Investigation of the role of β1 integrins in cell-cell adhesion

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SUMMARY

Various β1 integrins (VLA-2, VLA-3, VLA-4) have been suggested to bind directly to themselves or to each other, thus mediating cell-cell adhesion. Here we expressed the human α2 and α3 subunits in three different cell lines (human erythroleukemia K562, human rhabdomyosarcoma RD and Chinese hamster ovary CHO cells). Although cell surface α2β1 and α3β1 in the transfectants mediated adhesion to matrix ligands (collagen or laminin 5, respectively), in no case did we observe enhanced cell-cell adhesion. In the presence of a range of different divalent cation concentrations, stimulatory anti-β1 antibodies or anti-α3 antibodies, VLA-2 and VLA-3 still did not appear to interact directly, through either heterophilic (i.e. VLA-3/VLA-2) or homophilic (i.e. VLA-3/VLA-3) mechanisms, to mediate cell-cell adhesion. Furthermore, in some but not all α3 transfectants we observed an unexpected decrease in cell-cell adhesion, suggesting a novel anti-adhesive function. This inhibitory effect was not observed for α2 transfection nor when the α3 cytoplasmic tail was exchanged with that of another integrin α subunit. Finally, no evidence for VLA-4/VLA-4 mediated cell-cell adhesion was observed using α4-transfected K562 and CHO cells. In conclusion, using many different combinations of cell lines, we found that cell-cell adhesion mediated by direct integrin/integrin interaction is not a widespread phenomenon, and is not observable in standard cell-cell adhesion assays. Furthermore, in some cell combinations, α3 expression may actually cause diminished cell-cell adhesion.

Key words: β1 integrin, cell-cell adhesion, integrin transfectant

INTRODUCTION

The integrin superfamily is a large group of heterodimeric receptors that play crucial roles in many cellular processes including immune defense, embryonic development, wound healing and metastasis (Hemler, 1990; Hynes, 1992; Springer, 1994). These phenomena require the careful regulation of multiple cell-cell and cell-matrix interactions. Integrins function in adhesion to extracellular matrix (ECM) proteins (e.g. laminin, fibronectin and collagen), as well as adhesion to cell-surface molecules, e.g. the immunoglobulin superfamily members ICAM-1 (intercellular cell adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) (Hemler, 1990; Hynes, 1992).

Among the β1 integrin dimers, some interact exclusively with one matrix protein (e.g. VLA-6 and VLA-5), some recognize more than one ECM ligand (e.g. VLA-1) and some interact with both ECM ligands and cell-surface counterreceptors (e.g. VLA-4). The β1 integrin VLA-2 (α2β1) can function as a receptor for laminin and/or collagen (Languino et al., 1989; Elices and Hemler, 1989). The binding specificity of VLA-2 depends on the cellular context (Chan and Hemler, 1993) and can be altered by manipulating extracellular cations (Santoro, 1986) or incubating with certain stimulating anti-β1 antibodies (Chan and Hemler, 1993). Defining the functional ligand binding specificity of VLA-3 (α3β1) has been controversial. Although early experiments implicated VLA-3 in adhesion to ECM proteins laminin, fibronectin and collagen (Carter et al., 1990b; Elices et al., 1991), it has been recently established that the predominant VLA-3 ligand is the laminin 5 variant (previously termed kalinin/epiligrin) expressed in the basement membrane (Carter et al., 1991; Wayner et al., 1993; Rousseau et al., 1991; Weitzman et al., 1993; Delwel et al., 1994). The β1 integrin VLA-4 (α4β1) has been well characterized as a receptor for fibronectin and the inducible cell-surface molecule VCAM-1 (Elices et al., 1990).

In addition to mediating cell-matrix adhesive interactions, it has been proposed that VLA-2 and VLA-3 may play roles in cell-cell adhesion (Kaufmann et al., 1989; Larjava et al., 1990; Carter et al., 1990b). Consistent with this, immunoelectron microscopy has localized VLA-2 and VLA-3 to points of cell-cell contact (Kaufmann et al., 1989) and anti-α3 monoclonal antibodies (mAb) have been shown to inhibit the Ca2+-induced aggregation of keratinocytes (Carter et al., 1990b). Furthermore, anti-α3 antibodies can induce homotypic aggregation of VLA-3 transfectant cells (Weitzman et al., 1993) and intercellular adhesion of keratinocytes (Symington et al., 1993) or fibroblast cells (Takeuchi et al., 1994). Recent work utilizing immobilized purified integrins, affinity chromatography, and blocking mAb, has suggested possible heterophilic (VLA-2/VLA-3) (Symington et al., 1993) and homophilic (VLA-3/VLA-3) (Sriramarao et al., 1993) interactions involving these
integrins. These studies implied that integrin/integrin interactions are easily observable and broadly generalizable phenomena. However, a potential problem with these studies is that they failed to demonstrate definitively the importance of integrin-integrin interactions in a strictly cellular context.

In addition, some studies have shown that VLA-4 plays a role in homotypic intercellular adhesion, perhaps suggesting additional VLA-4 counter-receptors (Campanero et al., 1992). Also VLA-4 expression appeared to suppress invasiveness of the metastatic B16 melanoma cell line, possibly by a mechanism involving direct VLA-4/VLA-4 adhesion (Qian et al., 1994).

