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Genetics

Increased adhesiveness of Down syndrome fetal fibroblasts *in vitro*

(morphogenesis/dysmorphology/cytogenetics/cellular aggregation/medical genetics)

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ABSTRACT **We compared the *in vitro* rate of divalent cation-independent aggregation of fibroblasts derived from abortuses with normal karyotypes and with trisomy 21 (Down syndrome). Fibroblasts from five lung and two of three cardiac cultures from subjects with Down syndrome aggregated more rapidly than matched fibroblasts from normal controls or lung fibroblasts from an abortus with trisomy 13. In contrast, skin fibroblasts derived from the trisomy 21 subjects had low rates of aggregation. The high rates of aggregation of trisomy 21 lung fibroblasts were not affected by hyaluronidase treatment. Lung fibroblasts from both normal and Down syndrome subjects had similar membrane polarization values in an assay using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. Thus, the increased aggregation rate we observed for trisomy 21 fibroblasts was restricted to specific fibroblast cultures, was not mediated by hyaluronic acid or gross membrane lipid alterations, and was specific for trisomy of chromosome 21. As illustrated in computer simulations presented elsewhere, increased intercellular adhesiveness during organogenesis could explain the frequent occurrence of malformations, including pulmonary hypoplasia and congenital heart defects, in Down syndrome.**

Down syndrome represents a constellation of inborn errors of morphogenesis that results from an extra (third) copy of chromosome 21 (1, 2). Dysmorphic features in Down syndrome result from localized failures in outgrowth of various tissues. For example, the upslanting palpebral fissures, brachycephalic head shape, midline hair whorl, and flat midface are secondary to deficits in brain outgrowth (3). Forty percent of individuals with Down syndrome have congenital heart defects, due mainly to deficits in outgrowth of the endocardial cushions (4–10). Pulmonary hypoplasia with spacious alveolar ducts and large acini has been reported as a feature of Down syndrome (11). The mechanisms whereby the excess genetic material of chromosome 21 result in the dysmorphology of Down syndrome have not been defined. However, it is known that genes on chromosome 21 are overexpressed in cells trisomic for this chromosome, demonstrating a phenomenon termed gene dosage (12–18). In a number of developmental systems, modulations of intercellular adhesiveness play a critical role in organogenesis. Based on this, we hypothesized that gene dosage for protein encoded by chromosome 21 yields increased intercellular adhesiveness, which in turn could mediate abnormal morphogenesis in Down syndrome. In this study, we show that specific types of Down syndrome fibroblasts derived from midtrimester abortus material manifest increased rates of divalent cation-independent cell adhesion *in vitro* compared to age-matched normal controls.

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MATERIALS AND METHODS

Cell and Culture Conditions. Fetal tissue was obtained from midtrimester elective abortuses under protocols approved by the Institutional Review Boards at Brigham and Women's Hospital and the University of Washington. For this study, five abortuses with a diagnosis of Down syndrome (designated TC 106, 108, 122, 143, and 151) and one abortus with a diagnosis of trisomy 13 (TC 13S) made on cells sampled at amniocentesis were obtained. In addition, eight age-matched normal abortuses (TC 118, 119, 123, 133, and 148 and DK 12, 13, and 17) were studied. Tissue was explanted under sterile conditions into Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and antibiotics. Organs selected for culture included skin, lung, and heart. Cardiac dissections to identify endocardial cushion-determined structures were performed by R. Van Praagh and R. Matsuoka (Departments of Cardiology and Pathology at Children's Hospital Medical Center). Two of the abortuses with Down syndrome (TC 106 and 143) had membranous ventricular septal defects identified by this analysis. Cardiac fibroblasts were more difficult to culture than skin or lung fibroblasts. Three cardiac fibroblast cultures were assayed from two of the Down syndrome abortuses (TC 106 and 108). For comparison, five cultures of cardiac fibroblasts from two normal abortuses (TC 118 and 119) were studied. Fibroblasts from each subject used in the study were karyotyped by using Q-, G-, or R-banding techniques (19). Each of the Down syndrome abortuses had a karyotype 47,+21 and the trisomy 13 abortus was 47,+13. All studies were performed on cells between the third and seventh passages.

