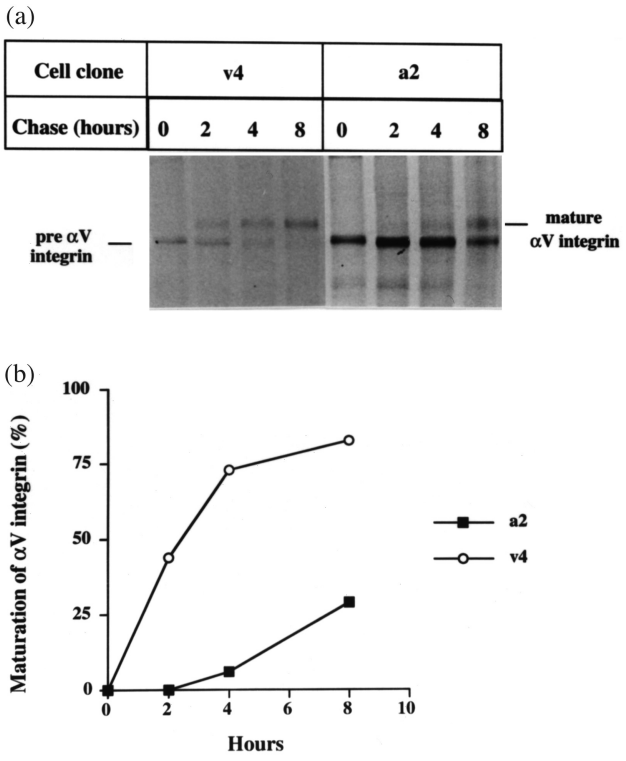

Fig. 3. Intracellular anti-αV single-chain antibody prevents the maturation of αV integrins as glycoproteins. Panel A shows the maturation of αV integrin as a glycoprotein in vector control and anti-αV transfected Saos-2 cells. Cells were labeled with [^{35}S]methionine for 1 h and harvested 0, 2, 4 and 8 h after the pulse. αV integrins were immunoprecipitated with a specific polyclonal antiserum, separated by SDS-page and visualized by autoradiography. Precursor αV and mature αV are indicated. Quantitative analysis of panel A shows the maturation of αV integrin (panel B). The curves indicate the amount of mature αV integrin as a percentage of the total (pre and mature) αV integrin.

rected to the expression of a house-keeping gene, GAPDH, used as an internal control (Fig. 7). Three days after seeding the cells alkaline phosphatase mRNA levels were already higher in a1 and a2 cell clones than in the control clones (Fig. 7A). They increased markedly in a1 and a2 cell clones up to day 6 (sixfold increase compared to levels on day 3) after which they began to fall again. A similar trend was found in alkaline phosphatase mRNA levels in v4 and v5 control clones but the levels were considerably lower (on average 81% lower on day 6). Thus the expression of osteoblast differentiation marker gene alkaline phosphatase seems to be higher when αV integrin function is inhibited. Osteopontin mRNA was not visible in vector controls at any time point (Figs. 6 and 7B). In anti-αV integrin expressing a1 clone osteopontin gene was turned on on day 3 (Fig. 7B), whereas in a2 clone its expression was detected on day 6, when a1 clone also showed the highest expression level. Osteonectin mRNA levels (Fig. 7C) were slightly higher in control cell clones than in anti-αV integrin transfected clones (on average 2.3-fold difference on day 6). The α1(I) collagen mRNA expression varied between the individual cell clones, but without correlation to anti-αV expression (not shown).

The direct role of αV integrins in the regulation of alkaline phosphatase was tested in an experiment in which confluent cells were exposed to exogenously added antibodies (L230, anti-β1 integrin mAb13, control IgG) for 3 days. L230, unlike anti-β1 integrin or control antibodies, could elevate alkaline phosphatase mRNA levels (twofold increase), but only in control cells and not in cells in which αV integrin expression was depleted by intracellular antibody expression. In these cells the basal level of alkaline phosphatase mRNAs was sixfold higher than in vector control cells and L230 had no effect (in L230-treated cells alkaline phosphatase mRNA levels were 1.05-fold when compared to control antibody-treated cells).