Because integrin mediated cell-cell adhesion has been suggested to provide evidence for new VLA-4 ligands (Campanero et al., 1992), mediate homotypic cell-cell aggregation (Weitzman et al., 1993; Takeuchi et al., 1994) and other cell-cell contacts (Kaufmann et al., 1989), guide keratinocyte interactions (Symington et al., 1993) and localization (Larjava et al., 1990), guide general epidermal cell differentiation (Carter et al., 1990b), and regulate melanoma cell metastasis (Qian et al., 1994), it is essential to understand the precise mechanisms involved. Thus, in this study we have utilized α2-, α3- and α4-transfectants, as well as cells endogenously expressing the VLA-2, VLA-3 or VLA-4 integrins, to investigate directly a role for these receptors in cell-cell adhesion in a totally cellular context.

As the cellular environment can drastically alter ligand binding activity during cell-matrix adhesion (Weitzman et al., 1993; Chan and Hemler, 1993; Kassner and Hemler, 1993), we have used three different cell types (K562, RD and CHO) as recipients for transfection. Also, we manipulated the integrin activity by varying extracellular cation concentrations or by adding stimulatory antibodies. Altogether we analyzed potential VLA-2/VLA-3 interaction using 15 different cellular combinations, VLA-3/VLA-3 interaction in 8 different cell combinations and VLA-4/VLA-4 in 4 different combinations. In no case did we obtain evidence for direct homophilic or heterophilic integrin/integrin interactions mediating cell-cell adhesion.

MATERIALS AND METHODS

Antibodies, ECM proteins and cell lines

The monoclonal antibodies used throughout this study have been previously described: anti-α2 5E8 (Zylstra et al., 1986), anti-α3 P1B5 and A3-1VA5 (Wayner and Carter, 1987; Weitzman et al., 1993), anti-α4 BS10 (Hemler et al., 1987), anti-human-β1 A-1A5 (Hemler et al., 1983), and TS2/16 (Hemler et al., 1984), anti-hamster-β1 7E2 (Brown and Juliano, 1988), negative control antibodies P3 (Lemke et al., 1978) and J2A2 (Hemler and Strominger, 1982). Rat tail collagen type I was purchased from Collaborative Research Incorporation (Bedford, MA).

Tissue culture and cell transfection

The human cell lines RD (rhabdomyosarcoma), K562 (erythroleukemia), HL-60, A431 (epidermoid carcinoma) and HT1080 (fibrosarcoma) were obtained from ATCC. All human cells were grown in RPMI 1640 medium supplemented with 10% FCS, 1 mM Hepes buffer and antibiotics. Chinese hamster ovary (CHO) cells negative for the dicydrofolate reductase gene were grown in MEM α + with 10% FCS and then switched to MEM α - medium with 10% dialyzed FCS (JRH Biosciences, Lenexa, KS) after transfection.

Full-length cDNA for the human VLA subunits α2 (Takada and Hemler, 1989), α3 (Takada et al., 1991) and α4 (Takada et al., 1989) in the mammalian expression vector pFneo (Ohashi et al., 1985; Saito et al., 1987) were used for stable transfection of K562, RD and CHO cells as previously described (Weitzman et al., 1993; Chan and Hemler, 1993; Kassner and Hemler, 1993). Transfectants were selected in Geneticin (G-418 sulfate, Gibco Laboratories) and analyzed by flow cytometry with anti-VLA mAb to select those expressing high levels of transfected VLA on the cell surface. Subcloning of RD cells immediately after transfection allowed isolation of RD-A3#3 and RD-A3#10 as separate cell lines. If needed, transfected cells were enriched by selection with immunomagnetic beads (Dynal Co.) incubated with the anti-VLA mAb or by fluorescence activated cell sorting (Epic, Coulter Corp., Hialeah, FL).

A chimeric cDNA construct (X3C4) was prepared containing the extracellular domain of the α3 subunit fused to the cytoplasmic domain of the α4 subunit. This was achieved by creating a site for the restriction enzyme HindIII in the α3 cDNA sequence, N-terminal of the GFRRK sequence at the equivalent position to that used for cloning of α2 and α4 chimeric molecules (Chan et al., 1992b; Kassner and Hemler, 1993). This was achieved by PCR using an antisense oligonucleotide with three mismatched bases so that AAGTCG was converted to AAGCTT, a HindIII site. The PCR-derived α3 fragment was ligated to a HindIII/ EcoRV fragment from the X4C4 cDNA plasmid (Chan et al., 1992b) encoding the α4 cytoplasmic domain. The cloned regions were sequenced before transfer into the pFneo vector for subsequent transfection. The resulting X3C4 chimera had the carboxy sequence....III-LWKLGLF GKKROYKSILOENRDRDSWYINSKSNDD with the underlined portion derived from the α4 molecule. The sequence retains the conservative alanine to leucine substitution which was required to engineer the HindIII cloning site but has been previously shown to have no effect on VLA-4 function (Kassner and Hemler, 1993).

Flow cytometry

For flow cytometric analysis, cells were preincubated in PBS buffer containing 5% human serum (HS, Gibco Laboratories, Grand Island, NY) for 30 minutes on ice, then washed 3 times in the same solution. Then aliquots of cells (2×10⁶) were incubated individually with mAb (undiluted hybridoma supernatants or ascites 1:500) for 45 minutes on ice. Cells were washed 3 times with PBS containing 1% HS and treated with goat anti-mouse IgG coupled to fluorescein (Cappel Laboratories, Malvern, PA) for 45 minutes on ice. Cells were washed three times and resuspended in PBS, 1% BSA and analyzed using a FACScan® machine (Becton Dickinson, Oxnard, CA).

