Journal of Supramolecular Structure 4:45 (45)–49 (49) 1976

NONRANDOM DISTRIBUTION OF RECEPTORS FOR MELANOCYTE-STIMULATING HORMONE ON THE SURFACE OF MOUSE MELANOMA CELLS

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An improved bubble method was developed for applying an ultrathin layer of nuclear track emulsion on the surface of cells labeled with I^{125}-MSH. The autoradiographs of I^{125}-MSH binding indicate a nonrandom distribution of receptors on the surface of mouse melanoma cells. It is suggested that MSH receptors are displayed in clusters previous to and independently of their exposure to the hormone.

INTRODUCTION

I^{125}-labeled melanocyte stimulating hormone (MSH) binds to specific receptors on the surface of cultured mouse melanoma cells in the G2 phase of the cell cycle (1). In a previous study we used an autoradiograph replica technique, which allowed us to distinguish between labeled and nonlabeled cells, but the resolution was too low for us to obtain information about the distribution of I^{125}-MSH binding cell surface receptors. In this paper we describe an improved method for light microscope autoradiography which is suitable for investigating the topography of cell surface receptors. We used a bubble of nuclear track emulsion for applying emulsion to the cell surface in an ultrathin layer so that we could obtain high resolution with I^{125}-labeled ligands.

METHODS

Culture Conditions

Cloudman mouse melanoma cells (NCTC 3960 CC153) were cultured in 30-ml Falcon tissue culture flasks at 37°C in an 5% CO_2-95% air atmosphere. Ham's F10 nutrient mixture, supplemented with 10% horse serum, 2% fetal calf serum, 1.2 mg/ml $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin, was inoculated with 10^5 cells. Cultures of 44 hr were used for the experiments reported here.

Binding of I^{125}-MSH to Melanoma Cells

We used a procedure similar to that we described previously (1). The culture medium was poured off, and the cells attached to the surface of the bottom of the flasks

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were rinsed with Hank's balanced salt solution (HBSS), pH 7.4, at 37°C. They were drained for 1 min and 50,000 cpm of I^{125}-MSH in 10 μl HBSS were pipetted onto a marked area. Following incubation at 0°C for 30 min, the unbound labeled MSH was removed by rinsing the flasks three times with 30 ml PBS. The cells were fixed with 2% (0°C) paraformaldehyde, rinsed in distilled water, and air dried. A microscope slide size portion of the bottom of the flasks was cut out with a red-hot razor blade and photographic emulsion was applied.

Autoradiography

Kodak OC filter safelight was used in the darkroom. To 1 ml liquefied Ilford L4 nuclear track emulsion (45°C), 1 ml solution containing 10% polyethylene glycol-20,000 (Fisher Scientific) and 2% Lubrol PX in water (45°C) were added and the mixture was kept at 45°C. 10 μl emulsion were withdrawn with a micropipette fitted with a mouthpiece. A bubble about 1.5 inches in diameter was formed on a flat, clean, cellulose acetate surface. In about 20 sec, silver/gold interference colors on the bubble indicated the formation of a monobilayer thickness as determined in preliminary experiments in daylight. The cutout plate of the flask bottom, cell-side down, was lowered horizontally onto the bubble 20 sec after it had been formed. In this way a thin layer of emulsion about 1 inch in diameter was brought up on the surface of the cell layer. Coated cells were kept in a light-tight box for 2–7 days. The gold-EAS method (2) was used to develop the slides.

Staining and Microscopy

Cells were stained for 2 min with a mixture of 0.01% methylene blue and 0.01% azure A in 1% sodium borate solution containing 1% glutaraldehyde. Microphotography was carried out with a Zeiss photomicroscope.

RESULTS AND DISCUSSION

We developed a technique in which light microscopy and autoradiography are used to visualize the binding of I^{125}-labeled ligands to cell surface receptors. The main point of the method involves the application of nuclear track emulsion as an upper segment of a bubble hemisphere. Shortly after the formation of the bubble, the thickness of the upper segment is reduced and forms an ultrathin layer of emulsion. In order to increase the mechanical stability of the bubble, we supplement the emulsion with polyethylene glycol and Lubrol PX. Bubble methods had already been used for the application of nuclear track emulsion (3). However, usually the bottom segment of a bubble sphere was applied an consisted of multiple layers of emulsion which quickly thickened after a bubble was formed.

