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Classification of 'activation' antibodies against integrin β1 chain

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Abstract We compared the effects of two anti-β1 integrin activating antibodies, TS2/16 and AG89, on K562 cell adhesion to fibronectin. Though both antibodies effectively induced cell adhesion, the EC_{50} for AG89 was more than 200-fold higher than that for TS2/16. Scatchard analysis of the data from [^{125}I]Fab fragment binding to the cells revealed that the TS2/16 epitope is exposed constitutively on all the β1 integrin molecules, while only 3% of the β1 integrins on resting K562 cells bear the AG89 epitope. Calculation of the actual number of each antibody bound to the cell during the cell adhesion assay revealed that induction of cell adhesion can be accomplished by binding much fewer AG89 molecules compared to TS2/16. Thus, AG89 and TS2/16 represent distinct classes of anti-integrin activating antibodies that show completely different binding characteristics as well as different activation effects on the integrin molecule upon binding.

Key words: Integrin; Cell adhesion; Stimulatory antibody; Fc receptor

1. Introduction

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits and mediate cell adhesion to extracellular matrix proteins as well as cell-cell interactions [1–3]. Integrin-mediated cell adhesion plays crucial roles in regulating the morphology, proliferation, migration and differentiation of cells. It is widely accepted that availability of integrins as adhesion receptors is dynamically regulated [4,5]. For example, integrins on floating cells such as leukocytes and platelets are usually inactive; they need to be 'activated' by various stimuli prior to displaying the binding to their extracellular ligands [6–8]. Such activating stimuli include activation of protein kinase C [9], cross-linking of several cell surface receptors [6,10], and binding of agonist to G protein-coupled receptors [8,11,12]. In addition, several 'activating antibodies' to integrin itself have been reported. These antibodies, most of them directed against β chains, are thought to cause conformational change in integrin molecules that results in conversion of resting integrins to active ones.

TS2/16 is a well-known anti-β1 activating monoclonal antibody (mAb) which recognizes an epitope in the putative ligand binding region [13]. This mAb can promote various β1 integrin-mediated cell adhesions at concentrations as low as 0.1 μg/ml. Recently, we have established an anti-β1 integrin mAb AG89 which also has adhesion-promoting activity [14]. It recognizes an epitope in the membrane proximal stalk-like region of β1 integrin, which is distant from the epitope for TS2/16. It was revealed that AG89 fell into a group of antibodies called anti-ligand induced binding site (or LIBS) antibody since binding of AG89 to cells is increased upon ligation of integrins. Most of the anti-LIBS mAbs against integrin β chains are reported to have adhesion-promoting activity, like AG89 [15–20]. In most cases, however, it has also been reported that high concentrations of these mAbs are required to achieve their activating effect. In order to get some insight into the difference between the activating effects of activating mAbs, we investigated the binding characteristics of TS2/16 and AG89 to K562 cells. By employing Scatchard analysis, it was revealed that only a limited number of β1 integrins on resting K562 cells bear the AG89 epitope while TS2/16 can bind to all the β1 integrins on the resting cells. Comparison of the binding data with the adhesion-promoting activity of these antibodies suggests that TS2/16 and AG89 can be distinguished as different classes of antibodies that activate β1 integrin.

2. Materials and methods

2.1. Materials

The preparation of activating antibody against human β1 integrin, AG89, has been described [14]. The hybridoma cell line secreting another anti-β1 activating antibody, TS2/16 [21], was obtained from the ATCC (#HB243). Both antibodies were obtained in ascites, and purified by ammonium sulfate precipitation and anion-exchange chromatography. Fab as well as F(ab')2 fragments of these antibodies were prepared using immobilized papain or pepsin (Pierce Chemical Co., Rockford, IL) followed by purification by a protein A-agarose. The absence of undigested IgG was confirmed by SDS-PAGE under non-reducing conditions. The function blocking antibody against FcγRII (IV.3) was purchased from Medarex Inc. (West Lebanon, NH). Bovine plasma fibronectin was purified as described previously [22].