Cell adhesion assay

Cell attachment to matrix proteins was studied as previously described (Elices et al., 1990; Chan et al., 1992a). Briefly, cells were labelled with the fluorescent dye BCECF (Molecular Probes, OR), and then attachment to protein ligands, coated onto 96-well microtiter plates (Flow Labs), was detected using a CytoFluor™ 2300 Fluorescent Measurement System (Millipore). Results are presented as mean number of bound cells per mm² ± s.d. from triplicate determination. The number of cells bound to BSA-coated control wells was subtracted from each value. Crude kalinin-ECM deposited by A431 cells was prepared as previously described (Carter et al., 1990; Weitzman et al., 1993). Cell-cell adhesion assays were carried out as above except that cell monolayers grown in full media for 2-3 days were used instead of immobilized ECM ligands.

RESULTS

Expression of α2β1 and α3β1 integrin heterodimers

Prior studies of VLA-2 and VLA-3 adhesive function (Weitzman et al., 1993; Kawaguchi and Hemler, 1993; Chan et al., 1992a) encoding the α3 and β1 integrin heterodimers (Chan et al., 1992a) encoding the α3 and β1 integrin heterodimers.
and Hemler, 1993) have been facilitated by the transfection of cDNA for the human α2 or α3 subunits into two different human cell lines (erythroleukemia K562 and rhabdomyosarcoma RD), and one hamster cell line (Chinese hamster ovary CHO), which normally express low levels of VLA-2 and VLA-3 on their cell surfaces. Drug-resistant transfectants (here designated RD-A2, K562-A2, CHO-A2, RD-A3, K562-A3 and CHO-A3) expressed high levels of α2β1 and α3β1 dimers, respectively, on the cell surface as assessed by flow cytometry using anti-α2 or anti-α3 mAb (Table 1) and as previously shown by immunoprecipitation analysis (Weitzman et al., 1993; Kawaguchi and Hemler, 1993). The levels of cell-surface β1 subunit were also elevated in some transfected cells as a result of a subunit expression. The levels of other α subunits remained unchanged as previously indicated.

**Adhesion of VLA-2 and VLA-3 transfectants to ECM ligands**

To confirm that the α2β1 and α3β1 receptors expressed by the transfected cells were functionally active, we studied the adhesion of each transfected cell to the appropriate ECM ligands. As previously described (Chan and Hemler, 1993; Kawaguchi and Hemler, 1993), VLA-2 expression correlated with increased adhesion to matrix protein collagen (Fig. 1). Although the VLA-2 was less than fully active in the K562 cell line and could be stimulated by the anti-β1 antibody TS2/16 (Fig. 1A), the VLA-2 in CHO and RD cells was already highly active and thus was not further stimulated by a 20-fold (Fig. 1B) or 10-fold (Fig. 1C) increase in manganese levels. Also, it has been shown elsewhere that VLA-2-mediated adhesion in K562 cells can be stimulated by manganese (Kawaguchi and Hemler, 1993), and in RD cells can be stimulated by TS2/16 (Bergelson et al., 1993).

VLA-3 expression correlated with increased adhesion to a crude ECM preparation (containing kalinin/epiligrin) deposited by carcinoma cell lines as previously reported (Weitzman et al., 1993). In contrast to the VLA-2 transfec-
tants, VLA-3 was highly active on K562-A3 transfected cells and thus not further stimulated by TS2/16 mAb (Fig. 1D).

### Table 1. Expression of VLA-2 and VLA-3 on different cell lines

<table>
<thead>
<tr>
<th>Cell</th>
<th>Negative</th>
<th>β1</th>
<th>α2</th>
<th>α3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>5.43*</td>
<td>99.65</td>
<td>38.33</td>
<td>75.49</td>
</tr>
<tr>
<td>HT1080</td>
<td>4.35</td>
<td>376.24</td>
<td>204.96</td>
<td>308.29</td>
</tr>
<tr>
<td>K562-M</td>
<td>3.52</td>
<td>70.17</td>
<td>3.30</td>
<td>3.14</td>
</tr>
<tr>
<td>K562-A2</td>
<td>4.23</td>
<td>92.47</td>
<td>39.61</td>
<td>3.74</td>
</tr>
<tr>
<td>K562-A3</td>
<td>4.96</td>
<td>120.80</td>
<td>5.64</td>
<td>78.66</td>
</tr>
<tr>
<td>RD-M</td>
<td>2.54</td>
<td>90.98</td>
<td>3.19</td>
<td>9.77</td>
</tr>
<tr>
<td>RD-A2</td>
<td>3.41</td>
<td>83.95</td>
<td>22.37</td>
<td>4.91</td>
</tr>
<tr>
<td>RD-A3</td>
<td>3.66</td>
<td>129.89</td>
<td>7.00</td>
<td>75.29</td>
</tr>
<tr>
<td>CHO-M</td>
<td>2.70</td>
<td>60.20*</td>
<td>2.71</td>
<td>2.80</td>
</tr>
<tr>
<td>CHO-A2</td>
<td>2.67</td>
<td>121.94*</td>
<td>86.19</td>
<td>2.97</td>
</tr>
<tr>
<td>CHO-A3</td>
<td>3.85</td>
<td>294.20*</td>
<td>3.46</td>
<td>437.25</td>
</tr>
</tbody>
</table>

*Cells were stained with mAb to human β1 (A-1A5) or hamster β1 (7E2), human α2 (5E8) or human α3 subunit (A3-IVA5), or a negative control (mAb P3). The numbers in the table represent mean fluorescent intensity. Numbers underlined indicate cells expressing high levels of α2 or α3 subunit on their cell surface.

