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Effects of Extracellular Matrix Macromolecules on the Differentiation of Plasma Membrane Structure in Cultured Astrocytes

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ABSTRACTS. **Orthogonal aggregates of small intramembrane particles, termed "assemblies," are concentrated especially in the cell membranes of astrocytic processes that form the glia limitans at the outer surface of the brain and the perivascular sheath surrounding the parenchymal blood vessels. As an initial step to clarifying the totally unknown biochemical nature of this intramembrane structure, we have devised a culture system which enhances the differentiation of assemblies in secondary cultures of astrocytes derived from neonatal mouse neopallium. Since assemblies are most concentrated in the plasma membranes attaching to the basement membrane, we expected that extracellular matrix molecules constituting the basement membrane would be suitable candidates for our aim. We report here that a mixture of type IV collagen, laminin, and fibronectin, major components of the extracellular matrix, has the potency to increase assembly density in cultured astrocytes. We also report that, in freeze-fracture electron microscopy of cultured cells, one can satisfactorily preserve membrane structure and reliably obtain large replicas by inoculating cells on aluminium foil and peeling it from the cells in a freeze-fracture apparatus.**

The plasma membranes of astrocytes apposed to the basement membrane investing vascular structures or to the basement membrane surrounding the brain surface contain a high concentration of orthogonally-arrayed intramembrane particle aggregates, termed "assemblies" (4, 7, 8, 11, 12, 13, 14, 16, 21). However, the biochemical nature or the function of the protein represented by these assemblies is totally unknown. Since astrocytes retain the capacity to support assemblies *in vitro* (5, 13, 15, 29), cultured astrocytes can be utilized to clarify the biochemical nature of assemblies. Hence, culture conditions have been searched which enhance assembly density in cultured astrocytes. To our knowledge, there have been so far three reports on this issue: (a) different lots of the fetal calf serum used to supplement the media (17), (b) co-culture of astrocytes with endothelial cells derived from brain (28), and (c) addition of dexamethason into the culture medium (18). On the other hand, because assemblies are most concentrated in the plasma membranes in direct contact with the basement membrane surrounding the blood vessels or the brain surface, we have attempted to ascertain whether or not astrocytes increase assembly density when they are layered on extracellular matrix (ECM) macromolecules-coated culture dishes.

Some preliminary data of the present study have been presented in abstract form (Neurosci. Res., Suppl., 17: 166, 1992).

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Abbreviations: ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; FCS, fetal calf serum.

MATERIALS AND METHODS

The present study was repeated in cultures from three different dissociations (Experiments 1, 2, 3). 5-day-old ICR mouse neonates were used (8 neonates for Exp. 1, 7 neonates for Exp. 2, and 8 neonates for Exp. 3).

Primary cultures. Astrocyte cultures were initiated according to the approach devised by McCarthy and de Vellis (19). Newborn mice were etherized prior to decapitation, and the cerebral hemispheres were removed. After the olfactory bulb, basal ganglia, hippocampus, choroid plexus, blood vessels, and meninges had been carefully removed, the neopallia were dissected out and minced. The minced neopallia were exposed to 0.25% trypsin/PBS for 15 min at 37°C. Dissociated cell aggregates were sedimented and were inoculated in 25 cm^2 Falcon flasks in 6 ml of D-MEM supplemented with 10% FCS. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air over 10–14 days with two feedings.

Secondary cultures. After growth to confluence, cells were dissociated with 0.25% trypsin, sedimented, rinsed, resuspended, and inoculated in Falcon culture dishes (75 cm^2). Thirty min after inoculation, cells were shaken manually for 15–

30 min at room temperature, and floating cells were discarded by exchange of medium. Cultures were fed every 7 days, and cells after 3 or 4 passages were used.

Immunocytochemistry. Cells to be examined with immunocytochemical techniques were grown in tissue culture chamber slides (Lab-Tek, Nunc, Inc., USA). For glial fibrillary acidic protein (GFAP) immunoreactivity, cells in the wells were briefly washed in PBS, and then fixed for 30 min in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 at room temperature. Fixed cells were incubated with rabbit antisera to GFAP (DAKO, Kyowa Medex, Japan) at dilution of 1 : 200 for 1 h at room temperature. Thereafter, cells were processed for avidin-biotin reaction using the DAKO ABC KIT (DAKO, Kyowa Medex, Japan). Cells were counterstained with hematoxylin and observed with light microscope.