Measurement of Aggregation. The rate of aggregation of suspended cells was measured as described (20). Cells were plated at a concentration of 10^5 cells per 100-mm tissue culture dish and allowed to grow for 48 hr prior to assaying the rate of divalent cation-independent aggregation. Cells were suspended by incubation in 0.02% (wt/vol) EDTA in phosphate-buffered saline, calcium- and magnesium-free, at pH 7.4 (CMF buffer) and resuspended in CMF buffer at a final concentration of 10^5 cells per ml. A 1.5-ml aliquot of the cell suspension was transferred to 35 × 10 mm non-tissue-culture Petri dishes and placed on a reciprocal shaker at 25°C. At designated times, the Petri dishes were removed and the extent of aggregation was determined by counting 300 successive cells with an inverted phase microscope using a 40× objective and scoring cells either as single cells or as aggregates of 2 or more cells. The net rate of aggregation was depicted by plotting the percent of cells in aggregates of 2 or more cells against time. Viability of the cells at the end of all assays was >90%, as judged by trypan blue exclusion. In some aggregation experiments, bovine testicular hyaluroni-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; GAG, glycosaminoglycan.

dase (Sigma type IV, 13,000 National Formulary units/mg) was added to the cell suspensions in CMF buffer immediately before the aggregation assay at a concentration of 0.66 or 6.6 μg per 10^5 cells. Ten micrograms of hyaluronidase showed no protease activity when assayed with azoalbumin as substrate (21).

Polarization Experiments. The aromatic fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to probe the lipid environment of the cell membranes (22). The polarization values (p values) were determined by exciting DPH with polarized light and measuring the depolarization of fluorescence, which depends on the rotation of the DPH molecules in the lipid environment. The p value was measured by the technique of Hoover *et al.* (23). DPH at a final concentration of 2 μM in CMF buffer was added to cells that were grown and removed from monolayer cultures as described for the aggregation experiments at a cell density of 1×10^6 per ml. The cells were incubated in DPH solution for 20 min at room temperature, pelleted by centrifugation, and resuspended in CMF buffer at 7.5×10^5 cells per ml. The p values were determined by using an Elscint microviscometer model MV-1A at 25°C.

Hyaluronate Metabolism. Glycosaminoglycan (GAG) biosynthesis was assayed by radiolabeling cell cultures with [^{3}H]glucosamine. Radiolabel in GAG in both cell layer and medium fractions was measured after differential digestions with GAG-specific enzymes (24) followed by precipitation with cetyltrimethylammonium bromide (25).

Hyaluronidase activity in both cell and medium fractions was measured by the colorimetric assay of Reissig *et al.* (26) as described previously (27) and also by a more sensitive radiochemical assay. For the latter assay, samples were incubated at 37°C with [^{3}H]hyaluronate substrate for periods of time up to 24 hr in formate buffer (0.1 M sodium formate/0.15 M sodium chloride) at pH 3.7 (27). After incubation, hyaluronidase activity was determined by precipitation of the digest with cetyltrimethylammonium bromide. In this assay, digested hyaluronate substrate remained in the supernatant after cetyltrimethylammonium bromide precipitation, whereas undigested material precipitated.

For studies on the binding and internalization of exogenous hyaluronate by cells, cultures were incubated at 37°C in complete medium with serum and [^{3}H]hyaluronate for 24 hr and the degree of binding and internalization was determined as described previously (28).