The RD and CHO cell lines a high background binding to the deposited ECM preparation was previously observed in untransfected cells (Weitzman et al., 1993). However, the VLA-3-dependent adhesion was highlighted when only low levels of divalent cation were present and thus non-specific background binding was largely eliminated (Fig. 1E and F). Elsewhere it was shown that VLA-3-mediated adhesion in K562 cells can be stimulated by manganese (Weitzman et al., 1993), and VLA-3 in RD cells can also be stimulated by TS2/16 (not shown). In all cases, integrin-mediated cell-matrix adhesion could be completely inhibited by anti-α2 and anti-α3 antibodies, respectively (not shown) (Weitzman et al., 1993; Kassner and Hemler, 1993). Also, transfectants and mock-transfected cells all adhered equally well to fibronectin independent of VLA-2 and VLA-3 expression (not shown).

Together these experiments confirmed that VLA-2 and VLA-3 transfectants exhibit integrin-mediated cell-matrix adhesion. Notably, although the degree of activation differed depending on the cellular environment, in all cell lines deficits in adhesive function could be readily overcome by optimizing the extracellular divalent cation levels or by adding stimulatory antibodies.

**CHO cells in homotypic and heterotypic cell-cell adhesion assays**

CHO cells transfected with the human α2 subunit have previously been reported to adhere to plastic-immobilized purified VLA-3 (Symington et al., 1993). Here we performed cell-cell adhesion assays to investigate whether CHO cells expressing...
VLA-2 would exhibit increased adhesion to VLA-3 on the surface of apposing CHO cells. The results (summarized in Table 2A) are expressed as a percentage of mock-transfectant adhesion. Adhesion ranged from 50 to 350 suspension cells bound per mm² of cell monolayer. Compared to mock-transfected cells (CHO-M), CHO cells expressing VLA-3 (CHO-A3) did not show a significant increase in adhesion to CHO-A2 or CHO-A3 cells, whether the CHO-A3 cells were used as an adherent monolayer or were added in suspension. Likewise, CHO-A2 cells also did not show significantly elevated cell-cell adhesion, either when presented as a monolayer or added in suspension.

In heterotypic cell–cell adhesion assays, the CHO-A2 and CHO-A3 cells did not differ from CHO-M cells in adhesion to monolayers of A431 cells. Adhesion of CHO-A2 and CHO-A3 transfectants is expressed as a percentage of mock-transfectant adhesion. Numbers represent average ± s.d. (n).

Table 2. CHO cells in homotypic and heterotypic cell-cell adhesion assays

<table>
<thead>
<tr>
<th>Monolayer Cell</th>
<th>mAb</th>
<th>Suspension CHO-M</th>
<th>Suspension CHO-A2</th>
<th>Suspension CHO-A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: CHO-M</td>
<td>None</td>
<td>100</td>
<td>98 (1)</td>
<td>102±30 (4)†</td>
</tr>
<tr>
<td>CHO-A2</td>
<td>None</td>
<td>111±14 (2)</td>
<td>100±27 (5)</td>
<td></td>
</tr>
<tr>
<td>CHO-A3</td>
<td>None</td>
<td>122±32 (2)</td>
<td>110±40 (5)</td>
<td></td>
</tr>
<tr>
<td>B: A431</td>
<td>None</td>
<td>103±44 (3)</td>
<td>93±18 (5)</td>
<td></td>
</tr>
<tr>
<td>A431†</td>
<td>Anti-α3</td>
<td>117±56 (4)</td>
<td>112±31 (5)</td>
<td></td>
</tr>
</tbody>
</table>

*CHO transfectants in suspension were tested for adhesion to monolayers of CHO cells or A431 cells. Adhesion of CHO-A2 and CHO-A3 transfectants is expressed as a percentage of mock-transfectant adhesion. Numbers represent average ± s.d. (n).

†In no case was adhesion of transfected cells statistically greater than mock-transfected cells (P>0.2; Student’s t-test).

Because the cellular environment in which integrins are expressed can have a dramatic effect on adhesive function (Chan and Hemler, 1993), we tested additional combinations of cell types expressing VLA-2 or VLA-3 integrins. Although VLA-2 and VLA-3 expressed in RD cells were functionally active with respect to ECM adhesion (Fig. 1), neither integrin caused increased RD cell adhesion to monolayers of RD itself in any combination tested (Table 3C). Also, RD transfectants did not show increased adhesion to A431 cells or HT1080 cells, both of which themselves express high levels of VLA-2 and VLA-3 (Table 3A and B). Furthermore, intercellular adhesion was unaltered in the presence of either the anti-β1 mAb TS2/16 or anti-α3 mAb (P1B5 or A3-IVA5) (Table 3).