2.5. Reduced expression of αV integrins suppresses matrix metalloproteinase (MMP) synthesis

MMPs play an important role in cancer cell invasion and recent evidence indicates that integrins may participate in the regulation of their production (reviewed in Heino, 1996). Gelatinase release and cellular mRNA levels were analyzed in anti-αV integrin single-chain antibody-expressing cells by using Northern blot hybridizations and gelatin zymography (Fig. 8). In vector control cell clones cultured for 24 h on fibronectin the mRNA levels of MMP-2 were on average approximately 12 times greater than in anti-αV integrin cell clones. On vitronectin the difference was even more dramatic: mRNA levels of MMP-2 in v4 and v5 cell clones were approximately 26-fold greater than in a1 and a2 cell clones. When the cells were removed from the vitronectin/fibronectin-containing matrix (FCS) to laminin-1 the difference was only fivefold (after 24 h on laminin; not shown). MMP-2 enzyme activity in medium was estimated by semiquantitative gelatin zymography and the results confirmed the difference between the cell clones (Fig. 8). In addition to the 72-kDa MMP-2 band a 92-kDa gelatinolytic protein was detected in zymography. This protein co-migrated with MMP-9 and its expression was also down-regulated in the anti-αV expressing cells (Fig. 8).

3. Discussion

The aim of this study was to analyze the role of αV integrins in the regulation of the osteosarcoma cell phenotype. Concomitantly it was possible to estimate the putative participation of αV integrins on osteoblast differentiation. The cell surface expression of

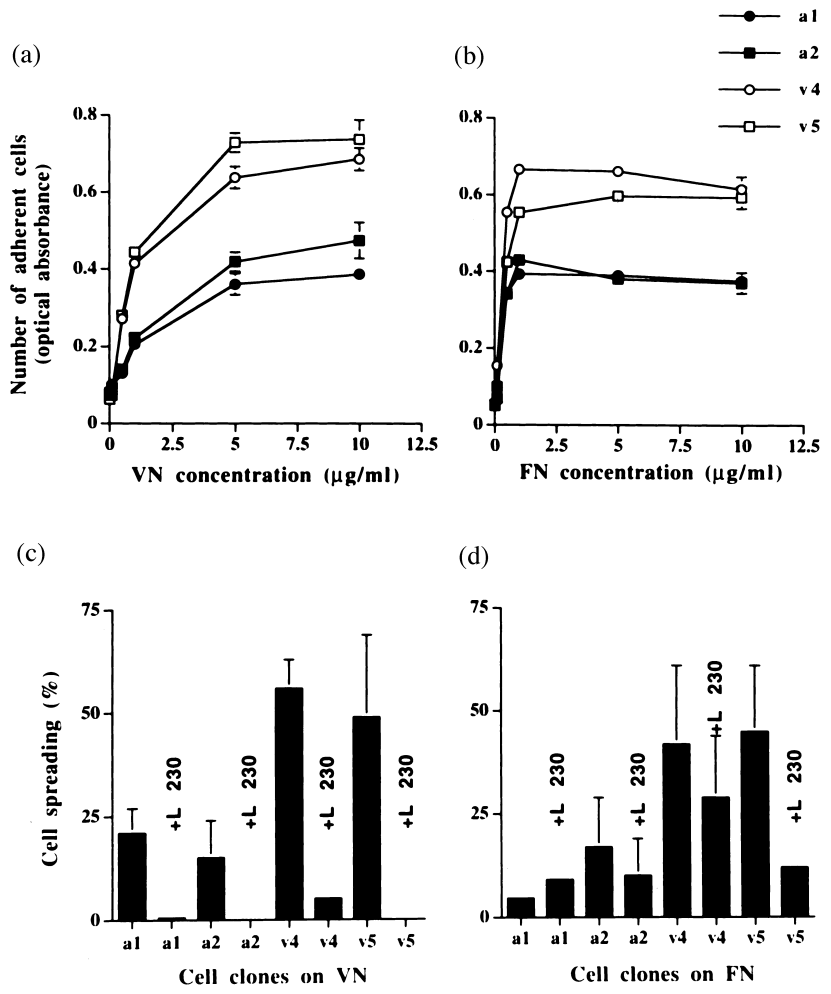

Fig. 4. Cell adhesion and spreading assays. Adhesion of anti-αV integrin intracellular antibody expressing Saos-2 cells (clones a1 and a2) and vector controls (v4 and v5 cell clones) on microtiter plate wells containing increasing concentrations of vitronectin (VN, panel A) and fibronectin (FN, panel B). Cell spreading on VN (panel C) and FN (panel D) in the presence and absence of L230 antibody against αV integrin. The same concentration (0.5 μg/ml) of both adhesion proteins was used. Results are percentages of well spread cells of all attached cells.