RESULTS

Aggregation of Lung Fibroblasts. The rates of divalent cation-independent aggregation of human fetal lung fibroblasts are summarized in Table 1. A significant difference between the rates of aggregation of normal and trisomy 21 lung fibroblasts was observed consistently at all of the time points (2.5, 5, and 15 min) in the assay. This difference corresponded to a 3- to 4-fold increase in the mean rate of aggregation of trisomy 21 cells over that seen for the normal controls. Lung fibroblasts obtained from a trisomy 13 abortus (TC 13S) aggregated at a low rate analogous to that obtained with the normal controls. Both parametric and nonparametric statistical tests (Table 1) showed the difference to be significant ($P < 0.01$). This difference between the Down syndrome and normal fibroblasts was not due to alterations in viability: at least 90% of cells excluded trypan blue at the end of the assay in all experiments.

Alterations in the lipid composition of the cell membrane have been shown to affect intercellular adhesiveness of several different cell types (23). We analyzed the lipid microenvironment of the cell surface by measuring p values of DPH in Down syndrome and normal lung fibroblasts. No significant differences in p values were found between the two groups of cells (data not shown).

Table 1. Aggregation of lung fibroblasts from abortuses with normal karyotypes, trisomy 21, and trisomy 13

	Percentage of cells aggregating		
Cell type	At 2.5 min	At 5 min	At 15 min
Trisomy 21			
TC 122	12.0 ± 3.7	20.8 ± 5.3	41.3 ± 5.0
TC 106	11.0 ± 1.8	24.0 ± 3.0	37.8 ± 4.0
TC 108	9.5 ± 2.5	16.8 ± 2.3	33.0 ± 4.0
TC 143	9.8 ± 1.2	16.8 ± 1.8	27.8 ± 1.3
TC 151	14.5 ± 0.7	26.3 ± 0.9	41.0 ± 2.3
Mean	11.4 ± 0.9	20.9 ± 1.9	36.2 ± 2.7
Normal			
TC 119	3.3 ± 1.1	6.0 ± 0.4	21.8 ± 2.4
TC 118	1.3 ± 0.8	0.3 ± 0.3	3.0 ± 2.4
TC 123	1.5 ± 0.9	4.3 ± 1.4	11.0 ± 1.9
TC 133	2.0 ± 0.4	2.5 ± 0.9	5.4 ± 1.5
TC 148	0.3 ± 0.3	1.3 ± 0.9	5.3 ± 3.4
DK 12	3.5 ± 1.4	7.5 ± 1.7	17.0 ± 3.2
DK 13	1.8 ± 1.0	10.3 ± 0.5	17.3 ± 1.4
DK 17	8.3 ± 1.3	10.8 ± 0.9	21.0 ± 0.4
Mean	2.7 ± 0.9	5.1 ± 1.5	12.7 ± 2.6
Trisomy 13			
TC 13S	1.3 ± 0.7	3.7 ± 2.0	5.3 ± 3.5

Lung fibroblasts were removed from Petri dishes with 0.02% EDTA, washed, and resuspended in CMF buffer. The extent of divalent cation-independent aggregation, expressed as the percentage of cells in aggregates of two or more cells, was determined at 2.5 min, 5 min, and 15 min after transfer of the dissociated cells to a reciprocal shaker. In each case, the mean ± SEM is given at each time point for the results from four separate experiments. The hierarchal design of these determinations corresponded to a two-factor experiment, having one factor nested under the other; using the corresponding F test (29), the rate of aggregation for trisomy 21 fibroblasts differed significantly from that of the normal fibroblasts ($P < 0.01$) at each time point. A nonparametric Mann–Whitney U test (30) also showed significant differences ($P < 0.01$) at each time point.