2.2. Cell adhesion assay

The human erythroleukemic cell line K562 was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan) and cultured in RPMI 1640 medium containing 10% fetal calf serum and non-essential amino acids. The cell adhesion assay was carried out by a method essentially described previously [23]. In brief, wells of 96-well microtiter plates were coated with 3 μg/ml bovine plasma fibronectin in PBS and incubated at 4°C overnight and non-specific binding sites were blocked by incubation with 1% bovine serum albumin for 30 min at room temperature. K562 cells (10^5 cells/well) in 100 μl of serum-free RPMI 1640 were added to the wells and incubated for 1 h at 37°C in the presence or absence of varying concentrations of mAbs. After removing the unbound cells by rinsing the wells three times with PBS, the bound cells were quantified by incubation with 100 μl of the phosphatase assay buffer (50 mM sodium acetate, 1% Triton X-100, 6 mg/ml *p*-nitrophenylphosphate, pH 5.2) for 1 h followed by addition of 50 μl of 1 N NaOH and measurement of absorbance at 405 nm.

2.3. Fab binding assay

The Fab fragments were labeled with ^{125}I using Iodo-Beads (Pierce) according to the method described previously [24]. Iodinated proteins were separated from free ^{125}I by gel filtration on desalting column DG-10 (Bio-Rad) equilibrated with 5 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, pH 7.2 (HEPES-Tyrode's buffer; HT). The specific activity of the radiolabeled Fab

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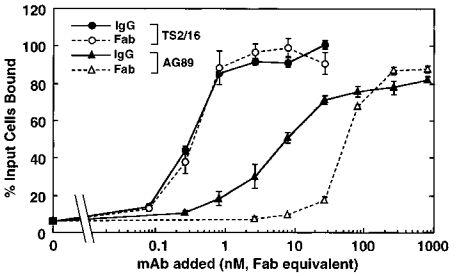

Fig. 1. Effect of activating mAbs on the K562 cell adhesion to fibronectin. K562 cells suspended in serum-free RPMI 1640 medium were treated with increasing concentrations of TS2/16 IgG (●), TS2/16 Fab (○), AG89 IgG (▲), or AG89 Fab (△) for 30 min and added to the wells that had been coated with 3 μg/ml bovine plasma fibronectin. After 1 h incubation at 37°C, unbound cells were removed by washing and bound cells were quantitated using the phosphatase assay. Data are from a representative experiment in which triplicate determinations were made and are expressed as mean ± S.E.M.

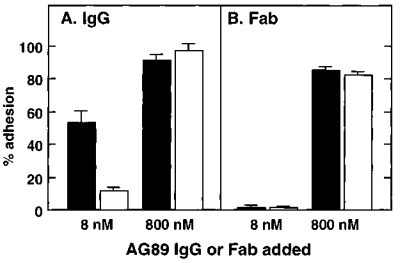

Fig. 2. Effect of anti-FcγRII antibody on the activating ability of AG89. K562 cells were preincubated in the absence (closed columns) or presence (open columns) of 2 μg/ml anti-FcγRII mAb IV.3 for 30 min and subjected to the cell adhesion assay in the presence of either AG89 IgG (A) or its Fab fragments (B) as in Fig. 1. Note that the increased activating effect of AG89 IgG compared to the Fab fragment was diminished upon treatment with anti-FcγRII function-neutralizing mAb IV.3.

fragments was in the range of 400–725 MBq/mg protein. Aliquots were stored at 4°C after the addition of BSA to a final concentration of 1%. K562 cells were washed once with HT and incubated with 5 mM EDTA at 37°C for 20 min in order to remove cell-bound divalent cations as well as fibronectin originating from the culture medium. After that, cells were washed three times, suspended in HT containing 1 mg/ml BSA, 0.42 mM $CaCl_2$, 0.2 mM $MgCl_2$ (the same divalent cation composition as that of RPMI 1640 medium) and mixed with the same buffer containing varying concentrations of [^{125}I]Fab fragments in a U-bottom microtiter well (Falcon #3910). The typical reaction mixture comprised 25 μl of cells (6×10^5 per tube), 25 μl of radiolabeled protein, and 10 μl of reagents (e.g. unlabeled Fab, divalent cations, EDTA, RGD peptide, etc.). The mixtures were incubated at 37°C with occasional agitation for 40 min, under which condition the binding of both Fab fragments reached plateau. After the incubation, 50-μl aliquots were layered onto 400 μl of 20% sucrose in the same buffer in microfuge tubes, and the cells were separated from unbound Fab by centrifugation for 4 min at 12000 rpm. The tips of the tubes were cut and the radioactivity associated with the cell pellet was counted in a well-type γ-counter. Non-specific binding was determined in the presence of a 100-fold excess of cold Fab fragment. The data were subjected to Scatchard analysis using RADLIG software version 4.0 (Biosoft, Cambridge, UK).

