Intercellular adhesion is fundamental to many diverse biological processes and therefore elucidating its molecular basis is of great interest. One approach to studying the mechanism of cell-cell adhesion in several systems (1-4) has recently focused on the observation that two systems appear to contribute to intercellular adhesion, one calcium-dependent and the other calcium-independent. The calcium-dependent system is well defined and in some cases, specific molecules have been implicated, whereas the calcium-independent system remains poorly defined.

A different approach to studying the mechanism of cell-cell adhesion has focused on the role of carbohydrate-binding proteins (CBPs, lectins; 5, 6). We have previously identified a CBP on the surface of teratocarcinoma stem cells (Nulli cell line). One component is divalent cation-dependent (Ca ++ or Mg ++) and the other involves a cell surface fucan/mannan-specific lectin, previously identified on stem cells by an erythrocyte rosetting assay. The existence of these two systems is inferred from the observation that reaggregation of stem cells was partially inhibited by the removal of divalent cations or by the presence of lectin inhibitors such as fucoidan, but reaggregation was completely blocked when the two conditions were combined. Our results are related to recent work describing a calcium-dependent system of intercellular adhesion in teratocarcinoma stem cells.

Materials:

Arbacia fucan was a generous gift of Dr. W. J. Lennarz (Johns Hopkins University School of Medicine, Baltimore, MD). Fucoidan was obtained from ICN K&K Laboratories, Inc. (Plainview, NY). Heparin (H3125), chondroitin sulfate (C2524), dextran sulfate (D7515), dermatan sulfate (C4259), hyaluronic acid (H1751), fetuin (F2379), ovalbumin (A2512), glycogen (G9251), bovine serum albumin (A4378) (BSA), DNase (D0876), trypsin (T8253), and egg white trypsin inhibitor (T2011) were obtained from Sigma Chemical Co. (St. Louis, MO). Fucoidan was desulfated and hydrolyzed as previously described (8).

Cell Harvesting and Reaggregation: The Nulli SCC-1 line of mouse teratocarcinoma stem cells was cultured as described previously (13). Stem cells were harvested as a single-cell suspension following 10-min incubation at 37°C in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). Viability as evaluated by trypan-blue exclusion was >90%. Cells were washed in CMF-PBS and allowed to reaggregate in rotary culture for 30 min at 37°C as previously described (7). Briefly, cells were added to 40 mM Tricine-buffered Eagle’s minimum essential medium (MEM) (with or without CaCl2 and MgSO4) that contained 1 mg/ml BSA and 20 μg/ml DNase. EDTA, EGTA, and the various glycoconjugates were added at concentrations stated in the text. 0.5 ml of these cell suspensions (1-2 × 10^6 cells/ml) was then dispensed into individual Linbro wells (Linbro Chemical Co., Hamden, CT) in duplicate, reaggregated by rotary gyration, and counted by Coulter counter (Coulter Electronics, Inc., Hialeah, FL), as previously described (7). Percent aggregation was calculated as the percent reduction in total particle number from time zero. All data expressed in terms of inhibition of aggregation are calculated on the basis of control...
aggregation assays performed in the presence of divalent cations.

Trypsin/Ca^{++}-harvested cells were obtained by incubating stem cells in 0.01% trypsin with 1 mM Ca^{++} in CMF-PBS for 20 min at 37°C, as described by Takeichi et al. (4). The cells were washed once in 1 ml/ml trypsin inhibitor in CMF-PBS and then twice in CMF-PBS containing 1 mM Ca^{++}. Reaggregation assays were performed as described above.