Substratum preparation. Mouse type IV collagen and laminin were purchased from GIBCO BRL, reconstituted as directed by the manufacturer, and frozen in −70°C in single-use aliquots. Mouse plasma fibronectin was purchased from Chemicon International, Inc., reconstituted as directed, and stored in single-use aliquots at −70°C. For our freeze-fracture study we chose aluminium foil as a plating substratum because it was more effective in rapidly freezing cells and preserving the cell membrane ultrastructure than plastic dish, coverslip or wrapping film. For coating aluminium foil, squares of sterilized aluminium foil (3 cm × 3 cm) were exposed for 60 min to 5 ml of 100 μg/ml type IV collagen in 0.05 N HCl and subsequently for 2 h to 5 ml of a mixture of 20 μg/ml laminin and 10 μg/ml fibronectin in PBS at room temperature. With their shiny surface downward, the aluminium foil squares were layered on the bottoms of 60 mm diameter petri dishes. Uncoated aluminium foil was used as controls. Cell suspension of 4×10^4/ml was plated into the foil-containing dishes in a medium supplemented with 10% FCS, and was grown for 5 days.

Freeze-fracture Electron Microscopy. Cells grown on aluminium foils were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 60 min, and stored in buffer for several hours. 3 mm × 3 mm squares were cut out from various parts of the aluminium foil to minimize the effect of the regional heterogeneity in the cultures. Being cryoprotected with 30% glycerol (30 min), one aluminium foil rectangle was carefully picked up with forceps and inverted onto the drop of 5% polyvinyl alcohol solution placed on a specimen carrier so that the cells were facing the polyvinyl alcohol. All samples were frozen in liquid Freon 22 and transferred to a JFD-7000 apparatus. The fracture was done by peeling the aluminium foil from the frozen underlying polyvinyl alcohol, which enabled us to constantly obtain well preserved membrane structure and a large fracture face. The fracture surface was rotally shadowed at a 25° angle with platinum-carbon. Cleaned replicas were picked up on uncoated copper grids with 400 mesh.

The freeze-fracture replicas were constantly photographed by an electron microscope (JEOL100C) at a primary magnification of 25,000× and enlarged photographically to 80,000×.

The largest fractured membrane surface was chosen in one grid, and at least 5 unobserved grids were interspersed between the two neighboring grids examined so that the field of the photographed area would have been biased as little as possible. The density of assemblies was determined in 1 to 5 fields for each cell; the total size of the field varied from 10.1 μm^2 to 74.0 μm^2 (Table I).

Morphometric techniques. In this study, we defined one assembly as one with a size equal to or larger than the rectangle of 2 × 3 subunits although it is generally accepted that one single assembly contains at least four subunits in replicas with optimal shadowing (7, 16). Consequently, we did not count an assembly composed of four or five subunits because it is sometimes difficult to distinguish it from common globular intramembrane particles. Furthermore, a cluster having more than four corners was counted as one single assembly. The area of membrane fracture surfaces was measured with a planimeter.

The morphometric data was presented as histograms (Fig. 2), and the detailed quantitative data for all measurements were given in Table I. Statistical comparisons were made by Student's t-test (95% confidence limit) for each experiment. We also applied a Mann-Whitney U-test (99% confidence level) by putting together all the numerical data obtained from the three sets of experiments (Fig. 3).

RESULTS

Since assemblies in cultured astrocytes revealed by freeze-fracture electron microscopy have been well documented in the literature (5, 13, 15, 29), morphological features distinct from those hitherto described are mainly focused upon here. In disagreement with the observation made by Anders and Brightman (5), we were able to find that assemblies persisted in the cultured astrocytes even after 4 passages.

Generally, the membrane structure in freeze-fractured astrocytes was qualitatively very similar between the control and experimental sides. The presence of assemblies is the only morphological criterion of membrane structure in freeze-fractured cells by which one can unequivocally identify a cell as an astrocyte. Cells which lack assemblies may also be astrocytes, or may be oligodendrocytes, fibroblasts or endothelial cells. Some 85–90% of the cultured cells were GFAP-immunopositive in our materials (data not shown).

Two types of fractured membrane surface were recognized: the outwardly facing cytoplasmic leaflet (P-face) of the membrane attaching to the culture dishes and the inwardly facing extracytoplasmic leaflet (E-face) of the membrane facing the culture medium (Fig. 1). P-face membranes were encountered as much as 10 times more than E-face membranes. In fact, we found assemblies both in the P-face and in the E-face, but the assembly

was consistently much more numerous on the P-face than on the E-face. E-face membranes were thus omitted in the present quantitative study. The size of the assemblies varied from a small rectangle (2 × 3 subunits) to a large rectangle (8 × 12 subunits) (Figs. 4, 6). The assemblies were also varied in form. In most instances, it was extremely difficult to discriminate or discern constituent particles comprising each assembly. On the other hand, assemblies appeared rather uniformly distributed over the fractured membrane surface. On close inspection, however, multiple small patches of assemblies were infrequently seen. In addition, there was no correlation between the assembly density and the presence or absence of an adjacent cell. Single globular particles, 5–12 nm in diameter, were scattered in the regions not occupied by the assemblies. Caveolae were sometimes seen. Small, punctate gap junctions were rarely encountered at the margins of the membrane fracture face at the early stage of culture.

