^{125}I-Thrombin Binds to Clustered Receptors on Noncoated Regions of Mouse Embryo Cell Surfaces

DARRELL H. CARNEY and JOHN S. BERGMANN
Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT We used electron microscope autoradiography (EMAR) to visualize the interaction of ^{125}I-thrombin with its surface receptors on mouse embryo (ME) cells. Autoradiographic grains were spaced over the surface of cells in a periodic nonrandom pattern, indicating ^{125}I-thrombin association with clusters of thrombin receptors. The grain spacing varied slightly from cell to cell, indicating subpopulations of cells with different numbers of thrombin receptors. The average distance between grains on ME cells after binding ^{125}I-thrombin (125 ng/ml) at 37°C was 1.65 ± 0.49 μm. The average distance between grains on prefixed cells and cells incubated with ^{125}I-thrombin at 4°C was not significantly different from that observed at 37°C. This indicates that thrombin receptors are clustered before thrombin binding and that the thrombin receptor aggregates do not redistribute into large aggregates on the surface of cells subsequent to thrombin binding. The number of grains per cluster also does not change under these three binding conditions. Thus, the number of occupied receptors in each cluster appears to be constant. On the basis of the average grain number and spacing, we estimate that each cluster is ~400 nm in diameter containing approximately 550 thrombin-binding sites. These receptor-clusters are not associated with specialized structures or coated regions of the membrane. Additionally, grains observed within cells were not found associated with coated vesicles. Therefore, neither the clustering patterns nor internalization of ^{125}I-thrombin are characteristic of molecules which bind to receptors and are internalized by receptor-mediated endocytosis.

A number of molecules including low density lipoprotein (LDL), asialoorosomucoid, α_2-macroglobulin, insulin and epidermal growth factor (EGF) bind to cell surface receptors which aggregate in coated membrane regions and are internalized by the process referred to as receptor-mediated endocytosis (1, 2). It is not clear, however, whether this type of endocytosis plays a role in the biological activity of hormones and growth factors or whether it merely serves as a degradative pathway to regulate the levels of these molecules or their receptors.

Some unoccupied receptors are aggregated on the cell surface before ligand binding. Fibroblasts prefixed in formaldehyde or chilled to 4°C before incubation with LDL appear to have a large proportion of their LDL receptors preclustered (3). In addition, up to 58% of the asialoorosomucoid receptors may be clustered before ligand binding (4). In both cases, these clusters associate with coated pits for internalization. Hormonelike molecules have also been reported to initially bind to aggregated receptors. For example, acetylcholine receptors appear to be aggregated on the surface of muscle cells prior to inner-

vation or incubation with acetylcholine (5). In addition, EGF and insulin may bind initially to both diffuse (6, 7) and clustered (8–10) receptors. The mechanism of this clustering and its significance to the biological action of these molecules remains to be determined.

We approached these questions by visualizing the interaction of thrombin with fibroblast cell surfaces. Our previous studies demonstrated that the action of thrombin at the cell surface is sufficient to initiate cell division (11) and that this initiation correlates with binding to specific thrombin receptors (12). Proteolytic activity of thrombin is required for this initiation (13), suggesting that cleavage of either the receptor or some adjacent molecule may be necessary to initiate cell proliferation. We recently reported immunofluorescence evidence suggesting that thrombin binds to clustered receptors on the surface of mouse, human, and hamster fibroblasts which have been fixed with 3% formaldehyde before thrombin binding (14).

For this study, we used EM autoradiography (EMAR) to

visualize the interaction of ^{125}I-thrombin with mouse embryo (ME) cell surfaces to further investigate both the physical and functional nature of these clustered receptors.

MATERIALS AND METHODS

Materials

Dulbecco-Vogt modified Eagle's medium (DV medium) and medium supplements were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY) calf serum from Irvine Scientific (Santa Ana, CA), and tissue culture dishes from Lux Scientific Corp. (Newbury Park, CA). Highly purified human thrombin (3,000 National Institutes of Health [NIH] U/mg) was generously provided by Dr. John W. Fenton II. Sodium iodide (IMS-30, ~17 mCi/μg) was purchased from Amersham Corp. (Arlington Heights, IL). Chemicals for EM and EMAR were obtained from Polysciences Inc. (Warrington, PA) and from Ted Pella, Inc. (Tustin, CA).

Cells and Cell Culture

Primary cultures of ME cells were prepared from the body walls of 11-13-day-old mouse embryos and cultured in DV medium supplemented with 10% calf serum (12). After 3-5 d, the primary cultures were subcultured into 60-mm Permalux dishes (5.2×10^6 cell cm^{-2}). After 16 h, the cells were rinsed and the medium was changed to serum-free DV medium. EMAR receptor localization experiments were performed 2 d later. At this time the cells are quiescent (90% G_0/G_1) and are mitogenically responsive to human thrombin at a concentration of 125 ng/ml (12).