RESULTS

Divalent Cation Requirement for Reaggregation

Cells of the teratocarcinoma stem cell line Nulli SCC-I were harvested in CMF-PBS as a single-cell suspension and allowed to reaggregate in rotary culture for 30 min at 37°C. Aggregation was quantified by measuring the percent reduction in total particle number as described in Materials and Methods. The 30-min period was sufficient for near maximal aggregation (50-60%) under control conditions in Tricine-buffered MEM containing physiological levels of Ca^{++} and Mg^{++} (1.8 and 0.8 mM, respectively). When CMF-MEM was used to ensure that divalent cations would be effectively absent from the assay mixture, aggregation was reduced to ~40%, indicating a 32% inhibition of reaggregation (Fig. 1). Addition of EDTA to the CMF-medium did not further inhibit aggregation (data not shown). That there was as much as 40% reaggregation in the absence of divalent cations indicated the existence of a divalent cation-independent system of intercellular adhesion. The increase over this baseline aggregation in the presence of divalent cations implied the existence of a divalent cation-dependent component. To further demonstrate this, we restored either Ca^{++} or Mg^{++} to the CMF-MEM assay mixture. Fig. 1 shows that in the presence of either of these divalent cations, reaggregation approached control levels (0% inhibition). Mg^{++} was more effective than calcium at promoting reaggregation.

Relationship between Lectin-dependent and Divalent Cation-independent Adhesion Systems

As noted in the Introduction, we have previously identified a lectin on the surface of teratocarcinoma stem cells that recognizes fucans and mannan. Evidence that this lectin plays a role in the intercellular adhesion of teratocarcinoma stem cells came from our study showing that mannan can interfere with the reassociation of stem cells in rotary cultures. Our goal here was to determine whether this lectin plays a role in the divalent cation-independent system or the divalent cation-dependent system. If the former were the case, one would expect that the effect of removing divalent cations and adding a lectin inhibitor would be additive, and that reaggregation could be completely inhibited. If, however, the lectin were involved in the divalent cation-dependent system of aggregation, then adding a lectin inhibitor to an assay mixture lacking Ca^{++} and Mg^{++} should not increase the extent of inhibition.

We first investigated the effect on the reaggregation of stem cells of several lectin inhibitors including fucoidan (a sulfated fucan of ~100,000 daltons [14] containing minor amounts [6%] of galactose, xylose, and uronic acid) in divalent cation-containing medium. Fig. 2a shows that fucoidan at concentrations of 50 μg/ml inhibited the reaggregation of stem cells to a maximum of 20%. We also found that mannan inhibited reaggregation up to 20% but at a 100-fold higher concentration than fucoidan (data not shown). Thus, fucoidan is a more potent inhibitor of reaggregation than mannan, as was the case for the rosetting and hemagglutination assays of lectin activity (8).

The Arbacia fucan, which contains only fucose and sulfate (15), also inhibited aggregation, producing 20% inhibition at a concentration of 250 μg/ml. The effectiveness of the Arbacia
Desulfated fucoidan (95% sulfate removal, as determined by titration on a mass basis as the intact molecule (data not shown). Sulfitated fucoidan (95% sulfate removal, as determined by rhodizonate assay) retained its ability to block stem cell reaggregation, although 50–100-fold higher concentrations were required (Fig. 2b). Sulfation, therefore, appears to contribute to the activity of fucoidan. However, sulfation alone was not responsible for fucoidan’s inhibitory effects, since several sulfated polysaccharides (chondroitin sulfate, dextran sulfate, and dermatan sulfate) were without inhibitory activity in the reaggregation assay as well as in the rosette and hemaggulutination assays. Perhaps because of similar charge distribution and/or structural homology to fucans, xylan and heparin caused a 10–15% inhibition of reaggregation in divalent cation-containing medium (Fig. 3a). This observation was consistent with the fact that both of these glycoconjugates are effective rosette inhibitors, although only xylan is also a hemaggulutination inhibitor (8). With all inhibitors tested, inhibition in the presence of divalent cations was partial, never exceeding 20–40%. Another series of glycoconjugates lacking fucose and activity in the hemaggulutination and rosette assays (hyaluronic acid, fetuin, ovalbumin, and glycogen) did not inhibit aggregation at 1 mg/ml.