In several experiments VLA-3 expression resulted in decreased intercellular adhesion. This was most notable in RD-A3 and K562-A3 experiments (Tables 4 and 5) but not in CHO-A3 experiments (Tables 2 and 4A). On various RD cell monolayers, K562-A3 cells bound 30-47% less than mock-transfected K562-M cells irrespective of whether the stimulatory anti-β1 mAb TS2/16 was present (Table 4D). In contrast, the adhesion of K562-A2 cells and mock-transfected cells were not significantly different (Table 4D). A similar decrease was observed when the RD-A3 cells were compared with RD-mock or RD-A2 monolayer cells in their ability to support adhesion by various K562 cell lines (Table 5A). The decrease correlating with VLA-3 expression was most notable in RD-A3 and K562-A3 experiments (Tables 4 and 5).
was reproducible and statistically significant. Also, it was observed with multiple α3-transfected RD and K562 clones. As indicated in a representative experiment (Fig. 3), two different K562-A3 clones showed less adhesion to RD cells and two different RD-A3 clones supported less K562 cell adhesion. Importantly, comparison of assays using K562-A3 cells on RD-A3 monolayers with K562/RD or K562/A2/RD-A3 combinations indicates an additive effect resulting in a 54-

Table 3. RD cells in heterotypic and homotypic cell-cell adhesion assays

<table>
<thead>
<tr>
<th>Monolayer Cell</th>
<th>mAb</th>
<th>Suspension Cell</th>
<th>Suspension Cell</th>
<th>Suspension Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: A431</td>
<td>None</td>
<td>100</td>
<td>92±45 (4)</td>
<td>108±42 (6)</td>
</tr>
<tr>
<td>A431</td>
<td>TS2/16</td>
<td>100</td>
<td>106±27 (3)</td>
<td>109±21 (4)</td>
</tr>
<tr>
<td>A431†</td>
<td>Anti-α3</td>
<td>100</td>
<td>106±67 (5)</td>
<td>110±27 (7)</td>
</tr>
<tr>
<td>B: HT1080</td>
<td>None</td>
<td>100</td>
<td>nd</td>
<td>87±21 (3)</td>
</tr>
<tr>
<td>HT1080</td>
<td>TS2/16</td>
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<td>nd</td>
<td>81±11 (3)</td>
</tr>
<tr>
<td>HT1080‡</td>
<td>Anti-α3</td>
<td>100</td>
<td>nd</td>
<td>85±9 (2)</td>
</tr>
<tr>
<td>C: RD-M</td>
<td>-‡</td>
<td>100</td>
<td>97</td>
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<tr>
<td>RD-A3</td>
<td>-‡</td>
<td>100</td>
<td>101</td>
<td>88±28 (9)</td>
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</table>

*RD cell transfectants in suspension were tested for adhesion to monolayers of A431 carcinoma cells (A), HT1080 fibrosarcoma cells (B), or RD cell transfectants (C). Adhesion of RD-A2 or RD-A3 transfectants is expressed as a percentage of mock-transfectant adhesion. Numbers represent means ± S.D. (n).

†In no case was adhesion of transfected cells statistically greater than the mock-transfected cell (P>0.09; Student’s t-test).
‡Anti-α3 subunit mAb P1B5 or A3-IVA5 were both used with similar results.
§nd, cell combination not tested.
‡§Since it had no effect, results ± TS2/16 have been pooled.

Table 4. K562 cells in heterotypic cell-cell adhesion assays

<table>
<thead>
<tr>
<th>Monolayer Cell</th>
<th>mAb</th>
<th>Suspension Cell</th>
<th>Suspension Cell</th>
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<tbody>
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<td>106±54 (6)</td>
<td>133±31 (4)</td>
</tr>
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<td>100</td>
<td>106±14 (2)</td>
<td>118±41 (3)</td>
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<tr>
<td>CHO-A3</td>
<td>None</td>
<td>100</td>
<td>105±52 (6)</td>
<td>132±22 (4)</td>
</tr>
<tr>
<td>B: A431</td>
<td>None</td>
<td>100</td>
<td>65±24 (10)</td>
<td>83±37 (11)</td>
</tr>
<tr>
<td>A431</td>
<td>S2/16</td>
<td>100</td>
<td>87±43 (12)</td>
<td>82±56 (13)</td>
</tr>
<tr>
<td>A431</td>
<td>mAb 13</td>
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<td>nd</td>
<td>79±34 (3)</td>
</tr>
<tr>
<td>C: HT1080</td>
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<td>100</td>
<td>89±12 (2)</td>
<td>76±45 (5)</td>
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<tr>
<td>HT1080</td>
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<td>129±31 (3)</td>
<td>57±45 (6)*</td>
</tr>
<tr>
<td>D: RD</td>
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<td>100</td>
<td>102±46 (7)</td>
<td>69±54 (15)*</td>
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<td>95±43 (9)</td>
<td>58±31 (15)**</td>
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<td>100</td>
<td>83±36 (4)</td>
<td>70±41 (7)</td>
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<tr>
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<td>TS2/16</td>
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<td>76±62 (6)</td>
<td>54±44 (7)*</td>
</tr>
<tr>
<td>RD-A3</td>
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<td>100</td>
<td>101±54 (15)</td>
<td>58±50 (20)**</td>
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<td>TS2/16</td>
<td>100</td>
<td>109±34 (11)</td>
<td>53±41 (18)**</td>
</tr>
</tbody>
</table>

†K562 cell transfectants, were tested for adhesion to monolayers of CHO transfectants (A), A431 carcinoma cells (B), HT1080 fibrosarcoma cells (C) or RD transfectants (D). In each case, adhesion of K562-A2 and K562-A3 transfectants is expressed as a percentage of mock-transfectant adhesion. Numbers represent mean ± S.D. (n).