Aggregation of Skin and Cardiac Fibroblasts. The aggregation assays of endocardial- and conal-cushion-derived fibroblasts are summarized in Table 2. Two of the cultures, derived from two different Down syndrome abortuses [one

Table 2. Aggregation of cardiac fibroblasts from abortuses with normal karyotypes and with trisomy 21

	Percentage of cells aggregating		
Cell type	At 2.5 min	At 5 min	At 15 min
Trisomy 21			
TC 106 ALMV	9.3 ± 2.3	20.3 ± 2.8	46.3 ± 11.5
TC 108 CS	16.0 ± 5.0	26.0 ± 3.0	27.3 ± 6.5
TC 108 ALMV	1.7 ± 0.9	2.3 ± 1.2	7.3 ± 5.5
Normal			
TC 118 MVS	3.3 ± 2.0	0.7 ± 0.7	2.0 ± 1.2
TC 118 IAS	0.0 ± 0.0	1.7 ± 0.9	2.7 ± 2.7
TC 119 IAS	5.3 ± 1.8	7.3 ± 2.8	9.7 ± 2.9
TC 119 MVS	4.0 ± 0.6	3.3 ± 0.9	6.7 ± 3.6
TC 119 ALMV	1.7 ± 1.7	2.3 ± 1.2	5.3 ± 2.9
Mean	2.9 ± 0.9	3.1 ± 1.1	5.3 ± 1.4

The mean ± SEM is given for the results of three replicate determinations at each time point. ALMV, anterior leaflet of mitral valve; CS, conal septum; MVS, membranous ventricular septum; IAS, inter-atrial septum.

with a membranous ventricular septal defect (TC 106) and one with normal gross cardiac morphology (TC 108)], showed high rates of aggregation similar to the values seen with the Down syndrome lung fibroblasts. These cells were from endocardial cushion material (anterior leaflet of mitral valve from TC 106) and conal cushion material (aortic valve from TC 108), respectively. In contrast, fibroblasts derived from another explant from Down syndrome abortus TC 108 (anterior leaflet of mitral valve) and five separate cultures from two normal abortuses (TC 118 and 119) showed low rates of aggregation similar to the values seen for normal lung fibroblasts. The structures explanted from normal controls included anterior leaflet of mitral valve, inferior portion of inter-atrial septum, membranous ventricular septum, and base of aortic valve. Thus, fibroblasts with high rates of aggregation were found in one endocardial and one conal cushion explant from Down syndrome abortuses, but not in cardiac explants from normal controls.

The rapid aggregation observed for lung and cardiac fibroblasts from abortuses with Down syndrome was not observed in skin fibroblasts. As summarized in Table 3, both normal and Down syndrome skin fibroblasts showed low rates of aggregation.

Hyaluronate Metabolism. Previous studies on divalent cation-independent cell aggregation of fibroblast cell lines have shown that hyaluronic acid plays an important role in mediating the aggregation of transformed cells (31). This was evidenced by a marked reduction in the rate of aggregation upon addition of hyaluronidase to the assay mixture. In contrast, addition of hyaluronidase to the Down-syndrome fetal-lung fibroblast lines in this study did not alter their rate of divalent cation-independent aggregation. For example, in one series of experiments, untreated fibroblasts from Down syndrome abortus TC 108 showed 37% aggregation at 15 min in the absence of hyaluronidase. The level of aggregation was not altered after addition of 0.66 μg of hyaluronidase per 10^5 cells (40% aggregation) or 6.6 μg of hyaluronidase per 10^5 cells (39% aggregation). Similar results were obtained with the other four Down-syndrome fetal-lung cell lines (data not shown).

Other aspects of hyaluronate metabolism were assessed in lung- and endocardial-cushion-derived fibroblasts obtained from normal and Down syndrome abortuses. We studied endogenous hyaluronate and sulfated GAG synthesis, hyaluronidase activity, and binding and internalization of exogenous hyaluronate substrates.