αV integrins was depleted by the use of intracellular single-chain antibodies. The intracellular expression of single-chain antibodies is an established method to prevent the transport and consequently the function of specific proteins (Deshane et al., 1994; Richardson et al., 1995; Yuan et al., 1996).

We used an anti-αV integrin hybridoma and a well documented PCR strategy (Richardson et al., 1995) shown to preserve the properties of the original monoclonal antibody to create an expression construct coding for single-chain antibodies. Integrin subunit αV is produced in excess compared to its β subunit partners and the complex formation is critical for the transport from endoplasmic reticulum to Golgi complex and further to the cell surface (Sheppard et al., 1992). A similar mechanism regulates the transport of β1 integrins (Heino et al., 1989). Therefore it was suggested that the cell surface expression of integrins can be prevented by blocking the α/β complex formation inside endoplasmic reticulum or their transport to cell surface. In our experiments the cell surface αV integrin levels were reduced in all four independent single-cell clones analyzed, without significant changes in αV integrin mRNA levels. We could see the decelerated maturation rate of precursor the αV subunit pool, suggesting that the single-chain antibody can affect the transport of the α/β complex from the endoplasmic reticulum to Golgi but it cannot completely prevent it. Thus the transfection of cells with anti-αV antibody construct was an effective way to reduce the cell surface expression of αV integrins, despite the fact that complete depletion of αV integrins on cell surface was not seen. It is possible that osteosarcoma cell clones without αV integrins are not viable but go to apoptosis. Importantly, the growth rate of cells expressing single-chain antibody construct was not affected. This fact allows the use of the αV integrin-deficient cells in various in vitro and in

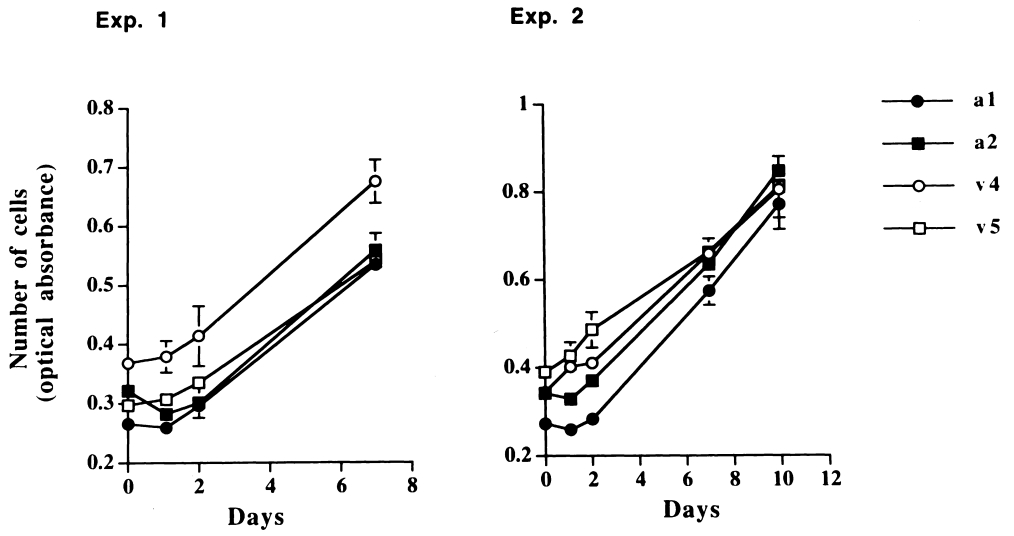

Fig. 5. Two independent growth curves of anti-αV integrin intracellular antibody expressing Saos-2 cells (clones a1 and a2) and vector controls (v4 and v5 cell clones). Cell clones were cultured in the presence of 10% FCS and the cell numbers were optically determined with the help of an enzymatic reaction.

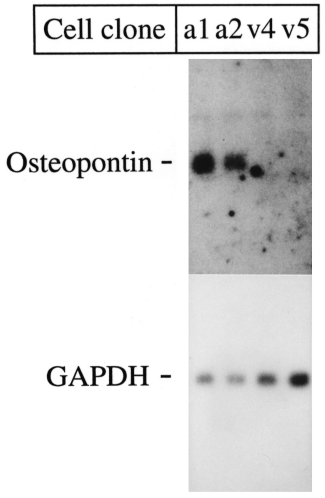

Fig. 6. The mRNA levels of an osteogenic differention marker gene, osteopontin, in Saos-2 cells expressing anti-αV integrin intracellular antibody (clones a1 and a2) and in vector controls (clones v4 and v5). Cells were cultured for 9 days in the presence of 10% FCS. Total cellular mRNA was isolated, separated on agarose gel and hybridized with cDNA probe specific for osteopontin. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was used as a control.