§All results not marked with ‘*’ or ‘**’ are not significantly different from the corresponding K562-M results (P>0.07).

Role of VLA-4 in cell-cell adhesion assays

It has been proposed that VLA-4 may act as a counter-receptor for itself to promote homotypic adhesion of melanoma cells and thus may contribute to the metastatic potential of these

Table 5. VLA-3 expression leads to decreased K562/RD intercellular adhesion

<table>
<thead>
<tr>
<th>Suspension Cell</th>
<th>Monolayer RD-M</th>
<th>Monolayer RD-A2</th>
<th>Monolayer RD-A3</th>
<th>Monolayer RD-X3C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: K562</td>
<td>100</td>
<td>102±19 (11)</td>
<td>84±41 (31)*</td>
<td>105±41 (10)</td>
</tr>
<tr>
<td>K562-A2</td>
<td>100</td>
<td>86±49 (7)</td>
<td>72±45 (21)*</td>
<td>nd</td>
</tr>
<tr>
<td>K562-A3</td>
<td>100</td>
<td>96±44 (9)</td>
<td>53±36 (25)**</td>
<td>116±49 (9)</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>96±37 (27)</td>
<td>71±42 (77)**</td>
<td>108±43 (19)</td>
</tr>
<tr>
<td>B: None</td>
<td>100</td>
<td>109±28 (3)</td>
<td>46±40 (15)**</td>
<td></td>
</tr>
<tr>
<td>TS2/16</td>
<td>100</td>
<td>92±25 (1)</td>
<td>41±45 (15)**</td>
<td></td>
</tr>
</tbody>
</table>

In A, adhesion of K562 cells to RD-A2, RD-A3, or RD-X3C4 transfectants is expressed as percentage of RD-M adhesion, regardless of which K562 cell line is utilized. In B, adhesion by specific cell combinations (K562-A2 cells to RD-A2 monolayers, or K562-A3 cells to RD-A3 monolayers) is expressed as a percentage of K562-M adhesion to RD-M monolayers determined in the same experiment. Numbers represent mean ± S.D. (n).

*, **Adhesion of α3-transfected cells was statistically less than mock-transfected cells (P<0.005, **P<0.001; Student’s t-test). All results not marked with ‘*’ and ‘**’ are not significantly different from the corresponding RD-M results (P>0.3).
DISCUSSION

We have used cells transfected with $\alpha_2$, $\alpha_3$ or $\alpha_4$ subunits to investigate a role for $\beta_1$ integrins in cell-cell adhesion in a totally cellular context. Using many combinations of cells expressing different integrins we found no evidence that direct interactions between VLA-2, VLA-3 or VLA-4 integrins mediate cell-cell adhesion, and in fact $\alpha_3$ expression caused a reduction in cell-cell adhesion for some (but not all) cell combinations, suggesting a novel ‘anti-adhesive’ function.

Homotypic and heterotypic cell-cell adhesion assays

Human and hamster cell lines transfected with cDNA for $\alpha_2$, $\alpha_3$ or $\alpha_4$ integrin subunits were used to examine directly the contribution of $\beta_1$ integrins to cell-cell adhesion. Because integrin adhesive function varies greatly depending on the cellular environment (Chan and Hemler, 1993; Weitzman et al., 1993; Kassner and Hemler, 1995, and unpublished data), we used three different cell lines (RD, K562 and CHO) as recipients for transfection. The transfected integrin $\alpha$ subunits were functionally active at the cell surface, and mediated cell adhesion to appropriate matrix ligands (collagen, kalinin matrix and CS-1, respectively), that was entirely inhibited by the appropriate blocking anti-$\beta_1$ or anti-$\alpha$ mAb. In sharp contrast, transfected integrin $\alpha$ subunits made no contribution to cell-cell adhesion in any cell-cell combination tested. We analyzed potential heterotypic VLA-2/VLA-3 interactions using 15 different cellular combinations, and potential homotypic VLA-3/VLA-3 or VLA-4/VLA-4 interactions in 8 and 4 different cell combinations, respectively. Furthermore, no increases in cell-cell adhesion were observed when cells were incubated with different concentrations of Mn$^{2+}$, or activating anti-$\beta_1$ mAb. These agents are well known to stimulate cell-matrix adhesion dramatically, and can readily overcome any cell type-specific deficits in integrin adhesive function. Thus, it is not likely that we failed to observe enhanced $\alpha_2$-, $\alpha_3$-, or $\alpha_4$-dependent cell-cell adhesion simply because we used the wrong cell type expressing an inactive integrin. Also, it seems unlikely that integrins expressed on monolayer cells would be inaccessible. Previously it was shown that monolayers expressing $\alpha_2\beta_1$ were accessible for echovirus 1 binding (Bergelson et al., 1993), and both $\alpha_3\beta_1$ and $\alpha_4\beta_1$ were available for FcRII-dependent cell-cell adhesion (Weitzman and Hemler, 1995, and unpublished data).

Fig. 3. VLA-3 expression correlates with decreased cell-cell adhesion. K562 cells transfected with vector alone (-MOCK, □), $\alpha_2$ cDNA (-A2, □) or $\alpha_3$ cDNA (-A3), were studied for adhesion to RD monolayers expressing VLA-2 or VLA-3. The experiment was performed with two independent clones of K562-A3 (clone#3 (●) and clone#10, □) and two independent RD-A3 clones (clone#10 and clone#12). Results are expressed as cells bound per mm$^2$ and represent average ± s.d. of triplicate values.