We next investigated the effect of lectin inhibitors upon stem cells reaggregated in the absence of divalent cations. These conditions were met by adding 2 mM EDTA or 10 mM EGTA to “control” aggregation medium that contained divalent cations or by using Ca++ and Mg++-free aggregation medium (Fig. 4). Consistent with the observations described above, all three of these conditions inhibited stem cell reaggregation (Fig. 4). When divalent cations were absent from the aggregation mixture, adding from 50 to 500 µg/ml fucoidan progressively inhibited stem cell reaggregation until it was almost completely blocked (Figs. 2a and 4). When aggregation was assayed in the presence of fucoidan (100 µg/ml) and physiological concentrations of either Ca++ or Mg++, the extent of inhibition was similar to that in medium containing both divalent cations (Table I). Thus, adding either Ca++ or Mg++ markedly decreased the extent of inhibition by fucoidan.

The morphology of the aggregates formed under the various conditions is shown in Fig. 5. In the presence of divalent cations, large, tight aggregates were formed with indistinct cell-cell boundaries (Fig. 5a). In the presence of 500 µg/ml fucoidan the aggregates formed were generally smaller, but they retained their tight morphology (Fig. 5b). With divalent cations removed (2 mM EDTA) the aggregates appeared smaller and much looser, with individual cell boundaries now clearly discernible (Fig. 5c). When fucoidan (500 µg/ml) was added in the presence of EDTA, aggregation was almost totally blocked, leaving mostly single cells and small (2–3 cell) aggregates with individual cells distinguishable.

To determine whether the inhibition produced by fucoidan remained specific in the absence of divalent cations, we restested a series of polysaccharides under the same assay conditions. The inhibitory activity of desulfated fucoidan was promoted by EDTA, although once again, higher concentrations were required than for fucoidan (Fig. 2b). Although inhibition mediated by xylan was not significantly enhanced by the presence of EDTA, inhibition mediated by heparin was enhanced to the same extent as was inhibition mediated by fucoidan (Fig. 3a). The control substances, chondroitin sulfate, dextran sulfate, dermatan sulfate, hyaluronic acid, fetuin, oval-

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**TABLE I**

| Medium        | Inhibition in the presence of 100 µg fucoidan/ml % |
|---------------|---------------------------------------------------|
| CMF-MEM       | 34 (±10.8)                                         |
| +Ca++         | 18 (±2.6)                                          |
| +Mg++         | 9.3 (±0.9)                                         |
| +Ca++ and Mg++| 0                                                 |

Cells were reaggregated in CMF-MEM with and without 1.8 mM Ca++ and/or 0.8 mM Mg++. Percent inhibition was determined relative to cells reaggregated in control medium containing Ca++ and Mg++. The means ± SEM were computed from three independent determinations.
bumin, and glycogen at 1 mg/ml, however, still showed no inhibitory effect in the absence of divalent cations. Therefore, only those glycoconjugates that interacted with the lectin as defined by the rosette and hemagglutination assays could inhibit the reaggregation of stem cells. Charge alone did not appear to be responsible for inhibition since the charged polysaccharides chondroitin sulfate, dextran sulfate, and hyaluronic acid were not inhibitory.

To demonstrate that fucoidan has no toxic effect upon the reaggregating stem cells, we determined that the incorporation
of [³H]leucine into trichloroacetic acid-precipitable material was not significantly decreased by adding fucoidan in either the absence or presence of EDTA (data not shown).

Reaggregation of Trypsin/Ca ++ -harvested Cells

Takeichi et al. (4) have shown that teratocarcinoma stem cell lines (F9 and AT805), harvested with trypsin plus Ca ++, require the presence of Ca ++ but not Mg ++ for reaggregation. Nulli cells, when harvested by the trypsin/Ca ++ procedure (4) also exhibit calcium-dependent adhesion. As shown in Table II there was a low baseline level of aggregation that was enhanced to a much greater extent by Ca ++ than by Mg ++. This is in contrast with the results obtained for CMF-PBS-harvested cells, in which there was 30–40% aggregation in the absence of divalent cations, and either Mg ++ or Ca ++ enhanced this aggregation (see above). The aggregation of trypsin/Ca ++-harvested cells in the presence of calcium was not inhibited by fucoidan (Table II). Thus, neither the Ca ++/Mg ++-dependent system of CMF-PBS-harvested cells nor the Ca ++-dependent cell adhesion of trypsin/Ca ++ cells was sensitive to fucoidan.