Iodination of Thrombin

Thrombin was iodinated using lactoperoxidase as described previously (12). Using a 1:1 ratio of radioactive to nonradioactive iodine, thrombin was prepared with specific activities up to 4.3×10^4 CPM/ng, corresponding to 1.1 iodines/thrombin molecule. The proteolytic activity of each iodinated thrombin preparation was determined by its ability to convert fibrinogen to fibrin (15). All iodinated thrombin used for these experiments retained >80% of its proteolytic activity.

[125]I-Thrombin Binding

Experiments were performed on monolayer cultures of nonproliferating ME cells (12). Cultures were rinsed and allowed to equilibrate with binding medium (serum-free DV medium containing 0.5% albumin buffered with 15 mM HEPES at pH 7.0) for 30 min at 37 or 4°C. Medium was then changed to binding medium containing 125 ng/ml of ^{125}I-thrombin ±2.5 μg/ml (20-fold excess) of unlabeled thrombin, and incubation was continued at 4 or 37°C for 3.5 or 1 h, respectively.[1] Total and nonspecific binding was determined both by solubilizing cell monolayers in 0.5 N NaOH for radioactivity measurement and by counting the number of EMAR grains on thin sections through cells. In each case, specific thrombin binding was calculated by subtracting thrombin bound in the presence of excess unlabeled thrombin from the total binding with ^{125}I-thrombin alone.

EMAR

After incubation with ^{125}I-thrombin, cells were fixed in their culture dishes in 1.25% glutaraldehyde and 1% OsO$_4$ in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min at 4°C, stained for 30 min in 2% aqueous uranyl acetate, rinsed twice with sodium phosphate buffer and distilled water, dehydrated in graded ethanol, and embedded in Epon 812 (11). With this procedure, we previously showed that >90% of the ^{125}I-radioactivity was retained by the cells during the EM fixation process (11). Thus it is unlikely that preparation for EM autoradiography altered the amount of location of ^{125}I-thrombin in these cells. Thin sections (~70 nm thick) were cut perpendicular to the cell monolayer and mounted on Formvar-coated grids. To increase autoradiographic resolution, sections were stained with uranyl acetate and lead citrate before overlaying a 0.1 μm film of Ilford L4 emulsion (16). With this procedure 95% of EMAR grains are located within 0.2

[1] Reportedly, up to 8% of ^{125}I-thrombin binding and virtually all urokinase binding to ME cells can be attributed to formation of protease nexin complexes (19, 20). Using the present binding conditions, we do not observe any protease nexin-^{125}I-thrombin complexes on SDS gels and no specific urokinase binding. Thus, under these conditions ^{125}I-thrombin binding is not complicated by protease nexin binding.

μm of the source (11). Exposure was carried out for 2 mo at 4°C. The emulsion was developed in Kodak Microdol-X for 5 min, and sections were restained with uranyl acetate and lead citrate before examination. Electron micrographs of cells magnified 10,000-40,000 times were used to determine the distance between cell surface grains and the average number of grains per receptor location.

RESULTS

Visualization of ^{125}I-Thrombin Binding

In our initial experiments, ^{125}I-thrombin (125 ng/ml) was incubated with ME cells for 1 h at 37°C. Under these conditions a distinct periodic pattern of EMAR grains was observed with grains or grain clusters uniformly spaced along the cell surface (Fig. 1). As shown, most of the grains were associated with the cell surface (of 713 grains counted, ~11% were internalized, >0.2 μm from the surface) and were distributed ~1.6 μm apart. The number of grains observed on cells incubated with ^{125}I-thrombin alone or with a 20-fold excess of unlabeled thrombin was proportional to the amount of total and nonspecific ^{125}I-radioactivity determined on parallel cultures (see legend of Fig. 1). In both cases, nonspecific binding was <30%. Therefore, most of the evenly spaced grains represent ^{125}I-thrombin bound to specific thrombin receptors. On the basis of the average number of ^{125}I-thrombin molecules bound per cell and the average number of grains observed on each cell, we calculate that approximately one EMAR grain was developed for every 40 molecules of ^{125}I-thrombin (see legend of Fig. 2).