As Figure 2 clearly shows, assembly density was considerably different from one cell to another both in the control and experimental sides in each set of experiment. However, the average assembly density was significantly higher in the experimental than that in the control side. Furthermore, a Mann-Whitney U-test ($p < 0.01$ level) also clearly showed that cells in the experimental side were significantly much higher in assembly density than those in the control side (Fig. 3). In addition, assembly density did not appear to be directly related to assembly size.

Experiment 1 (Fig. 2A, Table I). All of the 25 cells examined manifested assemblies in the control side as did 24 cells in the experimental side. Exposed astrocytic membranes were crowded with a great number of intramembrane particles of various sizes (Fig. 4). These particles were not homogeneously distributed throughout the membrane, but tended to form loosely-packed aggregates or clusters, leaving many particle-poor or particle-free regions. These aggregates or clusters of particles were not separated but continuous with one another via lineally-arranged particles. Among these aggregates, assemblies of various sizes and forms were readily recognizable. Owing to the lower height of the constituent subunits (16), assemblies were intermediate in electron density between the ordinary large globular particles and the membrane matrix devoid of particles, and looked like stains on cloth. Assembly density varied widely from 2.2 to 46.0 per μm^2 in the control, and from 0.9 to 112.0 per μm^2 in the experimental side. The mean values of assembly density were $18.1 \pm 11.6/\mu m^2$ (mean ± SD) in the control and $33.4 \pm 21.5/\mu m^2$ in the experimental side. These two values were significantly different by Student's T-test at the $P < 0.05$ level. The value obtained in the control side was two times higher than that reported in previous in vitro studies (17, 18, 28).

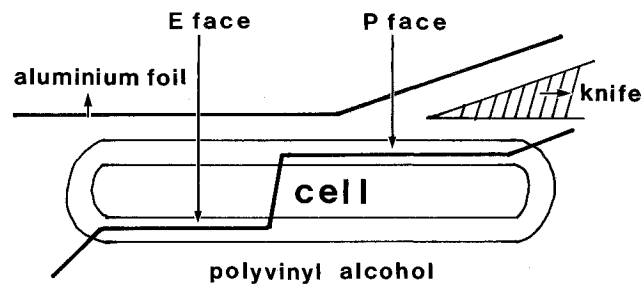

Fig. 1. Diagram illustrating the process of freeze-fracturing monolayered cells grown on the aluminium foil. A strip of aluminium foil covered with a monolayer of fixed cultured cells is inverted onto the drop of polyvinyl alcohol solution placed on a specimen carrier. The aluminium foil is fractured away by raising in with a knife. The dark line through the cell indicates a fracture plane. P-face: the outwardly facing cytoplasmic leaflet of the membrane attaching to the aluminium foil. E-face: the inwardly facing extracytoplasmic leaflet of the membrane facing the culture medium.

Experiment 2 (Fig. 2B, Table I). In sharp contrast to Exp. 1, of 25 cells studied, only four bore assemblies in the control side, resulting in a low mean value ($2.2 \pm 7.2/\mu m^2$). In some cells with no assemblies, particle-free domains were enlarged and surrounding intramembrane particles were rather few in number. Particles were lined in a row, and a small group of particles were seen to be scattered in this row, notably at the points where two rows cross-linked (Fig. 5). In other cells with no assemblies, particle-free domains were surrounded by a continuous band composed of many aggregated intramembrane particles of various sizes and heights. These particle-free domains were tortuous and rather uniform in width. Between these two extremes lay all sorts of variations. In the four cells expressing assemblies, the assemblies were generally small in size and tended to be distributed among the particle aggregates surrounding the particle-free domains (Fig. 6). On the experimental side, on the other hand, 16 out of 25 cells had assemblies ($14.0 \pm 19.9/\mu m^2$). The proportion of cells expressing assemblies was clearly increased, and the assembly density was much higher than that in the control side.

Experiment 3 (Fig. 2C, Table I). The pattern of the histogram obtained from Exp. 3 was intermediate be-

Fig. 2. Histograms of assembly density (number per μm^2) obtained from the three sets of experiments (A for Exp. 1, B for Exp. 2, and C for Exp. 3). The control side is on top in each set of histograms. The 25 cells examined are arranged according to the order of observation (in abscissa). In each histogram dotted transverse line indicates the mean value of the assembly density. In each set of experiment assembly density was significantly higher in the experimental than in the control sides (Student's t-test, <0.05).