The distribution of silver grains, related to cell-bound I^{125}-MSH, was patchy on cells from 2-day-old nonsynchronized cultures (Figs. 1–3). None had a uniform distribution of grains. However, the degree of clustering of grains showed a great difference (c.f. Figs. 1–3). In order to determine whether or not the clustering of MSH receptors was an artifact, we studied the effect of fixation and temperature on the pattern of distribution of grains. The main points of our findings were: (1) Prefixation with acetone produced a random distribution. However, mouse 3T3 cells and Hela cells showed similar labeling patterns

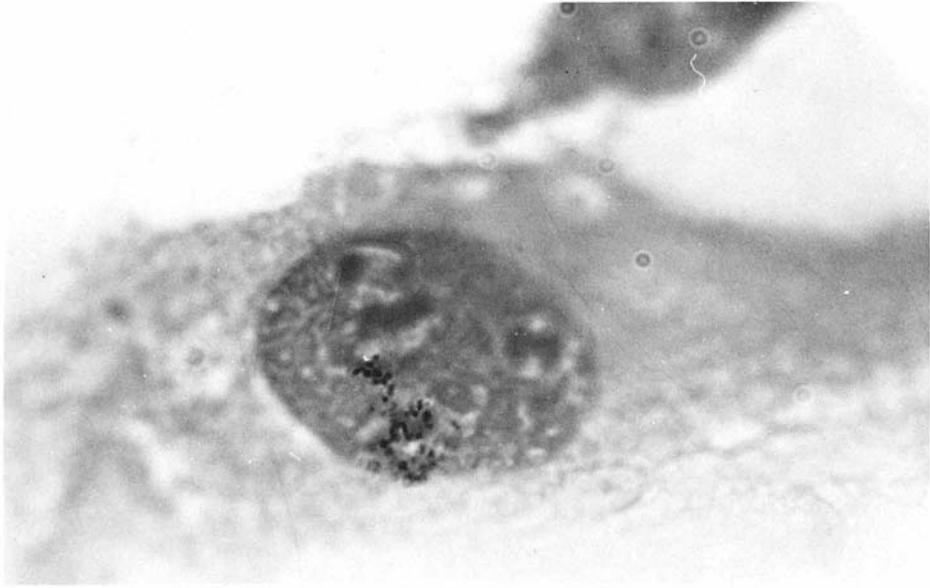

Fig. 1. A single large cell is shown with clustered grain distribution. The dark area at the top of the figure is part of a small cell, which is unlabeled.

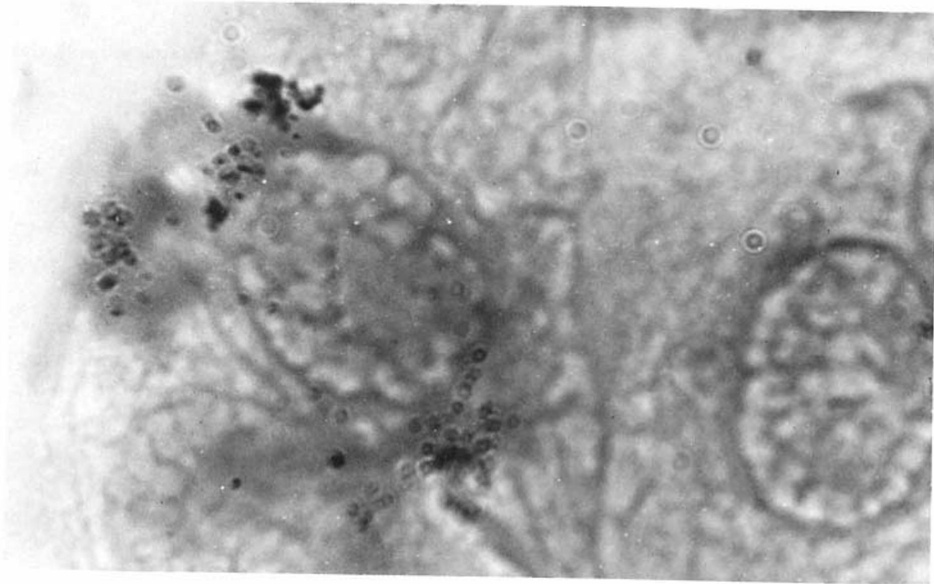

Fig. 2. The picture shows part of a colony made up of seven cells. One peripheral cell is labeled at the two poles of its nucleus. The rest of the cells in the colony are unlabeled.