As shown in Fig. 2, if thrombin were bound to randomly diffuse receptors we would expect EMAR grains to also be randomly distributed over the surface. In contrast, if receptors were aggregated in evenly spaced clusters containing a large number of receptors, then, as one examined a thin section through these clusters, EMAR grains would be evenly spaced. Under the binding conditions shown in Fig. 1, an average of approximately 140,000 ^{125}I-thrombin molecules were bound specifically to each cell. Radioactive decay of these iodinated molecules produced approximately 3,500 autoradiographic grains, or about eight grains per cell section as viewed in the EM. If thrombin receptors were diffuse, the grain spacing on a given cell should vary from 0.5 μm (grains closer than 0.4 μm were considered as arising from the same location) up to perhaps 4 or 5 μm. This was not the case. As shown in Fig. 1 and Table I, the grains on a given cell were quite regularly spaced with standard deviations averaging ~15% of the mean. Since the resolution of EMAR under these conditions is ~0.2 μm (12), this amount of variation could be accounted for by the variation in grain location relative to the ^{125}I-thrombin molecules. Statistical analysis of 100 measurements from 17 different cells incubated with ^{125}I-thrombin for 1 h at 37°C showed several important facts: (a) 76% of the variance was due to differences in average spacing between cells rather than within measurements taken from individual cells. (b) The pooled estimate of variance between grains on all cells was 0.17. This is clearly different from an estimated variance of approximately 1.9 for totally random spacing or 1.0 for uniform single receptor distribution (see legend of Fig. 3). (c) 14 of the 17 cells examined showed variance levels of approximately 0.1, indicating even spacing of the grains. The upper limit (95% confidence interval) for estimated cell variance on these 14 cells is 0.66. Thus the EMAR grain spacing is significantly different from that expected for thrombin binding to random or uniform nonclustered single receptors. Each of the other three cells had one large space which might have resulted from the internalization of a receptor cluster. Even with these three
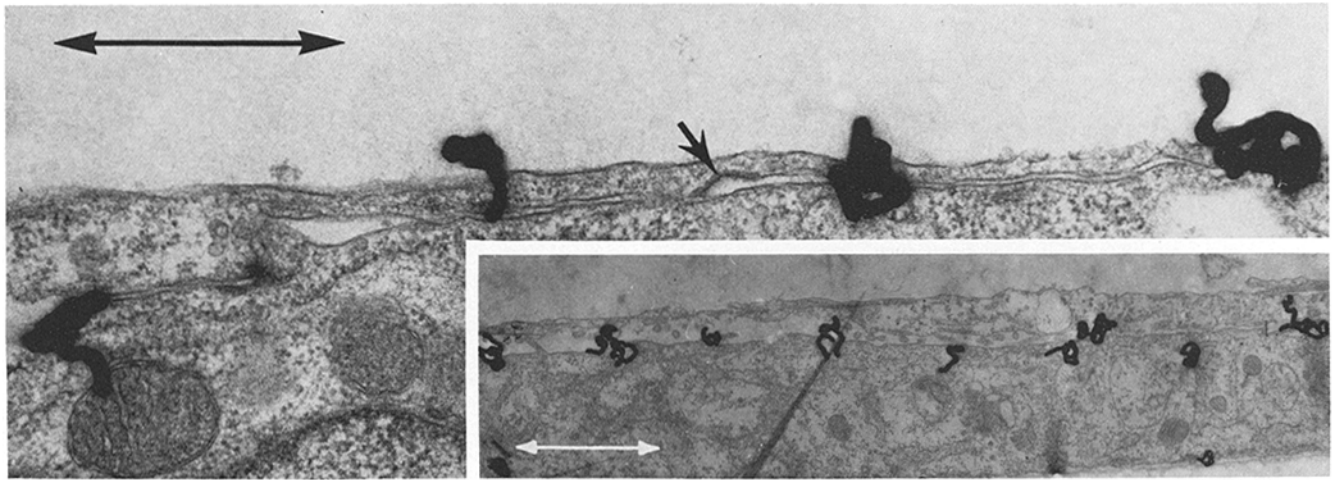
FIGURE 1 Visualization of ^{125}I-thrombin binding to ME cells. Cells were incubated with ^{125}I-thrombin (125 ng/ml) for 1 h at 37°C and prepared for EMAR. Of 18.8 grains per section total binding (38 cells), 5.0 grains per section (56 cells) were nonspecific. Thus, 73% of the grains represent specific binding. This corresponds to the percent specific binding determined by counting radioactivity on parallel plates. Arrow denotes coated pit. Bar, 1 μm. × 40,000. Insert, 2 μm. × 10,000.

Diffuse Receptors   **Clustered Receptors**
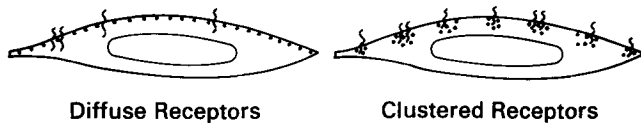
FIGURE 2 Diagram of EMAR grain distribution on cells with clustered and diffuse receptors. On the basis of the number of grains observed per cell section and number of ^{125}I-thrombin molecules bound per cell in parallel cultures (five experiments), we calculate that each EMAR grain represents 38.9 ± 13.0 thrombin molecules. As shown, if receptors (shown as dots) were diffuse the grain pattern (squiggles) would be completely random. In contrast, if receptors are clustered in large aggregates the grain pattern will reflect the location and spacing of these aggregates.

