Adhesive Multiplicity in the Interaction of Embryonic Fibroblasts and Myoblasts with Extracellular Matrices

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ABSTRACT Neff et al. (1982, J. Cell Biol., 95:654–666) have described a monoclonal antibody, CSAT, directed against a cell surface antigen that participates in the adhesion of skeletal muscle to extracellular matrices. We used the same antibody to compare and parse the determinants of adhesion and morphology on myogenic and fibrogenic cells. We report here that the antigen is present on skeletal and cardiac muscle and on tendon, skeletal, dermal, and cardiac fibroblasts; however, its contribution to their morphology and adhesion is different. The antibody produces large alterations in the morphology and adhesion of skeletal myoblasts and tendon fibroblasts; in contrast, its effects on the cardiac fibroblasts are not readily detected. The effects of CSAT on the other cell types, i.e., dermal and skeletal fibroblasts, cardiac muscle, 5-bromodeoxyuridine-treated skeletal muscle, lie between these extremes. The effects of CSAT on the skeletal myoblasts depends on the calcium concentration in the growth medium and on the culture age. We interpret these differential responses to CSAT as revealing differences in the adhesion of the various cells to extracellular matrices. This interpretation is supported by parallel studies using quantitative assays of cell-matrix adhesion. The likely origin of these adhesive differences is the progressive display of different kinds of adhesion-related molecules and their organizational complexes on increasingly adhesive cells. The antigen to which CSAT is directed is present on all of the above cells and thus appears to be a lowest common denominator of their adhesion to extracellular matrices.

Adhesive differences among embryonic cells are thought to play a prominent role in directing morphogenesis (2, 32). Numerous examples of such differential adhesions have been described and evidence has been presented that relates them to morphogenic phenomena. The histotypic sorting out of embryonic cells is rationalized as reflecting the graded, differential adhesive interactions of the constituent cells (24, 26, 30, 31). In the nervous system, the high specificity of retinotectal interactions appears to reflect the adhesive gradients generated by the retinal and tectal cells (12, 24). The migration of neuronal processes to their peripheral targets is also highly specific and is hypothesized to reflect the differential affinities of the processes for extracellular substrates (7, 23).

Recently membrane proteins have been identified and isolated using adhesion-perturbing monoclonal antibodies whose antigens localize in the region of cell-substratum adhesions (13, 27, 28). One of these antibodies, called CSAT, is directed against an antigen that participates in the adhesion of embryonic skeletal muscle to extracellular matrices (17, 18, 27). The active determinant appears to be part of an integral membrane protein complex located in the vicinity of transmembrane assemblies involved in adhesion. The identification of such adhesion-related molecules allows an investigation of their role in regulating the adhesion and morphology of different cell types. Here we describe the effects of CSAT on the morphology and matrix adhesion of a group of avian embryonic cells. We find that the antigen is present on skeletal, cardiac, and 5-bromodeoxyuridine (BrdU)-treated skeletal myoblasts and on skeletal, tendon, dermal, and cardiac fibroblasts. The effect of the antibody on their adhesion, however, is different and characteristic. The tendon fibroblasts

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and skeletal myoblasts lie near one extreme while the cardiac fibroblasts lie near the other. The morphology and adhesion of the former are perturbed markedly by CSAT; the latter in contrast are not altered detectably. These observations are interpreted as revealing a hierarchy of morphologic and adhensive determinants (10, 16). We hypothesize that this hierarchy arises from a progressive display of additional adhesion-related molecules and their organization complexes on increasingly adhesive cells.

MATERIALS AND METHODS

Avian Cell Cultures: Skeletal muscle cultures were explanted, dissociated, and cultured as described elsewhere, except that chicken egg white Conalbumin (Sigma Type II, iron complex) replaced the chick embryo extract (27). The dissociated cells were plated onto Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) coated with 0.1% gelatin (BBL Microbiology Systems, Cockeysville, MD) at a density of 3.5 x 10^5 cells per 35-mm dish. 0.2-0.3 mM EGTA was added to calcium-free medium to prevent fusion of myoblast cultures; otherwise the cells were grown in normal (1.8 mM) calcium concentrations. BrdU-treated myoblasts were grown similarly except that the cells were grown in medium containing BrdU at a concentration of 1.6 x 10^{-6} M and was readied either every day or every other day to cultures growing in normal calcium concentrations.

Cardiac myoblasts were prepared as described by DeHaan (11) from 7- to 8-day embryonic heart ventricles. The cells were plated at a density of 5 x 10^5/ml on 0.1% gelatin coated Falcon tissue culture dishes and cultured in Dulbecco’s modified Eagle's medium (DME) containing 10% horse serum, 1% albumin.