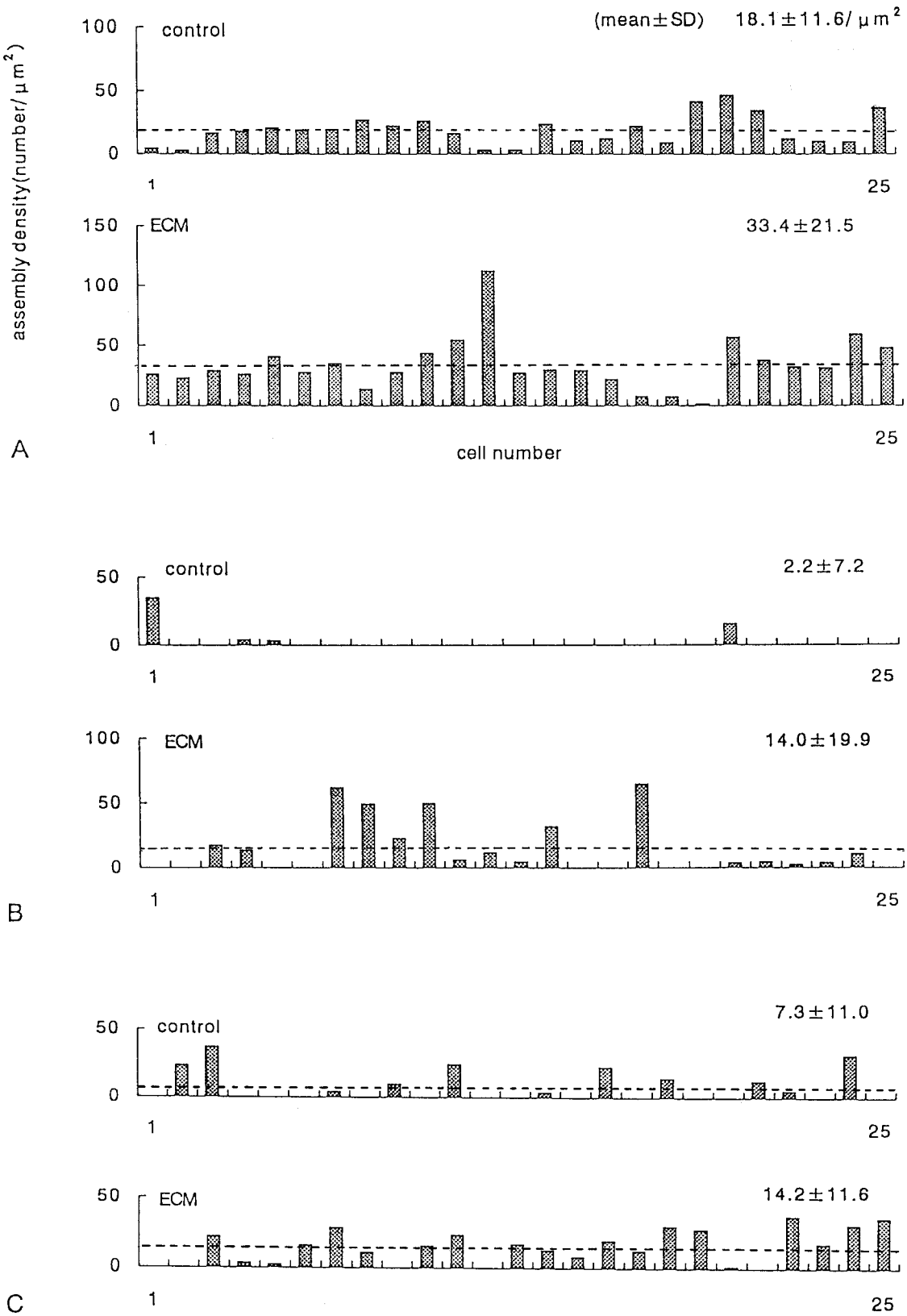
assembly density(number/μm²)
100
50
0
control
(mean±SD) 18.1±11.6/μm²
1
25
150
100
50
0
ECM
33.4±21.5
1
25
cell number
A
50
0
control
2.2±7.2
1
25
100
50
0
ECM
14.0±19.9
1
25
B
50
0
control
7.3±11.0
1
25
50
0
ECM
14.2±11.6
1
25
C

Fig. 2.

Table I. QUANTITATIVE DATA OF INDIVIDUAL FREEZE-FRACTURED CELLS.