TABLE I

Examples of Mean Distance between EMAR Grains on the Surface of Individual ME Cells after ^{125}I-Thrombin Binding *

Binding conditions	No. of grains measured per cell	Mean distance between grains
		μm ± 1 S.D.
1-h incubation at 37°C	10	1.35 ± 0.20
	6	0.99 ± 0.12
	8	1.45 ± 0.30
	7	1.85 ± 0.15
4-h incubation at 4°C	10	1.60 ± 0.29
	8	1.15 ± 0.24
	6	1.06 ± 0.15
	7	2.00 ± 0.27

* ME cells were incubated with ^{125}I-thrombin (125 ng/ml) as described in Figs. 1 and 2. Distance between grains was measured on individual cells.

exceptions, the data clearly indicate that individual grains (or grain clusters) are evenly spaced.

To graphically demonstrate the statistical analysis of the even grain spacing, we standardized each measurement by dividing by the average distance between grains on each cell and plotted the frequency of these standardized values (Fig. 3). As shown, the actual measurements have a distribution with very little variance, reflecting the average standard deviation

of ~15% of the mean. For comparison, if the grains were located totally randomly the standardized plot would appear as a straight line. This type of analysis also shows that if single thrombin receptors were evenly spaced and if one random grain was detected for every 40 molecules of ^{125}I-thrombin, the frequency distribution would represent a skewed pattern with a standard deviation approximately equal to the mean. Thus this quantitative and statistical evaluation of the EMAR grain pattern clearly shows that the grains are evenly spaced and not random. Further, since only one EMAR grain was observed for every 40 ^{125}I-thrombin molecules, the regular spacing of the
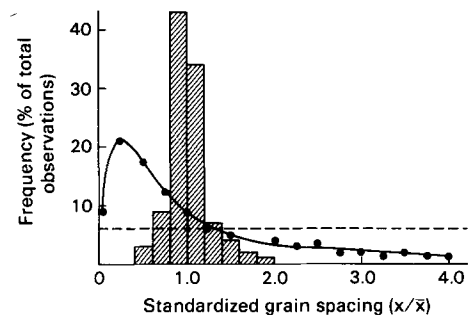

FIGURE 3 Frequency distribution of standardized grain spacing observed on ME cells compared to hypothetical distribution for random and uniformly spaced single receptors. 103 individual grain measurements (X) from 17 cells incubated for 1 h at 37°C with ^{125}I-thrombin were standardized by dividing by the average grain spacing ($\bar{X}$) for each cell. The frequency distributions of these measurements are shown as a bar graph. For comparison, the hypothetical distribution for totally random distances between any two grains is drawn in (- - -). Distribution for nonclustered uniformly spaced single receptor binding (●) was computer-generated to fit a mean spacing of ~1.6 μm. Assuming a uniform single receptor spacing where one-fortieth of the molecules would randomly generate an EMAR grain, the computer selected 1,000 random numbers out of 40,000, ordered them, and determined the difference representing the distance between each pair. As shown, after dividing by the mean, the frequency distribution of this type of single receptor binding appears as a skewed pattern with a mean of 1.0 and a standard deviation approximately equal to the mean. Thus this pattern is significantly different from the observed EMAR grain spacing.
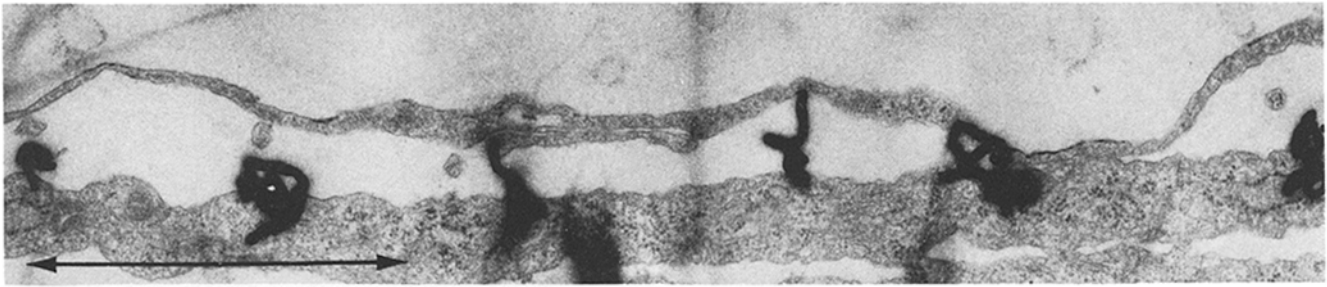
FIGURE 4   Visualization of ^{125}I-thrombin binding after 4°C incubation. Cells were chilled to 4°C and incubated with ^{125}I-thrombin (125 ng/ml) for 3.5 h and prepared for EMAR as described. Bar, 2 μm. × 25,000.