DISCUSSION

We report here evidence for the existence of two separable systems of intercellular adhesion in teratocarcinoma stem cells. One component requires divalent cations—either Ca ++ or Mg ++. The other component is divalent cation-independent and appears to involve a fucan/mannan-specific cell surface lectin. This conclusion is based on the observation that either the presence of lectin inhibitors or the absence of divalent cations caused a partial reduction of reaggregation, whereas a combination of these two conditions caused complete inhibition of reaggregation.

Several laboratories have described dual calcium-dependent and calcium-independent components of intercellular adhesion for various cell types including fibroblasts (1), embryonic neural retinas cells (2, 3), embryonic liver (16), and teratocarcinoma stem cells (4). Takeichi et al. (4) have investigated the calcium-dependent system of teratocarcinoma stem cells in detail. A 145,000-dalton cell surface protein (17) and a possibly related 84,000-dalton cell surface protein (18) have been implicated in this system. It has also been demonstrated that the same Ca ++-dependent aggregation system that plays a role in stem cell adhesion is involved in the adhesion of early mouse embryos (19). When Nulli cells are harvested according to the trypsin/Ca ++ procedure of Takeichi et al. (4), we also observed Ca ++-dependent reaggregation. This aggregation was fucoidan insensitive and, therefore, did not depend on the fucan-specific lectin. The calcium-dependent component of cell adhesion exhibited by the trypsin/Ca ++ cells was presumably related to the divalent cation-dependent cell adhesion of CMF-harvested cells. However, there is one significant discrepancy in that reaggregation of CMF-PBS harvested cells was stimulated by either Mg ++ or Ca ++, whereas reaggregation of trypsin/Ca ++ cells was selectively stimulated by Ca ++. Perhaps the trypsin/Ca ++ cells required an additional Ca ++-dependent step to recover from the trypsinization and then, like the CMF-PBS harvested cells, would be able to aggregate in the presence of either divalent cation. We are presently investigating this possibility.

In terms of specificity, Takeichi et al. (4) present evidence that the Ca ++-dependent system of adhesion can mediate selective adhesion of stem cells in mixtures with fibroblasts. Brackenbury et al. (16), however, claim that, for neural retina cells and liver cells of the chick embryo, calcium-dependent adhesion is nonspecific, whereas the calcium-independent system is tissue-specific. It remains to be determined what role the divalent cation-independent adhesion system, which appears to depend on the fucan/mannan-specific lectin, plays in the selective adhesion of stem cells. It is of interest that lectins apparently mediate specific recognition events in a number of other systems including species-specific adhesion of slime molds (20), symbiosis of rhizobium and legume (21), and uptake of circulating glycoconjugates by the liver (22).

The effectiveness of fucans as inhibitors of a component of teratocarcinoma stem cell intercellular adhesion implies the existence of cell surface glycoconjugates that act as receptors for the CBP. Two recently described carbohydrate-containing antigens on teratocarcinoma stem cells are of particular interest as possible receptors for the lectin. First, Muramatsu et al. (23) have described the presence on the surface of teratocarcinoma stem cells of a family of large molecular weight, fucose-containing glycopeptides including the F9 antigen. Second, α-1-3 linked fucose, which is found in fucoidan, is a critical part of the recognition determinant of stage-specific antigen SSEA-1 detected on mouse embryos and teratocarcinoma stem cells (24, 25). Further studies will aid our understanding of the role of the stem cell lectin and its potential receptors in intercellular adhesion.

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