vivo assays. When compared to other approaches to study the significance of integrin subunits for cell function, the use of intracellular antibodies has obvious advantages. First, the treatment of cells with anti-integrin antibodies may either block integrin function or mimic ligand binding. Secondly, in many in vivo assays it is not possible to constantly block integrin function by the use of externally added antibodies. Instead methods to prevent the expression of the selected integrin on cells are required. Finally, the cDNAs coding for effective intracellular single-chain antibodies can be ligated to viral gene transfer vehicles, which opens new in vitro and in vivo applications and might lead to putative gene therapy approaches.

The selected cell clones expressing intracellular anti-αV antibodies also showed increased expression rates of α4β1 integrin. The mechanism of this phenomenon was not studied, but the increase in the α4 integrin expression may be a compensatory response. Interestingly, the levels of another fibronectin receptor, α5β1 integrin, did not change.

Here we found that the expression of intracellular single-chain antibody against αV integrin, concomitantly with decrease of αV integrin on cell surface, causes an increase in alkaline phosphatase expression and turns on the osteopontin gene expression. Thus our data indicates that this receptor is an important regulator of the phenotype of the osteosarcoma cells. Since alkaline phosphatase has been used as a marker for osteoblast differentiation and osteopontin is a gene expressed in bone, we suggest that as a result of the depletion of αV integrins the osteosarcoma cells are closer to osteoblasts than the original malignant sarcoma cells were. The direct role of αV integrins in the regulation of alkaline phosphatase was confirmed in an experiment in which cells were exposed to exogenous anti-αV antibodies (L230). L230, unlike anti-β1 integrin or control antibodies, could elevate alkaline phosphatase mRNA levels, but only in control cells not in cells in which αV integrin expression was depleted by intracellular antibody expression. In vivo αV integrins are not necessary for osteoblast differentiation (Bader et al., 1998). It is, however, possible that αV integrin is a negative regulator of the differentiation of osteosarcoma cells and even the

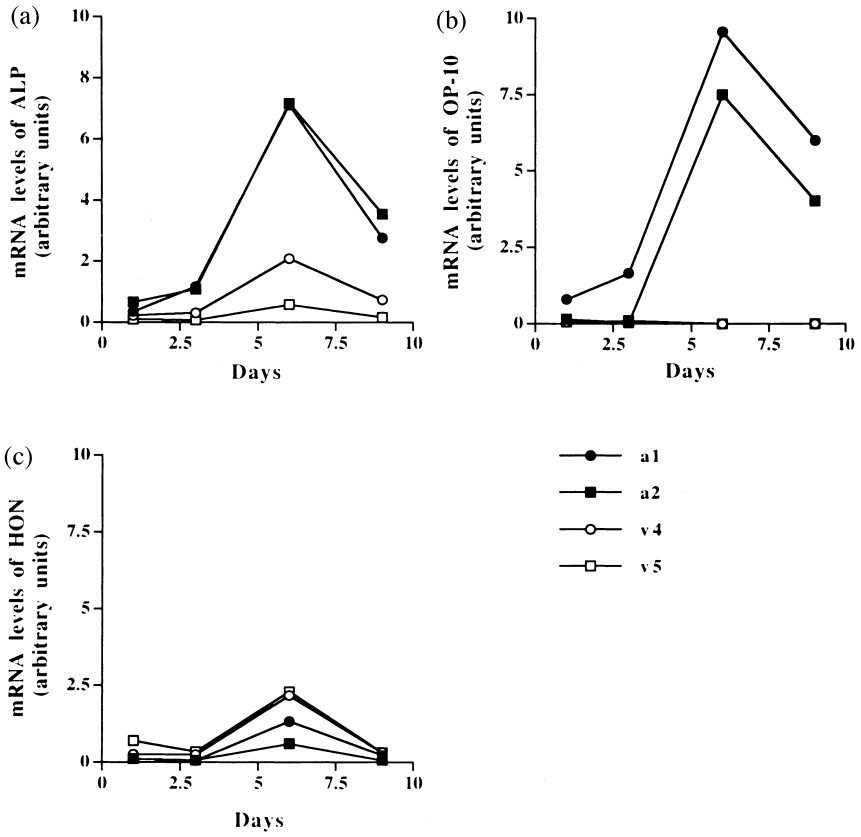

Fig. 7. The mRNA levels of osteogenic differention marker genes in Saos-2 cells expressing anti-αV integrin intracellular antibody (clones a1 and a2) and vector controls (clones v4 and v5). Quantitative analysis of mRNA levels of alkaline phosphatase (ALP) (A), osteopontin (OP-10) (B), and osteonectin (HON) (C) mRNA levels by correlation to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. Confluent cells were cultured in the presence of 10% FCS. Total cellular mRNA was isolated, separated on agarose gel and the Northern blots were hybridized with specific cDNA probes.