EMAR grains indicates that the ^{125}I-thrombin molecules are associated with clusters of receptors rather than single receptors. These data also predict that each cluster must be large enough to insure that at least 40 molecules of ^{125}I-thrombin would be present in the portion of the cluster included in each thin section. It should be noted that this even spacing was observed over all regions of cells and on both upper and lower cell surfaces. Thus this clustered pattern appears to be characteristic of thrombin receptors, and there are no areas such as those over the nucleus or near the cell periphery which are devoid of thrombin receptor clusters.

Do Thrombin Receptors Redistribute after Thrombin Binding?

To determine the location of thrombin receptors prior to thrombin binding, ME cells were chilled to 4°C, rinsed, and incubated with ^{125}I-thrombin (125 ng/ml) for 3.5 h at 4°C. As shown in Fig. 4, EMAR showed the same type of uniform periodicity and spacing between grains as observed with binding at 37°C (compare to Fig. 1). In this case, ~98% of the grains were located at the cell surface. Thus with binding at 4°C and maintaining the cells at 4°C throughout our manipulations, there was little if any internalization and presumably no receptor redistribution within the membrane.

If receptor aggregation occurred subsequent to binding, one would expect a diffuse pattern after 4°C incubation where membrane fluidity is decreased. Even if some artifactual clustering occurred subsequent to binding at 4°C, we would predict a 4°C pattern with a large number of small, closely spaced receptor groups which would further aggregate at 37°C to form large, evenly spaced aggregates. To determine whether altered membrane mobility would affect aggregation, we measured both the distance between grains and the number of grains per receptor location on over 150 photographs of cells where at least three grains were visible to determine the average grain spacing and grain density for each cell. The averages for each cell were then plotted as a scattergram to allow comparisons between average grain spacings on a large number of cells (Fig. 5). In these experiments, we also included cells which were rinsed and incubated with 3% formaldehyde at 25°C for 15 min prior to binding ^{125}I-thrombin. It should be noted that this fixation restricts redistribution and internalization of ^{125}I-thrombin (97% of EMAR grains are associated with the cell surface following binding at 37°C) but does not decrease the degree of specificity or quantity of ^{125}I-thrombin bound (see legend of Fig. 5).

As shown in Fig. 5, the average center-to-center spacing between grains on individual cells after incubation at 4°C or at 37°C to normal or prefixed cells was very similar, ranging from 0.74 to 2.97 μm with a mean of 1.54 ± 0.49 μm at 4°C,
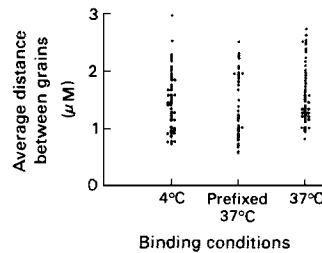

FIGURE 5   Average EMAR grain spacing on ME cell surfaces. ^{125}I-thrombin (125 ng/ml) was incubated at 4 or 37°C as previously described. Binding to prefixed cells was determined by rinsing cells with PBS, fixing them in 3% formaldehyde (Tousimis) for 15 min at 25°C, rinsing, and then binding ^{125}I-thrombin for 1 h at 37°C as described. Under these conditions, ^{125}I-thrombin binding was ~95% specific with 1.58 × 10^5 ^{125}I-thrombin molecules bound per cell. After EMAR, micrographs of 150 cells (each with three or more grains) were measured to determine the average center-to-center distance between grains for each cell.
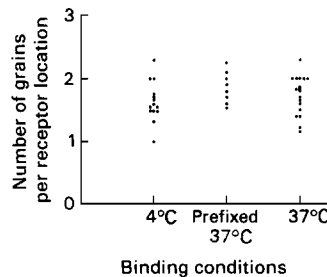

FIGURE 6   Average number of grains per receptor cluster location. After the determination of cluster spacing described in Fig. 4, the average number of grains per cluster location was determined. Spaces without grains which fall into the spacing pattern for cell were counted as 0.

from 0.85 to 2.76 μm with a mean of 1.65 ± 0.49 μm at 37°C, and from 0.58 μm to 2.53 μm with a mean of 1.40 ± 0.69 μm for prefixed cells. Thus, the EMAR grains are quite evenly distributed with no significant difference ($P < 0.05$) in average distance between grains on cells where thrombin was bound at 4°C, 37°C or to prefixed cells. There was also no significant difference ($P < 0.05$) in the average number of grains observed per receptor location under the three binding conditions (Fig. 6). As shown, there were approximately 1.6 grains per location in all cases. These results demonstrate that the thrombin receptor clusters appear to be the same in size and are approximately the same distance apart whether or not receptor redistribution within the membrane is restricted. Thus it appears that these clusters do not coalesce into larger clusters even after incubation for 1 h at 37°C. These results also indicate that most if not all of the thrombin binds to the clustered receptors. For example, if even part of the receptors were diffuse prior to thrombin binding, we would expect either the spacing or number of thrombins bound per cluster to change under the different binding conditions.