Fig. 4. Surface expression of $\alpha_3$ on RD-A3 or RD-X3C4 transfectants. RD cells transfected with vector alone (RD-M), wild-type $\alpha_3$ cDNA (RD-A3) or a chimeric cDNA construct expressing the $\alpha_3$ extracellular domain fused to the $\alpha_4$ cytoplasmic domain (RD-X3C4), were stained with anti-$\beta_1$ mAb A-1A5 (small dotted line), anti-$\alpha_3$ mAb A3-IVA5 (solid line), or a negative control mAb P3 (dotted line) and then measured by flow cytometry.
Previous evidence for a role in cell-cell adhesion

A number of studies have implicated αβ1 and α3β1 integrins in cell-cell adhesion (Symington et al., 1993; Sriramarao et al., 1993; Kaufmann et al., 1989; Takeuchi et al., 1994; Larjava et al., 1990), implying that this may be a widespread and readily observable phenomenon. VLA-2 and VLA-3 expression has been noted at cell-cell contact sites (Larjava et al., 1990; Carter et al., 1990b) and Ca²⁺-induced keratinocyte aggregation could be partly inhibited by anti-α3 or anti-β1 mAb (Carter et al., 1990b). Also, studies using immobilized purified integrins, affinity chromatography, and blocking mAb, suggested possible heterophilic (VLA-2/VLA-3) (Symington et al., 1993) and homophilic (VLA-3/VLA-3) (Sriramarao et al., 1993) interactions. However, these studies did not demonstrate the importance of integrin/integrin interactions in a totally cellular context.

Here we failed to obtain any evidence that integrin-mediated cell-cell adhesion is a generally widespread event. Even though one previous study did show that CHO cells transfected with α2 displayed increased adhesion to immobilized, purified VLA-3 (Symington et al., 1993), our α2-transfected CHO cells did not show increased adhesion to α3-transfected CHO or any other α3-positive cell. Another study examining the effect of anti-integrin mAb on calcium-dependent keratinocyte adhesion also failed to find evidence for integrin involvement in cell-cell adhesion (Tenchini et al., 1993).

We propose that experiments using purified integrins and in vitro biochemical techniques may not reflect true interactions that occur when receptors are presented on the cell surface. For example, integrin/integrin interactions detected by biochemical means may reflect potential lateral interactions, of the sort that promote integrin clustering in the plane of the membrane on the same cell (Van Kooyk et al., 1994), rather than counter-receptor interactions on apposing cells. Also in this regard, integrin/ligand interactions observed on chromatography columns have not always reflected the adhesive properties of the receptor in a cellular environment (Elices et al., 1991).

A recent study has shown that VLA-4 on a B16 melanoma cell line might participate in direct VLA-4/VLA-4 mediated adhesion, not involving VCAM-1 (Qian et al., 1994). Also, transfection of VLA-4 was shown to increase homotypic adhesion which could be blocked by anti-α4 mAb (Qian et al., 1994). In spite of these results, we have found no evidence for VLA-4/VLA-4 interaction in our experiments using 3 different VLA-4-positive cell lines. Thus, the B16 melanoma model may be an unusual case.

Although our results do not favor an obvious role for VLA-2/VLA-3, VLA-3/VLA-3 or VLA-4/VLA-4 interactions during primary adhesive events, it is possible that they could play a secondary role. For example, they could stabilize cell-cell adhesive junctions, consistent with the observed localization of VLA-2 and VLA-3 at cell-cell borders (Kaufmann et al., 1989; Larjava et al., 1990).

### Antibody-induced cell-cell adhesion

Various anti-α3 mAb can induce homotypic aggregation of keratinocytes (Symington et al., 1993), fibroblasts (Takeuchi et al., 1994) and transfected K562-A3 cells (Weitzman et al., 1993; Takeuchi et al., 1994). However, since aggregation could be triggered by anti-α3 mAb regardless of their ability to block adhesion to ECM, and aggregation was not blocked by EDTA (Weitzman et al., 1993), VLA-3 itself may not actually mediate the cell-cell adhesion occurring during homotypic aggregation.

We also found that anti-α3 mAb induced heterotypic cell-cell adhesion even when VLA-3 and VLA-2 were not present on both cells (Weitzman and Hemler, 1995), arguing against mAb stabilization of VLA-2/VLA-3 interactions as was proposed in other studies (Symington et al., 1993). Further arguing against a specific VLA/cell-counter-receptor interaction, many mAb have been found to trigger homotypic aggregation of various leukocyte cell lines. These include mAb against other integrin subunits α2 (Campanero et al., 1992), α5 (Caixia et al., 1991), α6 (Wuthrich, 1992) and β1 (Caixia et al., 1991; Bednarzycz et al., 1993) and against CD2 (Elenstrom-

### Table 6. Contribution of VLA-4 to cell-cell adhesion*

<table>
<thead>
<tr>
<th>Cell</th>
<th>Antibody</th>
<th>Suspension K562-M</th>
<th>Suspension K562-A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M</td>
<td>None</td>
<td>100</td>
<td>149±26 (3)</td>
</tr>
<tr>
<td>CHO-M</td>
<td>TS2/16</td>
<td>100</td>
<td>115±17 (3)</td>
</tr>
<tr>
<td>CHO-A4</td>
<td>None</td>
<td>100</td>
<td>104±15 (3)</td>
</tr>
<tr>
<td>CHO-A4</td>
<td>S2/16</td>
<td>100</td>
<td>110±11 (3)</td>
</tr>
</tbody>
</table>