normal osteoblasts. Interaction of α5β1 integrin with fibronectin is essential for rat calvarial osteoblast differentiation (Moursi et al., 1996, 1997). Thus αV integrins, instead of directly affecting osteoblast gene expression, may compete with α5β1 integrin in ligand binding and indirectly suppress osteogenic differentiation.

Osteopontin expression was seen only after depletion of αV integrins. Interestingly, αVβ3 integrin is a cellular receptor for osteopontin. Many cell types, including osteoblasts and smooth muscle cells, use osteopontin in migration (Panda et al., 1997; Faccio et al., 1998). Furthermore, osteopontin may regulate integrin signaling and the differentiation of cells, including osteoblasts (Liu et al., 1997). Thus, in osteosarcoma cells the interplay between αVβ3 integrin and osteopontin may regulate features, such as phenotype, differentiation, and movement.

To test further the idea that the depletion of αV integrins in the osteosarcoma cells reduces some features typical for malignant cells we tested their MMP expression. Secretion of MMPs is essential for tumor cell invasion through basement membranes and connective tissues. Both cancer cells and activated connective tissue cells have been found to produce MMPs in different tumors (Birkedal-Hansen, 1993). Integrins have an important role in mediating signals that regulate the expression of MMPs. Our data demonstrate that the expression of MMP-2 is reduced in intracellular αV antibody expressing SAOS-2 cells. MMP-2 is able of degrading native and denatured interstitial collagens, as well as the basement membrane collagen (type IV). Previously MMP-2 was found to be upregulated by αVβ3 integrin in melanoma cells (Seftor et al., 1992). Likewise, a positive correlation between β3 integrin and MMP-9 expression in melanoma cells has been reported (Gouon et al., 1996).

In addition to αVβ3 many other integrins can also regulate MMP expression. The first reports indicated that in rabbit synoviocytes the interaction of α5β1 integrin with fibronectin is able to trigger signal transduction pathways activating MMP-1 (collagenase-1), MMP-3 (stromelysin-1), and MMP-9 (92 kDa gelatinase) expression (Werb et al., 1989; Huhtala et al., 1995), whereas ligation of fibronectin by α4β1 integrin inhibits the expression of the same enzymes

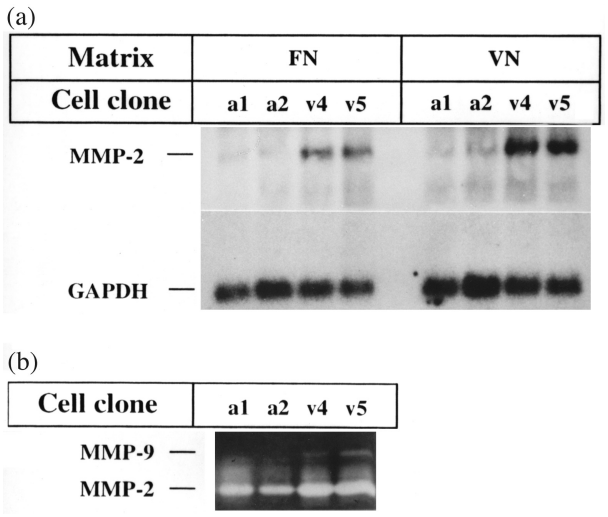

Fig. 8. MMP-2 expression in Saos-2 cells expressing anti-αV integrin intracellular antibody (clones a1 and a2) and vector controls (clones v4 and v5). Cells were cultured for 24 h on fibronectin (FN) or vitronectin (VN) in serum-free DMEM. Total cellular RNA was isolated. Panel A shows Northern blot analysis of MMP-2 mRNA levels. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Panel B shows semiquantitative gelatin zymography of medium proteins from cell clones cultured on VN. The position of MMP-2 and MMP-9 is indicated.