The somewhat large standard deviation for the average spacing between grains may be attributed to several factors. First, EMAR resolution itself can lead to variation. For example, some grains may be up to 0.3 μm from the ^{125}I-source although 95% are located within 0.2 μm (11). As shown earlier

in Table I, the variation on individual cells was ~15% of the mean or <0.25 μm. Thus the resolution can account for part of the larger variation seen in the whole cell population. Second, there appears to be variation in the spacing and number of receptor clusters on individual cells within the population (Fig. 7). This difference, observed by EMAR, was also demonstrated by labeling cells with peroxidase-conjugated antibodies following thrombin binding to glutaraldehyde-prefixed cells. With immunocytological visualization, it is possible to show that within the ME cell population some cells have a large number of receptor clusters which are closely spaced, whereas other cells have fewer receptor clusters which are farther apart. As clusters get very far apart, however, it is unlikely that our thin sections would cut through clusters consistently enough to allow visualizations of periodic spacing. Another source of variation is simply the arrangement of clusters and the angle of thin sections relative to the cluster pattern. For example, thin sections cut at different angles through the hypothetical cluster pattern shown in Fig. 8 vary from 0.8 to 2.4 μm with an average cluster spacing of ~1.6 μm. Thus this type of pattern and spacing approximate the EMAR data obtained from cell sections.

Size of Receptor Clusters and Number of Receptors per Cluster

From the average distance between grains and variability of spacing, we can approximate the pattern and numerical density for receptor clusters on the surface of ME cells (Fig. 8). Even though the cluster pattern on cells is likely to vary from such a rigid square lattice, we can use this type of pattern to
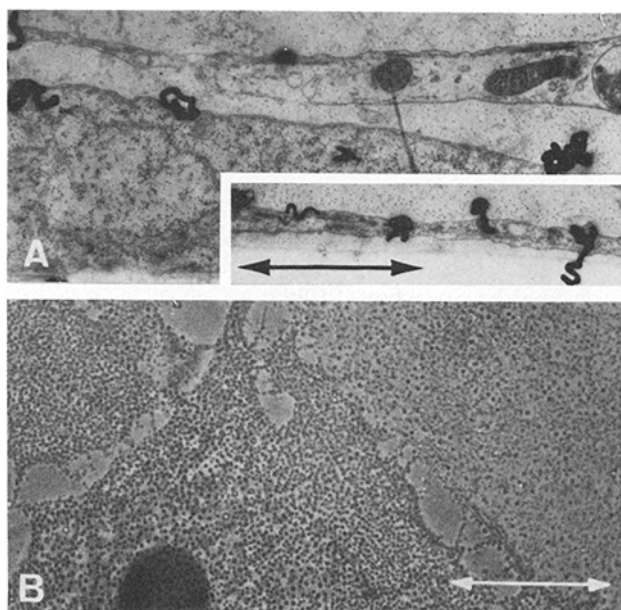
FIGURE 7 Variation in EMAR grain spacing and thrombin receptor cluster density. (Panel A and *Insert*) EM autoradiographs of two different ME cells after binding of ^{125}I-thrombin (125 ng/ml) at 37°C as described in Fig. 1. Magnification is the same for both cells × 12,500. Scale Bar, 2 μm. (Panel B) Micrograph of ME cells which were fixed in 1% glutaraldehyde for 7 min at 4°C, rinsed, and incubated with thrombin (200 ng/ml) for 2 h at 23°C, followed by incubation with affinity-purified antithrombin antibody, and immunoperoxidase stained with second antibody (14). It should be noted that parallel cultures incubated without thrombin showed no dots (14). Scale Bar, 30 μm. × 750.
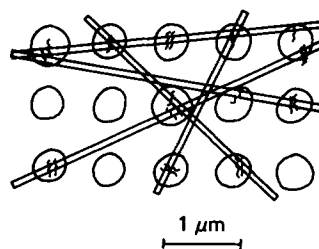
FIGURE 8 Approximation of cluster density and size. This pattern represents a hypothetical square lattice spacing of circular clusters with closest centers being 0.8 μm apart. Random sections through such a cluster pattern would have grains spaced from 0.8 μm to 2.4 μm apart which approximates the variation observed in EMAR sections (see Fig. 5). This pattern would have a cluster density of 1.56 clusters/μm^2. Using the stereometric formula $N_V = N_{AT}/(D + T)$ established by Abercrombie (28), we show N_V, the numerical density (1.56/μm^2) = N_{AT}, number observed per section (0.75/μm) divided by D, average cluster diameter + T, section thickness (0.07 μm). Solving for D, we find that the average diameter of these clusters is 0.41 μm.