Skeletal fibroblasts were prepared from preplates of myogenic cultures (28). The cultures were grown in DME containing 5-10% fetal calf serum on uncoated tissue culture dishes and used between the third and seventh passages. Tendon fibroblasts were obtained from 15-18-day-old embryos. After decapitation, the lower leg was removed at the knee and placed into calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). The toes were partially digested with 0.05 M diethylamine at pH 11.6.5 The samples were neutralized to pH 7.5 with 1 M HCl, dialyzed overnight at 4°C against TNC, concentrated using polyethylene glycol (PEG 8000, J. T. Baker Chemical Co., Phillipsburg, NJ) and Spectrapor tubing (3,500 molecular weight cut-off; Arthur H. Thomas Co., Philadelphia, PA), and diluted 1:1 into 0.05 M Tris-HCl, 2% SDS, 10% glycerol, and 5% mercaptoethanol, pH 6.8, heated at 95°C for 3 min, run on 7% polyacrylamide gels (22), and visualized by fluorography using ENHANCE (New England Nuclear).

Immunoprecipitations were performed on the Nonidet P-40 cell extract using Immunobeads (rabbit anti-mouse immunoglobulin; Bio-Rad Laboratories, Richmond, CA). The Immunobeads were first washed three times in wash buffer (PBS at pH 7.6 containing 0.1% bovine serum albumin, 0.02% NaN3, and 0.5% Nonidet P-40) and resuspended to a stock solution of 10 mg/ml. A 0.2-mg aliquot of beads was then blocked by incubating with 0.2-0.4 mg unlabelled lysate for 1 h on ice followed by two washes with wash buffer. The labelled lysate was preclutered by resuspending 2.0 mg of Immunobeads into 0.2-0.4 ml of the Nonidet P-40 cellular lysate and incubating for 1 h on ice. The beads were pelleted, and the supernatant saved. 0.05-0.2 ml preclutered lysate (0.75-8 x 10^6 cpm) was then incubated with the CSAT antibody (3 μg) for 3 h on ice. It was then added to 2 mg of the blocked beads and allowed to incubate for 1 h on ice. The Immunobeads were then sedimented in the Eppendorf centrifuge for 2 min and washed three times with wash buffer. The pellet was resuspended and transferred to a new centrifuge tube, and the beads washed one final time. The supernatant was aspirated, and the pellet resuspended into 50 μl of 0.07 M Tris-HCl (pH 8.6), 10% glycerol, 5% mercaptoethanol, and 3% SDS. The suspension was incubated for 30 min at room temperature, the beads sedimented, and the supernatant heated for 3 min at 95°C and run on 7% SDS polyacrylamide gels (22) and visualized by fluorography using ENHANCE (New England Nuclear).

RESULTS

A Comparison of the Effects of CSAT on the Morphology of Different Cell Types

We have reported previously (27) that the addition of CSAT to 2-d-old cultures derived from 10-11-d-old chick pectoral muscle explants induces a rapid rounding of the myogenic cells; nearly all of the cells are round and either weakly adherent or detached from the substratum (see also Fig. 2). The kinetics of this response is markedly dose dependent. At an antibody concentration of 20 μg/ml, morphologic changes are seen within 20-30 min. The effect of CSAT on the morphology of fibroblasts differs from that described for the myoblasts (Fig. 1). The general effect is to induce a retraction of the edges to produce a slightly less well spread morphology. The cardiac fibroblasts lie at one extreme; changes in their morphology, if they exist, are not detected by our methods. In skeletal fibroblasts, the effect is subtle, and while not readily apparent using phase

2 Abbreviations used in this paper: BrdU, 5-bromodeoxyuridine; CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; CMF-HH, calcium- and magnesium-free HEPES-Hank’s; DME, Dulbecco’s modified Eagle’s medium; TNC, 0.01 M Tris, 0.5% Nonidet P-40; 0.5 mM CaCl2, pH 8.0.
FIGURE 1 Effect of CSAT on fibroblasts. Cultures were seeded at a density of 2–8 × 10^4 cells/dish and grown for 16–96 h on uncoated 35 mm tissue culture dishes. CSAT was added at a concentration of 10–50 μg/ml, and the cells were observed under modulation contrast optics 4–28 h later. Bar, 20 μm × 125. Tendon: 24-h tendon fibroblasts (third passage) in DME containing 2% fetal calf serum. Control is the same as +CSAT except that 20 μg/ml CSAT was added to the latter after 20 h in culture. Dermal: 30-h dermal fibroblasts (primary cultures) were grown in DME containing 10% fetal calf serum. Control is the same as +CSAT except that 50 μg/ml CSAT was added to the latter after 24 h in culture. Skeletal: Skeletal fibroblasts (pass 6) were grown in DME containing 5% fetal calf serum for 16 h. Control is the same as +CSAT except for the presence of 20 μg/ml CSAT added to the latter at the time of plating. Cardiac: 96-h cardiac fibroblasts (pass 3) were grown in DMEM containing 5% fetal calf serum. Control is the same as +CSAT except that 20 μg/ml CSAT was added to the latter after 72 h in culture.