(Werb et al., 1989; Huhtala et al., 1995). The two fibronectin receptors recognize different fibronectin domains and cells binding to the intact fibronectin with both receptors show no changes in MMP expression (Werb et al., 1989; Huhtala et al., 1995). In addition we have shown that α2β1 integrin can upregulate MMP-1 and MMP-13 (collagenase-3) expression (Riikonen et al., 1995; Ravanti et al., 1999) and that α3β1 integrin related signals participate in upregulation of MMP-9 (Larjava et al., 1993).

The mechanism of integrin-dependent changes in metalloproteinase expression seems to be related to activation of specific signal transduction pathways after integrin binding to a ligand or to anti-integrin antibodies (Ravanti et al., 1999). Altered cell shape and the action of small GTPases, such as Rac-1, may also be an important regulation mechanism (Kheradmand et al., 1998). Here the exact mechanism of MMP-2 gene regulation was not studied. However, the direct participation of αV integrins in MMP regulation was suggested because cell adhesion to vitronectin was mediated entirely by them and the decrease in MMP synthesis was more extensive on vitronectin than on fibronectin. Furthermore, differences in MMP-2 expression were much smaller when cells were exposed to laminin. The clear difference between αV-depleted and control cells was also seen when MMP-2 accumulation was assayed by using gelatin zymography. The difference between the cell clones seemed to be, however, less dramatic than in mRNA levels. Gelatin zymography is only a semiquantitative method and we do not know whether there is an additional regulation mechanism between mRNA levels and protein secretion.

This report shows the effectiveness of the intracellular single chain antibody approach in the modulation of cellular integrin pattern. Here the approach was used to show the importance of αV integrins for the osteosarcoma cell phenotype.

4. Experimental procedures

4.1. Cell lines

Human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal calf serum (FCS, Gibco), 2 mM glutamine, 100 IU/ml penicillin-G and 100 μg/ml streptomycin.

4.2. Construction and transfection of cDNA coding for intracellular single-chain antibody

Anti-αV integrin intracellular single-chain antibody was constructed as described previously (Marasco et al., 1992, 1993; Richardson et al., 1995). Briefly, total RNA was isolated from 5×10^6 cells of hybridoma line L230 expressing anti-αV integrin monoclonal antibody (obtained from the ATCC) by using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc.). This RNA was used to prepare cDNA by using primers B (TGM GGA GAC GGT GAC CRW GGT CCC T) and D (ATT TGC GGC CGC TAC AGT TGG TGC AGC ACT). The primer sequences were from Richardson et al. (1995). Immunoglobulin heavy and light chain variable domains (VH and VL) were amplified from the cDNA by PCR using primers A (TTT AAG CTT ACC ATG GAA AGG CAC TGG ATC) and B or C (GAG CTC GTG CTC ACM CAR WCT CCA) and D. A DNA segment coding for the interchain linker (ICL) was amplified from an anti-tat 3 gene (a gift from Wayne A. Marasco, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA) by PCR using primers E (GGG ACC TGC GTC ACC GTC TCC TCA) and F (TGG AGA CTG GGT GAG CAC GAG CTC AGA TCC). The single-chain antibody gene was assembled from the VH, VL, and ICL fragments by overlap extension (Horton et al., 1989) followed by PCR amplification with primers A and I (TTT TCT AGA TTA TTA CAG CTC GTC CTT TTC GCT TAC AGT TGG TGC AGC ATC). The complete sequence of the assembled intracellular single-chain anti-αV anti-

body gene was determined by the dideoxy chain termination method (Sanger et al., 1977). The construct was digested with HindIII and XbaI and ligated into the vector pcDNA3 (Invitrogen), which carries the neomycin resistance gene.

Transfections were performed with calcium polypren/DNA method on confluent 60-mm dishes. Incubation with 5 μg of DNA and 5 μg of polypren in 1 ml of 10% FCS/DMEM per dish was carried out for 6 h agitating dishes every hour. DMSO (30% in FCS) shock was done for 3 min, cells were washed twice with PBS and culture medium was added. Neomycin analogue G418 (Life Technologies, Inc.) was added to the culture medium at a concentration of 400 μg/ml. G418-resistant cell clones were selected for 2–3 weeks, isolated and analyzed for their expression of αV integrin. Control cells were transfected with the pcDNA3 plasmid only. Transfected cells were cultured in 10% FCS/DMEM containing 2 mM glutamine, 100 IU/ml penicillin-G, 100 μg/ml of streptomycin and 200 μg/ml G418 (Life Technologies, Inc.).