3. Results

The human erythroleukemic cell line K562 expresses only α5β1 as β1 class of integrin on the surface, and K562 cell adhesion to fibronectin is exclusively mediated by α5β1, which was confirmed by complete inhibition by function blocking anti-α5 mAb (data not shown). When coated at a relatively low concentration (3 μg/ml), fibronectin cannot promote the adhesion of K562 cells unless the cells are treated with some activating agent such as activating mAbs and Mn^{2+} ion [14]. As clearly shown in Fig. 1, TS2/16 and AG89 induced K562 cell adhesion to fibronectin in a dose-dependent manner. However, there are two clear differences between the activating effect of these antibodies. First, AG89 IgG showed increased adhesion-promoting activity compared with its Fab fragment at concentrations between 0.8 and 30 nM, while the dose-response curves of TS2/16 Fab and IgG are identical. Second, much higher concentrations were required for both the IgG and Fab portions of AG89 to promote adhesion than for TS2/16.

The first difference seems to be due to the involvement of the Fc portion of AG89 IgG in the activation step, since the dose-response curve for the F(ab')2 fragment of AG89 was almost identical to that of the Fab fragment (data not shown). As it is well known that K562 cells express FcγRII (CD32) on their surface, we looked into the effect of anti-FcγRII antibody on mAb-induced cell adhesion. As shown in Fig. 2A, prior treatment of the cells with an anti-FcγRII antibody (IV.3) abolished the activating effect of 8 nM AG89 IgG but had no effect on the maximum adhesion induced by 800 nM AG89. Moreover, neither an inhibiting nor an enhancing effect of IV.3 was observed with cell adhesion induced by AG89 Fab (Fig. 2B). It is concluded that the increased activating effect of IgG relative to the Fab fragment is attributable to the increased binding of AG89 via interaction of the Fc portion of AG89 and Fc receptors (FcγRII) on K562 cells.

As the EC_{50} of TS2/16 and AG89 Fab for promoting K562 adhesion estimated from multiple experiments shown in Fig. 1 are ~0.3 nM and ~60 nM, respectively (Table 1), affinities of these mAbs seem to differ by more than two orders of magnitude. We then analyzed binding of the ^{125}I-labeled Fab fragments of each mAb to K562 cells. As shown in Fig. 3, both mAbs showed specific and saturable binding to resting K562 cells. Scatchard analyses of the binding data suggest that each mAb has a single class of binding sites with an apparent dissociation constant (K_d) of 12.7 nM (for TS2/16) and 41.5 nM (for AG89), which differ by only 4-fold (Table 1). On the other hand, it was revealed that AG89 can

Table 1
Comparison of the binding parameters for TS2/16 and AG89

Activating mAb	K_d[a] (nM)	B_{max} (sites/cell)	EC_{50}[b] (nM)
TS2/16	12.7 ± 0.8	178000 ± 9000	0.32 ± 0.07
AG89	41.5 ± 6.2	5500 ± 600	60 ± 13

[a]Estimated from the Scatchard analysis ($n = 5$).
[b]mAb concentration required to obtain half-maximal extent of cell adhesion to fibronectin ($n = 4$).

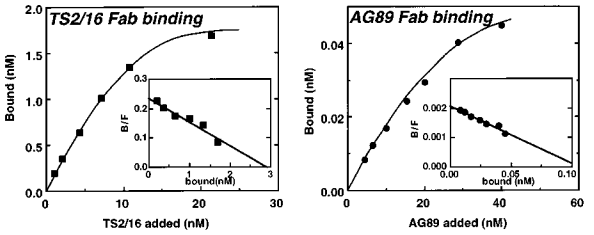

Fig. 3. Binding of ^{125}I-labeled Fab fragment of TS2/16 (left panel) or AG89 (right panel) to K562 cells. Increasing concentrations of ^{125}I-labeled Fab fragment of TS2/16 or AG89 were incubated with K562 cells in HEPES-Tyrode's buffer at 37°C for 40 min. Unbound Fab fragments were separated by centrifugation of the cell suspension through a 20% sucrose solution and the radioactivity associated with cells was counted. Amounts of bound Fab fragments were calculated by the specific activity. Data are from representative experiments in which duplicate determinations were made. Inset, Scatchard analyses of the data were performed using the LIGAND program [29].