contrast, it can be seen clearly using modulation contrast optics (3, 15). Both confluent and nonconfluent cultures are affected. In the former, the cell outlines are particularly prominent, and, in the latter, the cells show a clear retraction of edges. This is most easily visualized in cells growing in low serum concentrations since they tend to be initially flatter on the dish. Few cells appear rounded and only a small fraction remain flat. Plating the cells in the presence of antibody produces the same morphology. Fivefold higher antibody concentrations alter the kinetics but not the magnitude of these effects. The final morphology is apparent only after 2–6 h in antibody.

The effects of CSAT on the morphology of tendon and dermal fibroblasts, cardiac myocytes, and BrdU-treated skeletal myoblasts, like those effects observed with the skeletal fibroblasts, lie between the two extremes just described for the skeletal myoblasts and cardiac fibroblasts (Figs. 1 and 2). When incubated with CSAT, they all appear less well spread. In contrast to the skeletal fibroblasts, the edges of the majority of tendon fibroblasts are more fully retracted, and a larger fraction of the cells are affected. An additional 20–40% appear round, but they still remain weakly adherent to the substratum. Dermal fibroblasts respond analogously to tendon fibroblasts except that their response decreases with each subsequent passage. Tendon fibroblasts tend to reattach after extended exposure to CSAT. There is some variability among different cell preparations in their response. The effect of CSAT also depends on serum concentration: cells growing in lower concentrations show larger effects.

The response of cardiac myocytes is particularly striking (Fig. 2). They stand out as less well spread islands of synchronously contracting cells surrounded by the flatter, well-spread fibroblasts. Like the skeletal fibroblasts, the morphological changes in the cardiac cells require at least 2–6-h incubation. The altered morphology and synchronous contractions are apparent in cultures plated in the presence of antibody, and they persist for at least 9 d in the presence of antibody. The original morphology returns to that of the control upon removal of the antibody. When plated in the presence of antibody, a large population of cells do not plate as rapidly as do the others. If these suspended cells are harvested and replated, they form cultures highly enriched in synchronously
contracting cells. The cells remaining on the original dish are enriched in fibroblast-like cells.

**Effect of Calcium and Time in Culture on CSAT Action**

Some effects of CSAT on morphology depend on culture age and calcium concentration. We previously reported that skeletal myoblasts, when grown in a low calcium medium (0.4 mM) through at least 5 d in culture, are rapidly rounded by CSAT (27). In contrast, cultures grown in normal calcium concentrations (2.0 mM) display age dependent changes in their response to CSAT. Young, 24--48 hour cultures are readily rounded, whereas older cultures require higher concentration of antibody and longer incubation times.

BrdU-treated skeletal myoblasts, replicating putative myogenic precursors, respond to CSAT analogously. When grown in a low calcium medium, for 24--96 h, the BrdU-treated myoblasts are rounded by CSAT. In contrast, older (72--96 h) cultures grown in normal calcium levels are not rounded but display the less well spread morphology. Fewer than 10% of the cells display a round morphology. When normal calcium levels are restored to 72-h cultures simultaneously with antibody addition, the cells are no longer rounded by the antibody.

**Adhesive Alterations Parallel the Morphologic Changes**

The different morphologic effects of CSAT just described imply differences in the adhesion of these cell types. This can be demonstrated, for weakly adherent cells, by gently washing the cell cultures and counting the fraction of cells detached. Although the results depend on the number of pipettings and their force, once a procedure is established the determinations are reproducible. Using this assay we find that 48-h skeletal myoblast cultures adhere more weakly and slowly than do the skeletal fibroblasts (not shown); this difference has been used previously to fractionate myogenic from fibrogenic cells. Among BrdU-treated skeletal myoblasts grown for varying lengths of time in culture, we find that the ratios of the fraction of cells removed in CSAT-treated cultures grown for 48, 72, and 96 h is 13:5:1. The assay also reveals that six times as many 48-h skeletal myoblasts and four times as many BrdU-treated skeletal myoblasts are removed after CSAT treatment (20 μg/ml for 3-4 h) as are skeletal fibroblasts. These differences are pairwise statistically significant (P < 0.025) and parallel the relative degree of morphologic change described above.