Experiment 1

Control side				experimental side			
No. of cells	No. of assembleies	Area (μm^2)	No. of assembly per μm^2	No. of cells	No. of assemblies	Area (μm^2)	No. of assembly per μm^2
1	63	15.3	4.1	1	390	15.3	25.5
2	35	15.6	2.2	2	323	14.7	22.0
3	187	11.9	15.7	3	427	15.2	28.1
4	262	15.1	17.4	4	387	15.1	25.6
5	302	15.2	19.9	5	590	14.7	40.1
6	280	15.1	18.5	6	400	14.8	27.0
7	283	14.9	19.0	7	519	15.0	34.6
8	379	14.5	26.1	8	198	15.0	13.2
9	314	14.5	21.7	9	413	15.3	27.0
10	371	14.5	25.6	10	665	15.4	43.2
11	231	14.6	15.8	11	835	15.5	53.9
12	42	14.4	2.9	12	1751	15.6	112.0
13	40	14.6	2.7	13	416	15.6	26.7
14	234	10.1	23.2	14	453	15.5	29.2
15	138	13.5	10.2	15	425	14.9	28.5
16	180	15.4	11.7	16	271	12.6	21.5
17	328	15.1	21.7	17	115	15.7	7.3
18	110	12.9	8.5	18	108	15.6	6.9
19	502	12.3	40.8	19	14	15.6	0.9
20	663	14.4	46.0	20	890	15.7	56.7
21	446	13.2	33.8	21	578	15.5	37.3
22	168	14.6	11.5	22	495	15.6	31.7
23	134	13.9	9.6	23	467	15.1	30.9
24	116	13.1	8.9	24	895	15.2	58.9
25	541	15.2	35.6	25	707	14.9	47.4

* In this experiment only one field was photographed for each cell.

Experiment 2

Control side				experimental side			
No. of cells	No. of assembleies	Area (μm^2)	No. of assembly per μm^2	No. of cells	No. of assemblies	Area (μm^2)	No. of assembly per μm^2
1	1786	52.1	34.3	1	0	73.2	0
2	0	43.0	0	2	0	74.0	0
3	0	45.7	0	3	1207	72.0	16.8
4	255	72.2	3.5	4	912	68.9	13.2
5	167	57.0	2.9	5	0	57.7	0
6	0	44.1	0	6	0	42.6	0
7	0	43.1	0	7	2611	42.6	61.3
8	0	58.5	0	8	1919	39.6	48.5
9	0	73.0	0	9	932	42.0	22.2
10	0	43.9	0	10	2332	47.6	49.0
11	0	43.6	0	11	245	41.6	5.9
12	0	41.9	0	12	450	40.5	11.1
13	0	41.9	0	13	246	55.6	4.4
14	0	41.1	0	14	1245	39.8	31.3
15	0	41.9	0	15	0	41.7	0
16	0	41.2	0	16	0	41.6	0
17	0	41.6	0	17	2783	43.6	63.8
18	0	41.5	0	18	0	44.5	0
19	0	41.4	0	19	0	44.1	0
20	596	40.1	14.9	20	145	44.0	3.3
21	0	43.5	0	21	226	51.6	4.4
22	0	43.6	0	22	91	42.9	2.1
23	0	43.8	0	23	190	55.4	3.4
24	0	42.8	0	24	437	41.7	10.5
25	0	43.8	0	25	0	43.9	0

* In this experiment 3~5 fields were photographed for each cell.

Experiment 3

Control side				experimental side			
No. of cells	No. of assembleies	Area (μm^2)	No. of assembly per μm^2	No. of cells	No. of assemblies	Area (μm^2)	No. of assembly per μm^2
1	0	25.7	0	1	0	24.8	0
2	543	23.8	22.8	2	0	25.4	0
3	915	25.0	36.6	3	550	26.0	21.2
4	0	25.9	0	4	75	25.9	2.9
5	0	26.2	0	5	43	24.4	1.8
6	0	24.8	0	6	392	25.8	15.2
7	91	25.7	3.5	7	710	25.9	27.4
8	0	25.9	0	8	256	25.2	10.2
9	245	26.1	9.4	9	0	24.7	0
10	0	26.2	0	10	313	21.0	14.9
11	483	20.2	23.9	11	489	21.5	22.7
12	0	23.5	0	12	0	23.4	0
13	0	22.7	0	13	335	20.8	16.1
14	82	25.2	3.3	14	306	25.9	11.8
15	0	23.0	0	15	166	22.2	7.5
16	581	26.0	22.3	16	492	26.3	18.7
17	0	24.9	0	17	305	26.2	11.6
18	362	26.3	13.8	18	754	26.2	28.8
19	0	25.7	0	19	643	24.2	26.6
20	0	23.0	0	20	21	25.8	0.8
21	311	26.1	11.9	21	0	24.3	0
22	120	25.4	4.7	22	919	25.7	35.8
23	0	24.4	0	23	426	25.8	16.5
24	816	26.2	31.1	24	770	25.6	30.1
25	0	26.3	0	25	889	25.8	34.5

* In this experiment two fields were photographed for each cell.