Fibroblast cultures from normal (TC 119) and trisomy 21 (TC 106) abortuses had similar patterns of endogenous hyaluronate synthesis. After incubation for 24 hr at 37°C with

Table 3. Aggregation of skin fibroblasts from abortuses with normal karyotypes and with trisomy 21

	Percentage of cells aggregating		
Cell type	At 2.5 min	At 5 min	At 15 min
Trisomy 21			
TC 122	1.3 ± 0.7	3.7 ± 3.2	5.3 ± 2.9
TC 143	3.7 ± 1.9	6.0 ± 2.5	9.0 ± 1.0
TC 151	1.7 ± 0.7	0.0 ± 0.0	5.0 ± 1.5
Mean	2.2 ± 0.7	3.2 ± 1.8	6.4 ± 1.3
Normal			
TC 123	0.3 ± 0.5	1.8 ± 1.7	6.0 ± 0.9
TC 133	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Mean	0.2 ± 0.2	0.9 ± 1.2	3.0 ± 4.3

The mean ± SEM is given for the results of three replicate determinations at each time point.

[^{3}H]glucosamine, all cultures synthesized similar proportions of hyaluronate and sulfated GAG [mean cell-associated hyaluronate = 57%; trisomy 21 lung fibroblasts = 43%; trisomy 21 anterior leaflet mitral valve fibroblasts (which aggregated rapidly) = 64%; normal lung fibroblasts = 65%; normal anterior leaflet mitral valve fibroblasts = 55%]. In these experiments, radiolabel was not detected in hyaluronate in the medium. In addition, endogenous hyaluronidase activity was not detected in any of the cell cultures in either the cell layer or in the culture medium.

Further experiments investigating hyaluronate metabolism demonstrated differences between lung and cardiac fibroblasts. Specifically, lung and endocardial cushion fibroblasts differed in their abilities to internalize exogenous hyaluronic acid. These differences were consistent among cell lines from each organ so that the differences reflected tissue of origin rather than karyotype. Cell cultures were incubated with exogenous [^{3}H]hyaluronate and binding and internalization of the radiolabel was monitored. Most of the radiolabeled hyaluronate bound by lung fibroblasts was internalized, as determined by release of label after trypsin treatment (mean trypsin removable cpm = 11%; range, 0–33%). In contrast, a significant proportion of the hyaluronate was not internalized by anterior-leaflet-of-mitral valve-derived cell fibroblasts but instead remained bound to the cell surface (mean trypsin-removable cpm = 75%; range, 63–94%). Decreased internalization of hyaluronic acid has been observed in cultures of embryonic chicken endocardial-derived mesenchymal cells, which are the precursor cells of valve and septal tissues as is the case in man. These avian endocardial cells have also been found to retain exogenous hyaluronate on their cell surfaces and not to internalize or degrade exogenous hyaluronic acid (32).

DISCUSSION

The results indicate that selected fibroblasts derived from midtrimester abortuses with Down syndrome show increased rates of divalent cation-independent aggregation compared to fibroblasts derived from age-matched normal abortuses and to fibroblasts derived from an age-matched abortus with trisomy 13. The increased rate of aggregation was present in all five fibroblast cultures derived from lungs of abortuses with Down syndrome and in two of three fibroblast cultures derived from fetal cardiac endocardial- or conal-cushion tissue. Lung fibroblasts were distinguishable from cardiac fibroblasts by their hyaluronate binding and internalization properties. The high rate of aggregation observed with trisomy 21 lung and cardiac fibroblasts was tissue specific since it was not observed in skin fibroblasts.

The data in Table 1 represent the results of four independent determinations for each time point for each cell culture. As indicated by the standard errors (Table 1), the data were reproducible on cells cultured and assayed as outlined in *Materials and Methods*. We have frozen many of these fibroblast cultures and have observed that, although most cultures gave reproducible results after thawing consistent with the original data reported in Table 1, some cultures did not. The availability of frozen cultures with karyotype-specific aggregation characteristics mirroring the original data in Table 1 will permit future studies to determine the basis for the increased intercellular adhesiveness we observed among trisomy 21 fibroblasts. The finding that freezing may influence the aggregation properties of some cultures mandates the use of early-passage, unfrozen cultures from explanted tissue to generate primary data in future studies to confirm and extend these results.