The expression of the mRNA coded by the intracellular antibody construct in transfected cells was confirmed by RT-PCR of the total RNA isolated from both anti-αV plasmid and vector control cells. RT-PCR was performed by the Titan™ One Tube RT-PCR System kit (Boehringen Mannheim GmbH, Germany) using primers A and D. TM was 65°C.

4.3. Cell adhesion and spreading assays

Coating of 96-well immunoplates (Maxi Sorp, Nunc, Denmark) was done by exposure to 0.2 ml of phosphate-buffered saline (PBS, pH 7.4) containing different concentrations (0, 0.1, 0.5, 1.0, 5.0, 10.0 μg/ml) of fibronectin (Human plasma fibronectin, Chemicon International Inc., Temecula, CA, USA) or vitronectin (Purified human vitronectin, Chemicon) for 12 h at 37°C. Bovine serum albumin (BSA, 0.1%) was used to measure the non-specific binding or spreading. Residual protein absorption sites in all wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C. Confluent cell cultures were detached by using 0.01% trypsin and 0.02% EDTA and then rinsed in PBS containing 1 mg/ml of soybean trypsin inhibitor (Sigma); 15 000 cells/well were suspended in DMEM and transferred into each well and incubated for 80 min at 37°C in cell adhesion assay. In cell spreading assay 15 000 cells/well were suspended in DMEM and transferred into each well and incubated for 35 min at 37°C. In the adhesion inhibition assays functional monoclonal antibody against αV (L230) was incubated with cells in tubes for 15 min at RT before adding them to wells. Non-adherent cells were removed by rinsing the wells with medium and adherent cells were fixed with 2% paraformaldehyde, stained with 0.5% crystal violet in 20% ethanol and washed with distilled water. In cell adhesion assays the immunoplates were allowed to air-dry, crystal violet in cell layer was dissolved into 10% acetic acid and measured spectrophotometrically at 600 nm with Multiscan Plus (Lab-Systems). In cell spreading assays the wells were washed with PBS and fixed with 8% formaldehyde and 10% sucrose in PBS for 30 min. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus. The portion of spread cells was expressed as percentage of the number of adherent cells.

4.4. Northern blot hybridizations

Total cellular RNA was isolated by using the guanidium thiocyanate/CsCl method (Chirgwin et al., 1979) or Rneasy kit (Qiagen). RNAs were separated in formaldehyde-containing agarose gels, transferred to nylon membranes (Zeta-probe, Bio-Rad Laboratories, Richmond, CA, USA) and hybridized with ^{32}P-labeled (Amersham, UK) cDNA probes. The following cDNAs were used: human αV integrin (Suzuki et al., 1987), human matrix metalloproteinase-2 (Collier et al., 1988), human alkaline phosphatase (Weiss et al., 1986), human osteonectin (Young et al., 1990a), human osteopontin (Young et al., 1990b), human proα1(I) collagen (Vuorio et al., 1987; Mäkelä et al., 1988) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985). [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography and the mRNA levels were quantified by scanning densitometry of the X-ray films using MCID software (Imaging Research Inc., St. Catharines, Ontario, Canada). The levels of integrin mRNAs were corrected for the levels of GAPDH in the same RNA samples.

To analyze the expression levels of bone differentiation marker genes cells were plated on 60-mm dishes that reached the confluence already after cells had attached. They were cultured in DMEM supplemented with 10% FCS and harvested after incubation for 1, 3, 6, and 9 days. The total cellular RNA was isolated and mRNA levels of specific genes were measured by Northern blot hybridization as above. In some experiments confluent cultures were incubated for 3 days with 1.4 μg/ml anti-αV integrin antibody (L230), 1 μg/ml normal rat IgG (Sigma) or 1 μg/ml anti-β1 integrin antibody (mAb 13; Coulter) prior to RNA isolation.