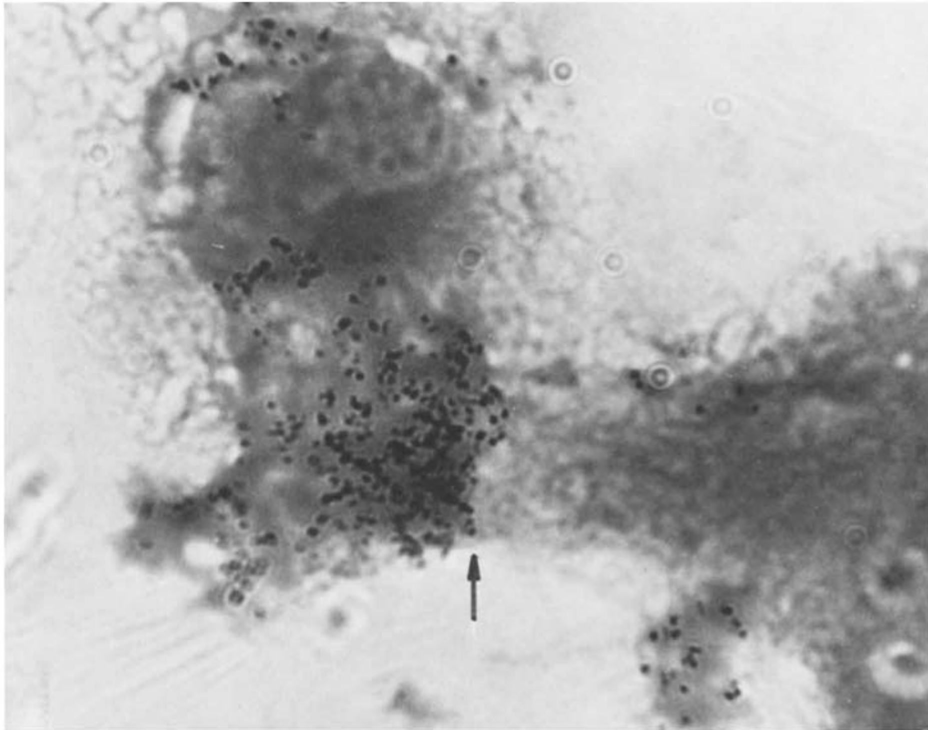

Fig. 3. A large labeled cell is shown with part of another cell carrying a surface structure with grains on it. Arrow indicates the location of interface between the two cells.

with I^{125}-MSH and showed that acetone treatment produced nonspecific labeling by destroying membrane permeability. (2) Prefixation with either paraformaldehyde or glutaraldehyde did not change the patterns of distribution of receptors. It did, however, reduce the degree of labeling and showed that part of the binding activity was destroyed by prefixation. For this reason, we used postfixation after labeling the cells at 0°C. (3) When labeling was carried out at 37°C, a more spread out pattern of receptors appeared and was similar to the pattern in Fig. 3. The same tendency was observed when labeled cells were incubated at 37°C for 30 min before fixation. These results suggest that a dissociation of labeled receptors was induced by exposing the cells to higher temperature (Varga, Fritsch, and Lerner, unpublished observations).

On a significant portion – about 10% – of the labeled cells, polar or bipolar distribution of grains (Fig. 2) was found. These observations are reminiscent of the "capping" phenomenon found with lymphocytes (4) and fibroblasts after those cells were exposed to bifunctional reagents (5). We assume that the clustering of MSH receptors is not a consequence of cross-linking of the receptors by the hormone. The essential pentapeptide segment of MSH which is responsible for its biological activity (6) and binding is located

in the middle portion of the octadecapeptide. This segment is probably too short to cross-link the receptors on the cell surface.

Frequently, grains accumulated at cell-to-cell interfaces with only one of the two cells bearing grains at junctions (Fig. 3). It is conceivable that this phenomenon may be a manifestation of cell-to-cell complementarity as part of intercellular recognition. It is possible that MSH receptors may be complementary to cell surface structures, in addition to the hormone. If this is so, one would expect MSH to displace MSH receptor-related cell-to-cell contacts and to facilitate cell detachment. Experiments are under way to investigate this point. Recent observations support this assumption. We found that besides MSH, increasing frequency of cell-to-cell contacts mimic the action of hormone, namely that of induction of tyrosinase and darkening of cells (7).

On the basis of these experiments we suggest that MSH receptors are displayed in clusters before the cells are exposed to the hormone. It is possible that the area on the surface of the cell carrying the hormone receptors is related to some intracellular organelle. A preliminary investigation made with light microscopy on single clusters of MSH receptors and enzyme stains, specific for the Golgi complex, showed that the two markers coincide (Varga, Moellmann, Fritsch, Godawska, Lerner, in preparation), suggesting that the action of MSH may proceed through channels which include the Golgi apparatus. This finding suggests that instructions by the hormone are carried out intracellularly in a compartmentalized manner.

ACKNOWLEDGMENTS

This research was supported by grants from United States Public Health Service AI-08614 and 5 RO1 CA 04679-15, by National Science Foundation grant GB 4382X, and by American Cancer Society grant BC-3F.

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