The magnitude of the difference between the rates of aggregation of normal and trisomy 21 cells exceeds the 50% difference that might be expected simply from gene dosage (12–18). An analogous nonlinear effect for increased sensi-

tivity of trisomy 21 fibroblasts to interferon, presumably mediated by the interferon receptor encoded by chromosome 21, has been observed (for review see ref. 15). In addition to the significant differences between normal and trisomy 21 cells, we observed differences among cell cultures from different individuals with the same karyotype (Table 1). Clarification of the nature and import of these differences will require additional investigation.

Cell–cell adhesion is a complex phenomenon that has been studied by a variety of techniques (33). Each of these techniques has advantages as well as limitations and is influenced by such variables as the presence or absence of serum proteins, concentrations of divalent cations, and the means by which cells are dissociated. Cell–cell adhesion appears to occur by at least two different and partially overlapping mechanisms (34), one of which depends on calcium and the other is divalent cation-independent. These two adhesive mechanisms may relate to different functions *in vivo* because they have been shown to be expressed differentially during embryonic chicken retinal development (35). Divalent cation-independent cell adhesion has been associated with the formation of neurite bundles (36) and organization of cell layers (37) in retinal tissue of chicken embryos. In addition, increases in divalent cation-independent cell adhesion occur after transformation of fibroblast cell lines (20). The mechanism by which divalent cation-independent adhesion occurs is unclear, but specific cell surface proteins (35) as well as cell-associated hyaluronic acid (36) may be involved. Further, lateral mobility of cell surface receptors may play a role in the increased rates of divalent cation-independent cell aggregation seen with transformed cell lines (38).

Our findings suggest that the increased aggregation of selected Down syndrome fibroblasts is not attributable either to changes in hyaluronic acid (31) or bulk changes in membrane lipid composition (38–42), both of which can affect cellular differentiation and adhesion. The aggregation rate of trisomy 21 fibroblasts was not altered by the addition of hyaluronidase. Further, trisomy 21 and normal fibroblasts showed similar patterns of GAG biosynthesis. No differences in the fluorescent polarization of DPH were found between trisomy 21 and normal lung fibroblasts, indicating that there are not bulk differences in membrane lipid composition between these fibroblasts. One explanation for the increased adhesiveness of trisomy 21 fibroblasts is that gene dosage for a locus (or loci) on chromosome 21 results in increased amounts of a cell surface macromolecule(s) (43, 44) that facilitates divalent cation-independent cell aggregation. We do not know why trisomy 21 fibroblasts from only selected organs exhibit high rates of aggregation, but this may reflect differences among embryonic tissues of origin or the developmental status of these organs.

The alterations in intercellular adhesiveness of trisomy 21 lung and cardiac cushion fibroblasts we observed *in vitro* could relate to the dysmorphology observed in Down syndrome subjects *in vivo*. Pulmonary acinar hypoplasia was observed in a morphometric survey of lung development in 6 of 7 subjects with Down syndrome (11) and 40% of children with Down syndrome have congenital heart disease (8–10). To illustrate how increased intercellular adhesiveness could mediate congenital heart defects in Down syndrome, we have performed computer simulations of endocardial cushion fusion (unpublished data), which defined levels of intercellular adhesiveness at which endocardial cushions always, sometimes, or never showed normal outgrowth. Increasing the adhesiveness of endocardial cushion cells in these simulations interfered with outgrowth of the cushions. Thus, these simulations demonstrated that quantitative alterations in a cellular phenotype such as adhesiveness could enhance the likelihood of abnormal morphogenesis in organs derived from cells exhibiting the alterations.

In this study we report an alteration in intercellular adhesive behavior associated with Down syndrome. The finding of increased adhesiveness in particular trisomy 21 fibroblasts establishes the potential for an *in vitro* system to detect the presence of a trisomy 21 phenotype at the cellular level. This *in vitro* model system should facilitate molecular and immunological studies to elucidate the pathogenesis of malformations in Down syndrome.

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