We developed a more quantitative assay of cell substratum adhesion that measures the initial binding events. This assay, based on that described by McClay et al. (25) for cell-cell adhesion, allows estimates of adhesive strength to different substrata. Cells from subconfluent cultures are removed with EDTA and added to tissue culture microwells, gently centrifuged to bring the cells into contact with the substratum, incubated for 20--45 min, inverted, the weakly adherent cells sedimented off the substratum, and the remaining cells counted. We used this assay to explore the adhesion of tendon skeletal and cardiac fibroblasts, cell types that show different responses to CSAT. These cells adhere best when incubated...
with a conditioned medium from the cells or to substrates containing an adhesion-promoting matrix molecule like fibronectin. The fraction of cells adhering to the substratum reaches a plateau after \(\sim 30-45\) min under the conditions described here.

Fig. 3 shows the adhesion of tendon, skeletal, and cardiac fibroblasts in the presence of conditioned medium and increasing concentrations of CSAT antibody. The adhesion of the tendon fibroblasts is inhibited \(\sim 90\%\) by \(10 \, \mu\text{g/ml}\) antibody. Half maximal inhibition occurs at an antibody concentration of \(\sim 1-2 \, \mu\text{g/ml}\). The adhesion of skeletal myoblasts shows a superimposable inhibition (data not shown). In contrast, the skeletal fibroblasts and cardiac fibroblasts are not inhibited half maximally even at 50–100-fold higher antibody concentrations. The modest inhibitions that do appear to result from a susceptible subpopulation of cells in the culture. These patterns of adhesive inhibition parallel the morphologic description of CSAT action: tendon fibroblast cultures are rounded by the antibody, and the cells remain only weakly adherent to the substratum; skeletal and cardiac fibroblasts show only modest morphologic effects of the antibody and remain adherent.

Since the conditioned medium may contain many different adhesion-promoting constituents, the adhesion to fibronectin, a defined adhesion promoting substratum, was studied (19, 29, 33-35). Both tendon and cardiac fibroblasts adhere to fibronectin-coated substrates. The adhesion of tendon fibroblasts to fibronectin was inhibited \(\sim 70\%\) when CSAT (20 \(\mu\text{g/ml}\)) was included in the assay: i.e., \(51 \pm 13\%\) vs. \(16 \pm 9\%\) of the cells initially added to the wells adhered in the absence and presence of antibody, respectively. In contrast, cardiac fibroblast adhesion was only slightly inhibited \((\sim 10\%): 35 \pm 14\%\) vs. \(32 \pm 10\%\) adhesion. Similar results were seen after a 5-h pretreatment with \(25 \, \mu\text{g/ml}\) cyclohexamide: \(48 \pm 9\%\) vs. \(17 \pm 3\%\) for the tendon fibroblasts and \(25 \pm 9\%\) vs. \(20 \pm 3\%\) for the cardiac fibroblasts.

These observations show that the differential responses of tendon and cardiac fibroblast cultures to CSAT are also seen in assays of the initial binding to fibronectin. This suggests that the differential responses to CSAT reside, at least in part, in the cell surfaces of the cell types themselves rather than in differences in the substrata to which they may be adhering.

### Antigen Comparison

Neff et al. (27) reported previously that the antigen purified by CSAT affinity chromatography from skeletal muscle ran as a broad band in the molecular weight range of 120,000–160,000 on SDS polyacrylamide gels. Polyclonal antibodies raised against this material detach myoblasts at high dilutions demonstrating the presence of the CSAT antigen in this molecular weight region (17).

The affinity-purified antigen isolated from tendon and cardiac fibroblasts, two cell types that respond differentially to CSAT, show indistinguishable patterns on SDS polyacrylamide gels (Fig. 4). These patterns are also indistinguishable from
that reported previously for the skeletal fibroblasts and from skeletal myoblasts (not shown). We also isolated the antigen by immunoprecipitation using the CSAT monoclonal antibody. This procedure omits the precipitation steps used in the affinity purification and may reveal co-purification of other proteins that might be associated with the CSAT antigen (Fig. 4). The antigen isolated from both cell types show similar electrophoretic patterns. In some experiments an additional band was observed in the 170,000-mol-wt range. The molecular weight of this band was consistently lower in the cardiac fibroblasts. The significance of this auxiliary band, if any, remains to be determined.