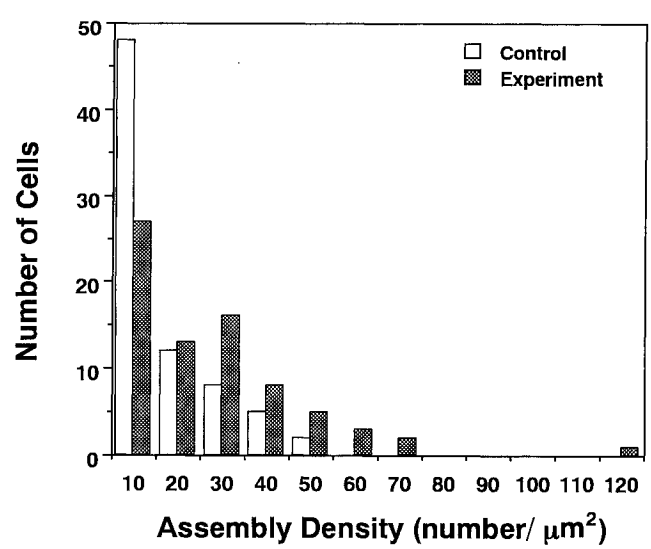

Fig. 3. Histogram showing the distribution of assembly density. The cell number was plotted against the assembly density. The scale for the assembly density is 10 per division. All the data from the three sets of experiments were put together. Assembly density was significantly higher in the experimental than in the control sides (Mann-Whitney U-test, <0.01).

tween Exp. 1 and Exp. 2. The mean value of assembly density was $7.3 \pm 11.0/\mu m^2$ in the control and $14.2 \pm 11.6/\mu m^2$ in the experimental side. The difference between control and ECM treatment reached statistical significance at the $P < 0.05$ level.

DISCUSSION

The present study indicates that the ECM macromolecules — type IV collagen, laminin, and fibronectin — remarkably increase the overall assembly density in astrocytes in vitro to a higher degree than thus far reported (17, 18, 28). Furthermore the results obtained strongly suggest that the ECM molecules also induce the formation of assemblies in some astrocytes with no assemblies. This increase in assembly density can be most adequately interpreted by aggregation of preexisting and/or newly membrane-inserted subunits of assemblies. Judging from the histograms illustrated in Fig. 2, the proportion of cells in the culture expressing assemblies is well correlated with the assembly density in individual cells.

The present study clearly shows that there was substantial variation in assembly density from dissociation to dissociation. According to Landis et al. (17), serum influences assembly density in cultured astrocytes; and supplementation of the culture medium with different lots of FCS leads to wide variations in the proportions of cells expressing assemblies, and in the densities of assemblies within a single culture. In this study, different lots of FCS were used from dissociation to dissociation, but the media were supplemented with the same lot throughout the primary and secondary cultures in each set of experiment. The considerable fluctuation of assembly density among the three sets of experiments found in this study can be partly ascribed to the effect of the different lot of FCS.

The present study also shows that assembly density fluctuated considerably from cell to cell even in cultures from a single dissociation. Moreover, we often encountered cells with few or no assemblies adjacent to cells with many assemblies, and vice versa. The reasons for this cell to cell variation are not obvious, but some explanations are possible. (1) It is generally accepted that astrocytes in vivo attach to the basement membrane underlying the pia mater or surrounding the blood vessels. However, it is not evident whether all of the astrocytes present in the central nervous system attach to the basement membrane or not. If a subgroup of astrocytes does not attach to the basement membrane and has no capacity to express assemblies, it is possible that astrocyte cell lines derived from this subgroup still express no assemblies in an in vitro system and that not all of astrocytes are equivalent in terms of the extent to which they can express assemblies. (2) The subpial glia limitans from mammalian brain generally consists of several layers of astrocytic processes. The astrocytic membranes directly attaching to the subpial basement membrane have the greater number of assemblies, and this number decreases with each successive layer of astrocytic processes (11, 12, 14). Astrocytes in vitro, on the other hand, are sometimes not grown in a purely monolayer fashion but multilayered on the plastic dish. Astrocytes growing over the confluent monolayer may contain a fewer number of assemblies than those in the monolayer because the latter cells are all in direct contact with the aluminium foil that might serve as a basement membrane. Hence, it is possible that, in addition to the cells attaching to the aluminium foil, the cells overlying the confluent monolayer are freeze-fractured although the cells in the monolayer are commonly, but not exclusively, freeze-fractured in our material. This possibility may explain to some extent the fluctuation in the number of assemblies from cell to cell observed in this study.