TABLE II

Location of EMAR Grains Relative to Coated Pits

Binding condition	No. of grains	No. of coated pits	No. of grains within 0.2 μm of pits
4°C	155	12	0
4°C plus 37°C for 1, 3, or 5 min	182	11	0
37°C binding	242	47	2
Total	579	70	2

approximate the average numerical density of the clusters. Using this numerical density, we calculate that the average diameter of these clusters should be ~400 nm (see legend of Fig. 8). Since the average thin section (70 nm thick) through these clusters had 1.6 grains and since each grain represents 40 ^{125}I-thrombin molecules, these sections contain about 65 bound thrombins. An average 70-nm section through a 400-nm circle would contain 16.9% of the total area (17). Thus, approximately 385 ^{125}I-thrombin molecules were bound to each receptor cluster. Our previous binding studies (12) have shown that at a ^{125}I-thrombin concentration of 125 ng/ml, ME cell receptors are ~70% saturated. This suggests that each cluster contains at least 550 thrombin receptors.

Do Receptor Clusters Associate with Coated Pits?

To determine whether thrombin receptor clusters were associated with coated pits, we examined EMAR micrographs of cells incubated with ^{125}I-thrombin at 4°C, 4°C plus 37°C incubation, or at 37°C. In these micrographs, we observed a total of 579 grains and 70 coated pits (Table II). There were no grains within 0.2 μm of the edge of pits after 4°C binding or after 4°C binding followed by 37°C incubation and only two grains that could possibly be associated with pits after incubation at 37°C. Interestingly, 23 of the 70 observed coated pits were located directly between two grains (see Fig. 1). Thus, coated pits appear to form between, but are not associated with, receptor clusters.

Grains which were inside of cells appeared either randomly in the cytoplasm or associated with large open vacuoles (Fig. 9). Even after 4°C binding, as cells were warmed at 37°C, we did not observe grains associated with coated vesicles. We also did not observe any vesicles with large numbers of grains which
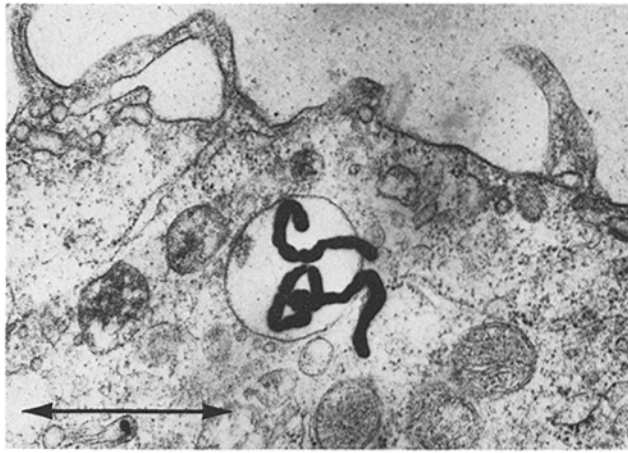
FIGURE 9 Association of autoradiographic grains with intracellular vesicles. EM autoradiograph of cell incubated with ^{125}I-thrombin (125 ng/ml) for 35 min at 37°C. Scale Bar, 1 μm. × 28,000.

would indicate collection of receptor clusters into single vesicles. The average number of grains per location within the cytoplasm was 1.45 which corresponds to the number of grains observed per receptor cluster on the cell surface. Thus any receptor-mediated internalization of thrombin would appear to involve a direct invagination of membrane with receptor clusters without further redistribution or involvement of coated pits.

DISCUSSION

A number of molecules including insulin, EGF, and α_2-macroglobulin appear to bind diffuse receptors which aggregate following ligand binding and subsequently move into coated pits (6). Other molecules such as LDL and asialoorosomucoid appear to have many of their receptors clustered in coated pits prior to ligand binding (3, 4). In both cases these molecules are internalized into coated vesicles (1, 2). Here we have shown that ^{125}I-thrombin binds to existing clusters of receptors on the surface of ME cells and that neither the stabilization nor the internalization of these clusters involves association with coated membrane regions.

It was recently reported that iodination of thrombin led to abnormally high binding affinities and binding to less than half of the available receptors (18). The present ^{125}I-thrombin preparations had approximately 1.1 iodines per thrombin molecule, yet the amount of ^{125}I-thrombin bound to ME cell surfaces after 37°C incubation with 125 ng/ml for 35 min at 37°C (5.8 ng/10^6 cells, trypsin sensitive) was approximately the same as the amount of noniodinated thrombin bound under similar conditions (18). Furthermore, the ^{125}I-thrombin binding presently observed indicates approximately 200,000 receptors per cell which corresponds to our original estimate for receptor number on ME cells (12). Thus, our iodination of thrombin does not appear to affect its receptor interaction.