DISCUSSION

The availability of monoclonal antibodies directed against adhesion-related membrane proteins allows investigations into their general presence and role in the adhesion of embryonic and adult cell types. The experiments reported here are the initial observations from such a study using CSAT, a monoclonal antibody that was first identified by its inhibition of the adhesion of embryonic skeletal muscle to extracellular matrices. The effects of CSAT on the cell types investigated here demonstrate a somewhat general role for the CSAT antigen in cell-matrix adhesion. The antigen participates in the matrix adhesion of different types of muscle and fibroblasts; however, its contribution to their adhesion is different. Whereas the antibody dramatically perturbs the adhesion and morphology of skeletal myoblasts and tendon fibroblasts, its effect on cardiac fibroblast adhesion and morphology is substantially less. The other cell types appear to lie between these two extremes. We interpret these observations as revealing a hierarchy of adhesive and morphologic complexity.

The molecular origin of this hierarchy is of particular interest. The nearly identical binding affinities of CSAT to skeletal fibroblasts and myoblasts (27), along with the similar SDS PAGE profiles of antigen purified from tendon and cardiac fibroblasts, suggests that CSAT is interacting with similar if not identical antigens on these different cells. Binding studies on myoblasts, myotubes, and skeletal fibroblasts, along with the similar intensity of fluorescent staining on the other cells studied here, suggests that they all possess a similar antigen density (27). However, 50–100-fold increases in antibody concentration neither round nor inhibit substantially the adhesion of cardiac and skeletal fibroblasts. Thus adhesive differences arising solely as the result of different amounts of CSAT antigen on these different cell types is unlikely. Finally, our observations that the adhesive differences are seen with tendon and cardiac fibroblasts plated onto a common substrate, i.e., fibronectin, further suggest that the adhesive hierarchy also does not arise solely from differences in extracellular materials.

The presence of additional, different kinds of adhesion-related molecules provides a more likely explanation for the differential responses to CSAT. In this interpretation, there exists a repertoire of molecules that participate in cell-matrix adhesion. The adhesion of any one cell type is then determined by the members of the repertoire that are present on it. The skeletal myoblast would represent one extreme. It uses only a small fraction of the repertoire, and hence it is a weakly adherent cell and highly responsive to CSAT. The cardiac fibroblast, in contrast, represents the other extreme. It uses a relatively large number of different adhesive molecules. Therefore, it is more strongly adherent and is not responsive to the presence of CSAT.

Further evidence for a multiplicity of adhesion molecules is also found in the work of others. Harper and Juliano (14) have described variants of Chinese hamster ovary cells that no longer adhere to fibronectin but do adhere to a more complex substratum. Interference reflection and immunoelectron microscopy reveal several morphologically distinct kinds of putative cell-matrix adhesion sites (6, 20). One of these, the focal contact, is the locus of shortest distance between the cell and substratum and is the site of stress fiber termination; when present, it corresponds to the strongest adhesive structure (1, 6, 20). Recently, Oech and Birchmeier (28) have described an adhesion-perturbing monoclonal antibody that localizes in the region of the focal contact. This localization and the molecular weight of the antigen distinguish it from the CSAT antigen.

Thus a picture of cell-substrate adhesion emerges in which more than one kind of adhesive molecule or complex of adhesive molecules mediates the adhesion of cells to extracellular matrices. In weakly adherent cells, for example, one anticipates that one or a very small number of different kinds of adhesion-related molecules would dominate with other members of the repertoire either absent or present in low concentrations. Strongly adherent cells, in contrast, would use additional members of the repertoire. The presence of the CSAT antigen on all of the cells studied thus far suggests that it is a lowest common denominator of matrix adhesion and that the hierarchy is not fully combinatoric. In this way, the adhesive differences of different cell types reflects a molecular hierarchy on these cells; that is the progressive display of additional kinds of adhesion-related molecules on increasingly more adhesive cells forms a nested set of molecular determinants.

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4 The cell surface organization of adhesive molecules will also contribute to adhesive stability. High local concentrations or clusters of adhesive molecules will provide a more stable and stronger adhesion than will a more uniform, diffuse, distribution (4, 5). The geometric details of this organization can further contribute to adhesive strength and stability. This organization could arise from interactions among the different adhesion molecules or from interactions with hypothetical organizing molecules, all of which would be members of the adhesive repertoire. As we reported previously (27), the fibroblast has highly organized sites of adhesion that are not evident in the myoblast.

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