4.5. Analysis of gelatinase expression

The coating of 60-mm dishes was done by exposure to 5.3 ml of PBS (pH 7.4) containing 1.5 μg/cm^2 fibronectin or vitronectin or 3.8 μg/cm^2 mouse

laminin-1 (Upstate Biotechnology, Lake Placid, NY, USA) for 12 h at 37°C. Residual protein absorption sites in all wells were blocked with 0.1% BSA in PBS for 1 h at 37°C. Confluent cell cultures were detached by using 0.01% trypsin and 0.02% EDTA. Trypsin was inhibited by DMEM supplemented with 10% FCS. Cells were washed with PBS, suspended in serum-free Optimem 1 medium (Life technologies) or DMEM and harvested after a 24-h culture. Medium collected from the cell cultures was subjected to SDS polyacrylamide gel electrophoresis using 10% gels containing 1 mg/ml MDPF (3-methoxy-2-4-diphenyl-3(2H)-furanone)-gelatin. The gels were washed in 100–200 ml of 50 mM Tris, 2.5% Triton X-100, 0.02% NaN_3 (pH 7.5), for 30 min at RT and then in 50 mM Tris, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.02% NaN_3 (pH 7.5). For detection of gelatinolytic enzyme activity the gels were incubated overnight at 37°C in 50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3 (pH 7.5), then fixed in 50% MeOH/7% HAc, and stained in 0.002% Coomassie blue G250 (Kodak). The gels were dried between Clear Cellophane Membranes (Bio-Rad) for 2 h at 58°C.

4.6. Cell proliferation assays

Confluent cell cultures were detached by using 0.01% trypsin and 0.02% EDTA; 5000 cells/well were suspended in 10% FCS/DMEM and transferred into wells of 96-well immunoplates (Maxi Sorp, Nunc, Denmark). The cell proliferation was analyzed after incubation for 4 h, 26 h, 48 h, 7 days and 10 days (only in Exp. 2) by using Cell Titer 96 (Promega) kit according to manufacturers instructions. Optical absorbance of the reaction was measured spectrophotometrically at 492 nm with Multiscan Plus reader (Lab-Systems).

4.7. Flow cytometry

Cells were grown to early confluence, detached with trypsin-EDTA, washed with PBS (pH 7.4) and then incubated with PBS containing 1% FCS for 30 min at 4°C. Cells were collected by centrifugation, exposed to saturating concentration of antibodies against α4 integrin (Pharmingen), α5 integrin (mAb 16, Akiyama et al., 1989), β1 integrin (R-322, rabbit polyclonal, Heino et al., 1989), αV integrin (24 B3-4, Weinacker et al., 1994), αVβ3 integrin (Pharmingen), αVβ5 integrin (PIF6, Weinacker et al., 1994) or β6 integrin (E7P6, Weinacker et al., 1994) in 1% FCS/PBS for 30 min at +4°C. For labeling, cells were incubated with rabbit anti-mouse (1:20 dilution), rabbit anti-rat (1:100 dilution) or swine anti-rabbit (1:20 dilution) IgG coupled to fluorescein (all from DAKO A/S, Glostrup, Denmark) for 30 min at 4°C, washed twice with PBS, and suspended in the same buffer. In order to measure the amount of integrins on cell surfaces, the fluorescent excitation spectra were analyzed by using a FACScan apparatus (Becton Dickinson). Control samples were prepared by treating cells without primary antibodies.

4.8. Immunoprecipitations

Cells were metabolically labeled with 100 μCi/ml of [^{35}S]methionine (Tran[^{35}S]-label, ICN Biomedicals Inc., Irvine, CA, USA) for 16 h in methionine-free minimum essential medium. Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM Tris–HCl (pH 7.4) and then detached by scraping. Cell pellets were obtained by centrifugation at $500 \times g$ for 5 min. Cells were solubilized in 200 μl of the same buffer containing 100 mM *n*-octyl-β-D-glucopyranoside (Sigma) on ice with occasional vortexing. Insoluble material was removed by centrifugation at $10\,000 \times g$ for 5 min at 4°C. Radioactivity in cell lysates was counted, and an equal amount of radioactivity was used in immunoprecipitation assays. Triton X-100 (0.5% v/v) and bovine serum albumin (0.5 mg/ml) were added to the supernatants, which were then precleaned by incubation with 50 μl of packed protein A-sepharose (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Supernatants were immunoprecipitated with anti-integrin antibody (L 230) for 12 h at 4°C. After incubation with secondary antibody (rabbit anti-mouse, DAKO) immune complexes were recovered by binding to protein A-sepharose and washing the beads four times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin and twice with 0.5 M NaCl and 25 mM Tris–HCl (pH 7.4). The immunoprecipitates were analyzed by electrophoresis on sodium dodecyl sulfate-containing 6% polyacrylamide gels under non-reducing conditions followed by fluorography.

In pulse chase assays cells were metabolically labeled for 1 h. Immunoprecipitation was performed with polyclonal anti-αV integrin antiserum (Bender MedSystems).

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