After binding of ^{125}I-thrombin at 37°C, EMAR grains were observed in an evenly spaced pattern on the surface of ME cells. This even spacing of grains (~1.6 μm apart) indicates the interaction of ^{125}I-thrombin with large clusters of thrombin receptors. Using the average distance between grains and variability of spacing, we were able to predict the numerical density of these clusters and, from this density, calculate the average size of each receptor cluster. These stereometric calculations indicate that each receptor cluster has a diameter of ~400 nm. Knowing the size of the clusters and number of EMAR grains observed per cluster, we calculate that each receptor cluster contains at least 550 thrombin-binding sites. Assuming closet spherical packing, we calculate the receptor-receptor spacing to be 16.7 nm. This may suggest that these receptor clusters also contain a number of molecules in addition to thrombin receptors.

The binding of thrombin to clustered receptors on ME cells has also been observed with immunofluorescence microscopy (14). Consistent with our EMAR results, the average spacing between immunofluorescent clusters is ~1.5 μm and the average diameter of the receptor clusters was 0.54 ± 0.18 μm. The immunofluorescence photographs showed variation in number of receptor clusters per ME cell, ranging from 20 to perhaps 800 or more (14). Assuming an average of ~2 × 10^5 receptors per cell (12), these studies also indicate that each receptor cluster has 500 or more thrombin receptors.

Most of the thrombin receptors appear to be clustered prior to thrombin binding. The average space between EMAR grains and number of grains per cluster observed after 37°C incubation were not significantly different from those observed after incubating thrombin with cells at 4°C or incubating thrombin with cells prefixed in 3% formaldehyde. If even part of the unoccupied receptors were diffuse and aggregated after thrombin binding, we would have expected a change in either the cluster spacing or number of grains per cluster.

Unoccupied receptors for LDL and asialoorosomucoid which are clustered prior to ligand binding are associated with coated pits (3, 4). In addition, 34% of EMAR grains were associated with coated pits after ^{125}I-EGF binding to human fibroblasts at 4°C (9). In contrast, after ^{125}I-thrombin binding a total of 579 EMAR grains was observed. Of these, only two were within 0.2 μm of coated membrane regions. Thus thrombin receptor clusters do not appear to associate with coated pits. These receptor clusters are therefore quite different from those of molecules such as LDL or EGF.

Recent reports (19, 20) indicate that internalization and degradation of ^{125}I-thrombin is mediated by protease nexin (PN). In our experiments, medium containing PN was removed prior to incubation with ^{125}I-thrombin to avoid PN-thrombin complex formation and binding to the PN receptor. Nevertheless, some EMAR grains were observed within the cytoplasm, suggesting internalization of ^{125}I-thrombin. None of these grains were associated with coated vesicles. There also was no evidence of accumulation of ^{125}I-thrombin into single vesicles as observed with ^{125}I-EGF or ^{125}I-LDL (9, 21). Thus any specific receptor-mediated internalization of ^{125}I-thrombin may involve a direct membrane invagination rather than coalescence into coated pits.

Previous studies have shown that ^{125}I-thrombin is internalized by chick embryo cells and remains intact and active for up to 20 h (22, 23). Most molecules associated with coated pit internalization are rapidly degraded. Thus our demonstration that thrombin receptors do not associate with coated membrane regions may explain how thrombin and perhaps other biologically active receptor-bound molecules avoid rapid intracellular degradation.

It has been suggested that receptor clustering may be involved in coupling hormone receptors to signal-generating molecules (24). Such local aggregation has also been suggested in the activity of EGF (25). Since thrombin receptor occupancy correlates with initiation of cell division on ME cells (12), the

clustering of thrombin receptors on these cells may be involved in their mitogenic responsiveness. Other clustered receptors may also be mitogenically active. For example, ferritin-labeled EGF which bound to clustered receptors on A431 cells was only able to compete for binding 10% as well as native EGF, yet this preparation initiated cell division equally as well as native EGF (8). In the case, ferritin EGF may have preferentially bound to mitogenically active clustered receptors. Whether or not clustering plays a causal role in generation of mitogenic signals remains to be determined.

If thrombin receptors are not held together in coated membrane regions by clathrin, one must envisage another type of receptor interaction which restricts their diffusion. We recently demonstrated that microtubule stabilization by taxol can inhibit the initiation of DNA synthesis by thrombin (26). It has also been shown that thrombin treatment can cause microtubule depolymerization in platelets (27). Thus it is possible that some form of cytoskeletal interaction is involved in both the anchorage of these receptor clusters and the generation of the signal to initiate cell proliferation. Studies are currently underway to pursue both of these possibilities.

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