Landis and Weinstein (15) observed that assemblies are not uniformly distributed over one single cell. In contrast, we know that cultured astrocytes exhibit assemblies more evenly as the passage time increases. The discrepancy as to the distribution of assemblies, therefore, may be due in part to the passage time. Increase in passage time may be advantageous for a more reliable estimation of assembly density in a cell population despite the recent report that there are fewer assemblies in the secondary than in the primary cultures (21).

An ECM comprises mainly proteoglycans, collagens, elastin, and adhesive glycoproteins (3). The basement membrane is a specialized ECM composed of three laminae: lamina lucida, lamina densa, and lamina fibroreticularis (2, 3). Type IV collagen forms a core mat of the lamina densa. Laminin, an adhesive glycoprotein present both in the lamina lucida and the lamina densa,

Fig. 4. Membrane structure in a cultured astrocyte under experimental conditions. There are many aggregates of orthogonally packed intramembrane particles ("assemblies") associated with the cytoplasmic half of the fractured plasma membrane (arrows). The individual particles constituting these assemblies are smaller than most ordinary intramembrane particles. The field from which this picture was taken had a density of assemblies $130/\mu m^2$. This picture was taken from the cell 12 at the experimental side in Exp. 1. The membrane structure was qualitatively very similar between the control and experimental sides. ×80,000.

Fig. 5. An example of the cell manifesting no assembly. This picture was taken from the cell 10 at the control side in Exp. 2. ×80,000.

Fig. 6. An example of the cell manifesting a small number of assemblies. This picture was taken from the cell 20 at the control side in Exp. 2. 12 assemblies are recognizable in this field (arrows). Two double arrows indicate the assemblies of which the size correspond to that of the rectangle of 2×3 subunits and which are the smallest identified in this study. ×80,000.