*In A, adhesion of K562-A4 cells is expressed as percentage of K562-M adhesion determined in the same experiment. In B, adhesion to CHO-A4 monolayers (by HL-60 cells) is expressed as a percentage of adhesion to CHO-M monolayers (by HL-60 cells). Numbers represent average ± s.d. (n). Results for the α4 transfectants are not significantly different from mock transfectants (P>0.07; Student’s t-test).
Magnarson et al., 1993), CD9 (Masellis-Smith et al., 1990), CD43 (Kuijpers et al., 1992; Cyster and Williams, 1992), CD44, CD45, CD46, CD55 (King et al., 1990), CD23 (Björck et al., 1993), CD19, CD20, CD21, CD39, CD40, MHC class I and class II (Bradbury et al., 1992, 1993; Kansas and Tedder, 1992; Wagner et al., 1993). Aggregation induced by these mAb includes both CD18-dependent and CD18-independent mechanisms.

Possibly, novel adhesion pathways could be responsible for Ab-induced cell-cell adhesion, but such assays should be interpreted with caution. For example, the apparent cell-cell adhesion could be a consequence of divalent Ab cross-bridging between cells, especially since the observation that purified Fab antibody fragments can self-associate on storage (Cyster and Williams, 1992) is often ignored. Also, the apparent Ab-induced cell-cell adhesion could be a consequence of molecular bridging mediated by Fc-receptors. We (unpublished data) and others (Horsewood et al., 1991) have found that Fc-mediated adhesion can sometimes be epitope-specific, Ab-specific and cell-specific, thus leading to quite elaborate, but misleading interpretations. Because mAb to so many different receptors induce aggregation, they may non-specifically trigger a general mechanism (see below), not involving interaction between the actual trigger molecule with a counter receptor. In conclusion, it is difficult to prove the existence of integrin/integrin homophilic interactions using Ab-induced cell-cell adhesion studies.

**VLA-3 may exert an ‘anti-adhesive’ effect on cell-cell adhesion**

In several experiments we observed a significant decrease in cell-cell adhesion as a result of α3 transfection, compared to mock-transfected or α2-transfected cells. The effect was observed using K562/RD-A3, K562-A3/RD, and K562-A3/HT1080 cell/cell combinations, but was not significant in any combination involving CHO or A431 cells. For the K562/RD combination, an ‘anti-adhesive’ effect was observed when the α3 subunit was expressed on either cell, and was additive if expressed on both. This may be the first report of such an ‘anti-adhesive’ role for an integrin receptor. A number of groups have reported anti-adhesive, or repulsive, functions for other cell-surface molecules and extracellular proteins (Calof and Lander, 1991; Chiquet-Ehrismann, 1991; Ardman et al., 1992; Rutishauser, 1992; Hilkens et al., 1992; Keynes and Cook, 1990; Sage and Bornstein, 1991) (also see below). For example, ECM proteins including the potential integrin ligands laminin, tenascin and thrombospondin have been shown to act as both pro-adhesive and anti-adhesive molecules (Sage and Bornstein, 1991; Calof and Lander, 1991; Chiquet-Ehrismann, 1991) by unknown mechanisms.

Loss of anti-adhesive function upon replacement of the α3 cytoplasmic domain with that of α4 clearly suggests a specialized function for the α3 tail. Aside from an involvement of the cytoplasmic domain, the mechanism of α3-mediated anti-adhesion is unclear. Possibly, α3 could differ from α2 or α4 in its influence on the distribution of known anti-adhesive surface molecules. Sialic acid-containing glycoproteins (sialomucins) including neural NCAM (Acheson et al., 1991; Rutishauser, 1992; Doherty et al., 1990), leukocyte antigens CD43 (leukosialin or sialophorin) and CD45 (Ardman et al., 1992; Manjunath et al., 1993; Wagner et al., 1993), and the endothelial molecules CD34 and epilasin (Hilkens et al., 1992; Delia et al., 1993) may play a regulatory role in cell-cell adhesion, due to their size and elongated shape and/or the negative charge of the sialic acid residues (Cyster et al., 1991; Hilkens et al., 1992). Hence, VLA-3 in some cells could conceivably alter the surface distribution of such sialoglycoproteins, causing an apparent reduction in adhesion. This redistribution may reflect a physical association, or altered surface distribution involving the integrin cytoplasmic domain.

Notably, α3-transfected RD cells showed markedly reduced tumorigenicity in nude mice (J. Weitzman et al., unpublished), possibly due to reduced cell-cell adhesion such as described here. The ‘anti-adhesive’ results observed here also could be related to recent findings that anti-α3 mAb enhance virus-induced cell fusion (Ohta et al., 1994; Ito et al., 1992).

In conclusion, we have used cells transfected with α2, α3 or α4 subunits to demonstrate: (i) that VLA-2, VLA-3 and VLA-4 do not play a generalized role in mediating cell-cell adhesion; and (ii) that in some cells VLA-3 may play an anti-adhesive role, specifically requiring the α3 cytoplasmic domain.

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