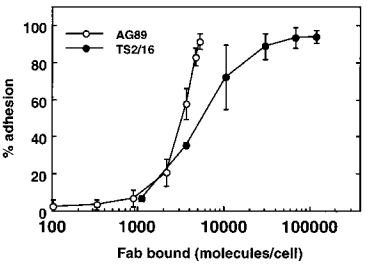

Fig. 4. Relationship between numbers of TS2/16 (closed circles) or AG89 (open circles) Fab bound to the cells and the extent of cell adhesion. Cell adhesion assays were performed as described in the legend to Fig. 1 and the numbers of Fab fragment bound were calculated by the equation described in the text. Data are mean ± S.E.M. from seven (AG89) or four (TS2/16) independent experiments in which duplicate determinations were made.

recognize only a limited population of β1 integrin on the resting cell surface; the maximum binding site numbers were 178 000 ± 9000 sites/cell for TS2/16 and 5500 ± 600 sites/cell for AG89. We can calculate the actual number of bound Fab molecules (N) using the following formula:

$$N = \frac{1}{1 + K_d/[\mathrm{Fab}]} B_{max}$$

where [Fab] is the concentration of Fab fragment added. According to this equation, the numbers of Fab fragments bound to the cells during the cell adhesion assay were estimated and data from multiple cell adhesion experiments were re-plotted with this parameter. As shown in Fig. 4, plotting the extent of cell adhesion induced by TS2/16 versus the number of TS2/16 molecules bound resulted in a typical sigmoidal curve consistent with the situation where the strength of the cell adhesion is dependent primarily on the density of activated β1 integrin on the cells. On the other hand, the situation was completely different in the case of AG89. Although the threshold numbers of bound Fab molecules required for induction of a low level of adhesion (< 20%) were almost the same for both TS2/16 and AG89, a very small number of AG89 Fab binding (~5000/cells) could induce more than 90% cell adhesion whereas binding of ~50 000 TS2/16 Fab molecules is required to induce the same degree of adhesion. This progressive increase in the adhesion-promoting ability of AG89 with a small number of bound Fab suggests a difference between TS2/16 and AG89 in the mechanism of activation of β1 integrin.

4. Discussion

We show here that anti-β1 integrin monoclonal antibody AG89, which has rather weak affinity (K_d=41 nM), binds and activates only limited numbers of β1 integrin on the surface of resting K562 cells in contrast to TS2/16. AG89 IgG could promote cell adhesion to fibronectin at a concentration much lower than that required for its Fab fragment (Fig. 1). This increased activating ability of IgG is attributable to the stabilizing effect of the Fc-FcγRII interactions on AG89-β1 integrin association, because pretreatment with a function blocking antibody to FcγRII abrogated the cell adhesion induced by a low concentration of AG89 IgG. As addition of control IgG at concentrations up to 1 mg/ml by itself did not induce K562 cell adhesion, it seems that the activation of the integrin by AG89 is exclusively mediated by the binding of the antibody to β1 integrin and the existence of the Fc portion merely increases the apparent affinity to the cell surface antigen.

In a previous paper [14], we reported that the ability of AG89 to induce FITC-fibronectin binding to cells in suspension is significantly lower than that of TS2/16. A similar difference was observed in the cell adhesion assay (Fig. 1). As the

Table 2
Activation antibodies against human integrin β1 chain

Activating mAb	Epitope location[a]	Conformation dependence[b]	Reference
TS2/16	207–218	no	[21]
8A2	207–218	no	[30]
A1A5	207–218	no	[13]
AG89	426–587	yes	[14]
15/7	355–425	yes	[20]
HUTS-21	355–425	yes	[31]
9EG7	495–602	yes	[19]
QE.2E5	426–587	n.d.	[32]
TASC(chickenβ1)	304–602	n.d.	[33]
JB1B	671–703	n.d.	[34]
B3B11	657–670	n.d.	[34]

[a]Expressed as residue number in the β1 chain primary structure.
[b]Antibodies that show increased binding upon activation and/or ligand binding to integrin are referred to as 'conformation dependent'. n.d., not described in the literature.