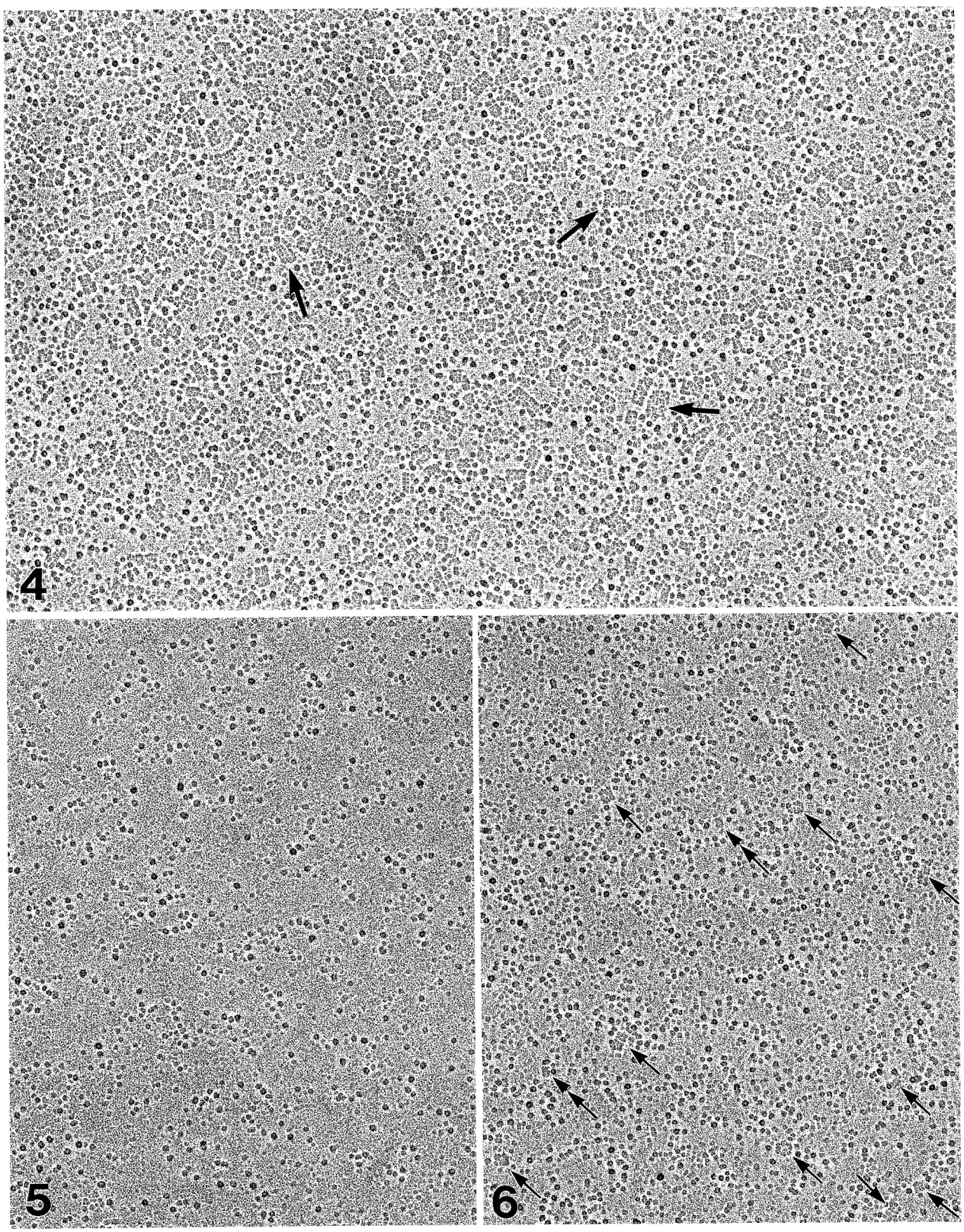

Fig. 4–6.

helps epithelial cells attach to the lamina densa (1, 26). Fibronectin, another adhesive glycoprotein, is involved in cell movements during embryological development, wound healing, and tumor metastasis, and bridges between cells and an ECM. In the central nervous tissue, like type IV collagen and laminin, fibronectin is found only in the basement membranes surrounding blood vessels and underlying the pia mater by immunocytochemistry (10, 24). In this study, we utilized these three major ECM components to obtain ECM-coated culture dishes but did not determine which of the three available ECM molecules is most effective in augmenting assemblies. However, considering the spatial arrangements of laminin and type IV collagen in the basement membrane, laminin appears to be the best candidate. On the other hand, Landis and Weinstein (15) pointed out that the number and appearance of assemblies in cultured astrocytes is not markedly influenced by the presence or absence of a collagen substrate, and we also confirmed their result (data not shown).

While the purification of the cell culture is significant in this kind of study, at the present time, it is impossible to obtain a 100% pure astrocyte culture, and no alternative culture conditions are now available for this aim. To our knowledge, neither astrocyte nor astrocytoma cell lines have been known which express assemblies.

To evaluate precisely an assembly density, it appears reasonable to take the size of assemblies into consideration. In fact, we counted assemblies regardless of their size. This is partly because it is almost impossible to discriminate and count subunits comprising each assembly on every occasion, as described in the previous studies (17, 18, 28) and partly because assembly density did not appear to be directly related to assembly size in our study.

Several speculations on the totally enigmatic function of the assembly have been made to date (4, 7, 8, 11, 17, 21, 28). We also here speculate about the possible functions of assembly. (1) Assemblies may be a given class of potassium channels. Recently, Newman (22, 23) has shown that the potassium conductances are most concentrated at the basal plasma membrane of Müller cell (a special type of astrocyte in the retina) attaching to the vitreal basement membrane. Assemblies are also most highly concentrated in this region of the same cell (30). At any rate, the high concentration of astrocytic assemblies at the interfaces with the blood and cerebrospinal fluid compartments highly suggests that they may be involved in transport of some unknown ions into or out of these compartments and thus in the regulation of intracerebral ionic milieu or of intracerebral pH. (2) Assemblies may be a given class of the integrins. Integrins are a widely expressed family of transmembrane ECM receptors. All integrins are heterodimers of α and β subunits; 12α subunits and 8β subunits have been sofar elucidated (9). For example, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins are receptors for collagen, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ integrins are receptors for laminin, and $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ integrins are receptors for fibronectin, although their binding specificity is somewhat overlapping (9, 20). Moreover, the $\alpha_6\beta_4$ integrin is specifically concentrated in hemidesmosomes in epithelial cells (27). Rat bladder carcinoma 804G cells expressing high levels of $\alpha_6\beta_4$ integrin adhere well to type IV collagen, laminin, and fibronectin. Hemidesmosomes or dense plaques are also recognizable in the astrocytic plasma membranes in direct contact with the subpial basement membrane (6, 25). As is well known, assemblies are most highly concentrated in this region of the plasma membrane.

The findings of our present study provide a means of substantially altering the assembly density in vitro, that may serve as a starting point for unraveling the function of assemblies.

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