EC_{50} for AG89 to induce cell adhesion was 200-fold higher than that for TS2/16, it was anticipated that the affinity of AG89 to β1 integrin was 200-fold lower. Upon performing Scatchard analysis of the binding data, however, it was revealed that the affinity of the two antibodies differed by only 4-fold. On the other hand, the numbers of maximum binding sites were quite different, though both antibodies recognize the integrin β1 chain. The number of binding sites on K562 cells for TS2/16 (~180 000 molecules/cell) fits very well with the reported number of fibronectin receptors on K562 cells (199 000/cell) [25] indicating that this antibody can bind to almost all β1 integrin molecules expressed on the cells. The number of biding sites for AG89, on the other hand, was much lower (~6000/cell), accounting for only 3% of the total β1 integrin molecules. It is obvious that this limited number of binding sites on the resting cells is responsible for the relatively poor effectiveness of AG89 to induce cell adhesion. As the addition of the RGD ligand peptide together with divalent cations to K562 cells greatly increased the number of AG89 binding sites leaving the K_d value unchanged (unpublished data), it is concluded that β1 integrin on resting K562 cells exists as two distinct classes; only 3% of the β1 integrins bear the AG89 epitope constitutively and the rest of the β1 integrin pool expose the AG89 epitope only after binding of the ligand. In spite of the full expression of the AG89 epitope, integrins in this 3% pool seem to be in the low affinity state because they cannot mediate cell adhesion until bound by the activating mAb (Fig. 1).

A number of activating mAbs against integrin β1 chain have been reported so far. Table 2 summarizes the epitope localization of those stimulatory antibodies. It is obvious that most of them fall into either of two groups with respect to their epitope localization: one recognizes a very short stretch of residues (residues 207–218) located in the predicted ligand-binding domain, and the other has the epitope in or near the cysteine-rich repeats (residues 442–629) located in the membrane proximal stalk-like region. It is noteworthy that activating mAbs that recognize LIBS on β1 integrin (i.e. 15/7, 9EG7, and AG89) all belong to the latter group. Interestingly, epitopes for a series of anti-β3 LIBS mAbs are also mapped to a similar region in the β3 chain [18,26] and most of these mAbs have a stimulatory effect when added at a high concentration. It is likely that these 'conformation-dependent' antibodies exert their activating effect by binding to the epitope that is exposed on certain populations of integrin receptors under physiological conditions. Although the extents of cell adhesions induced by both TS2/16 and AG89 increase as a function of the number of each mAb bound on the cells, the effect of AG89 was more pronounced than that of TS2/16 (Fig. 4). In other words, the increase in cell adhesion induced by AG89 was much more sensitive to the small increase in the number of bound mAbs than in the case of TS2/16-induced adhesion. There are several possible explanations for this phenomenon. (1) The β1 integrins that constitutively express the AG89 epitope are qualitatively different from the others and a very small number of integrins in this population can effectively mediate cell adhesion upon activation. (2) Two antibodies activate β1 integrin in different ways: the ligand-binding affinity of the β1 integrin bound by AG89 is higher than that bound by TS2/16. (3) Binding of AG89 but not TS2/16 can transduce certain signals into the cells resulting in 'inside-out'-type up-regulation of cell adhesion. It is also possible that the actual number of AG89 molecules bound to the cells during the cell adhesion assay is larger than the estimate shown in Fig. 4, because of the progressive expression of AG89 epitopes on integrin transiently interacting with immobilized fibronectin. Direct measurement of ligand-binding affinity of individual integrin molecules activated by each mAb will be necessary to clarify these points.

Integrin activation is accomplished by various mechanisms, but their mode of action is variable. For example, up-regulation of integrin-mediated cell adhesion by phorbol esters is exerted by increasing the mobility of the integrin on the cell membrane but not by increasing the affinity for ligands [27]. In contrast, the adhesion-promoting effect of Mn ion is ascribed to the direct binding of Mn ion to the integrin extracellular domain that results in increased ligand-binding affinity of individual integrin molecules [28]. Activation antibodies are also thought to facilitate cell adhesion by converting low affinity integrin molecules to a high affinity state through direct binding and induction of conformational changes. The present study suggests that activation antibodies can be subdivided into distinct classes with regard to their binding characteristics and activation mechanisms. Thus TS2/16 and AG89 represent two distinct prototypes of activating antibodies: one that binds and activates all the β1 integrin molecules and one that binds to a small fraction of integrin on the resting cells. Molecular determinants that define the differential expression of the AG89 epitope on the β1 integrin molecule, as well as the physiological importance of the existence of two different classes of receptors on resting cells remain to be elucidated.

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