Direct visualization of binding, aggregation and internalization of human growth hormone in cultured human lymphocytes

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Abstract. The distribution of human growth hormone (hGH) receptor complexes on IM-9 cultured lymphocytes was studied using fluorescein and $^{125}$I-labelled hGH (F-hGH and $^{125}$I-hGH). The cells labelled with F-hGH were visualized with a sensitive video intensification microscopic system which permitted direct observation of the location of the fluorescent hormone on the surface of the living lymphocytes. At $4^\circ$C F-hGH bound diffusely to the cell surface and remained dispersed but following incubation for 30 min at $37^\circ$C the hormone receptor complexes aggregated into patches on the cell surface and formed a single cap on one pole of the cell. Progressive internalization into the cell was demonstrated at $37^\circ$C with $^{125}$I-hGH. It is hypothesized that the aggregation and internalization of the hGH receptor complexes are associated with the action and degradation of the hormone and probably also with the mechanism of down-regulation of the receptors.

Binding of polypeptide hormones to a specific site on the plasma membrane of the target cell is the first in a chain of events culminating in the biological action exerted by each particular hormone (Pastan et al. 1966; Kahn 1976). A number of workers (Carpentier et al. 1978; Goldfine et al. 1978; Gorden et al. 1978; Schlessinger et al. 1978; Amsterdam et al. 1979; Bergeron et al. 1979) has demonstrated that a variety of hormones is internalized into the cell. Although the physiological role of this sequence of events is not completely understood internalization appears to be necessary for the biological action of certain ligands (Anderson et al. 1976). Whether this process also occurs with human growth hormone (hGH) remained to be determined.

Previous studies have shown that hGH binds to fresh (Eshet et al. 1975; Stewart et al. 1983) and cultured human lymphocytes (Lesniak et al. 1974). Hereby presented are the findings of a study carried out using fluorescent-labelled hGH (F-hGH) and $^{125}$I-labelled hGH with the purpose of determining the site of localization of hGH on the cell and to follow the process of internalization taking place.

Material and Methods

Cells and reagents

Human cultured lymphocytes of the IM-9 cell line were used in all the experiments (Fahey et al. 1971; Lesniak et al. 1974; Carpentier et al. 1978). The cells were grown at $37^\circ$C in RPMI 1640 medium containing 10% foetal calf serum, 2 mM glutamine and combined antibiotics (0.6%). Twice a week the culture was divided by 1:4 and each time fresh medium added.

Preparation of $^{125}$I-hGH

Purified human growth hormone (Kabi Diagnostica, batch No. 840901-65824-51) was iodinated at the specific activity of 100 µCi/mg by a modification of the chloramine-T method (Lesniak & Roth 1976). The labelled hGH was purified by filtration on G-75 Sephadex (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) before each experiment.
Preparation of F-hGH

To 50 µl hGH (Kabi Diagnostica – 20 µg in 0.05 M borate buffer, pH 9.3), there was added 50 µl of fluorescein isothiocyanate (FITC – 2 µg in the same buffer) over a period of 4 h at room temperature. After the addition of 100 µl glycine (375 µg) the solution was dialysed against 4 l of PBS, pH 7.0, for 24 h at 4°C. To determine its binding affinity the F-hGH was tested in the following radioreceptor assay with human liver microsomes.

Binding affinity test of F-hGH to human liver receptors

Liver tissue was obtained immediately after pronouncement of clinical death by traumatic causes from donors of kidney for transplantation. Following removal the tissue was kept in a 0.25 M sucrose solution at −20°C until used in the radioreceptor assay. Receptor preparation was carried out as described by Tsushima & Friesen (1973). One hundred µl of [125I]hGH was incubated with the liver microsomal pellet fraction in 25 mM Tris-HCl-10 mM MgCl₂ buffer, pH 7.4, containing 0.1% bovine serum albumin in a final volume of 0.5 ml, for 48 h at 4°C with constant shaking. Incubation was terminated by adding 2 ml ice-cold 0.1% bovine serum albumin Tris/magnesium buffer. Receptor-bound and free radioactivity were separated by centrifugation at 2000 × g for 30 min at 4°C. Radioactivity was measured in a Packard model 5260 Autogamma scintillation spectrometer. Parallel incubations were made in the presence of excess (5 µg/ml) unlabelled hormone (hGH or F-hGH): specific binding is the difference between the radioactivity bound in the absence (TB – total binding) and the presence (NSB non-specific binding) of excess unlabelled hormone and is expressed as per cent of the total radioactivity in the incubation.

Incubation conditions

Cells were removed from the growth medium by centrifugation at 600 × g for 5 min at 24°C and were then washed twice by resuspension in the assay buffer (50 mM sodium hepes, 120 mM NaCl, 12 mM magnesium sulphate, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 10 mg/ml bovine serum albumin, pH 7.6). For each experiment approximately 15–25 × 10⁶ cells/0.5 ml were incubated in the assay buffer in 17/100 plastic tubes. For experiments with fluorescent hGH, 50 ng/ml of F-hGH was added, and incubation was carried out at 4°C for 90 min or at 37°C for 30 min. In some of the tubes incubated at 4°C for 90 min incubation was then continued at 37°C for another 30 min. To stop the reaction 2 ml of assay buffer were added and the tubes centrifuged for 10 min at 600 × g. For fluorescent visualization the cells were resuspended in 0.5 PBS + 0.1% BS and observed directly.

For experiments with iodinated hGH, [125I]hGH was added at varying concentrations, in amounts of 1, 5, 10 or 20 ng/ml, and incubated with 15 × 10⁶ lymphocytes in assay buffer at 37°C for 30 min. After stopping the reaction as above, the cells were separated from the medium by centrifugation and the radioactivity in the cell pellet determined by gamma counting. Identical incubation was carried out with the addition of unlabelled hGH at a concentration of a 1000-fold that of the labelled hGH to determine non-specific binding.

Since the round structure of lymphocytes does not permit direct visualization of intracellular localization with F-hGH and since it has been found that following treatment of the lymphocytes with acetic acid the non-accessible radioactivity bound to the lymphocytes constitutes a reliable estimate of the internalized hormone (Yarden et al. 1981) another series of lymphocytes was treated by this method. The cells were incubated as above at 37°C to achieve hormone binding, the incubation being stopped at various intervals up to 180 min, following which the cells were treated with 0.5 M acetic acid, pH 2.7, for 4 min at 4°C, and the radioactivity counted in the pellet after centrifugation.

Image-intensified video fluorescence microscopy

The location of the fluorescent analogue was observed with a silicon intensified target (SIT) camera (RCS TC/1030 H) (Willingham & Pastan 1978), which detects very low levels of light and permits the use of low excitation intensities so that fluorescent molecules can be visualized. The phase and fluorescence micrographs were taken with a polaroid camera from a television screen projecting the intensified image.

Results

F-hGH was found to compete with [125I]hGH for its specific receptor to the same extent as unlabelled hGH in the radioreceptor assay using human liver microsomes (Eshet et al. 1984) (Table 1).

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<th>% Total binding</th>
<th>% Non-specific binding</th>
<th>% Specific binding</th>
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<tbody>
<tr>
<td>Untreated hGH</td>
<td>29.7</td>
<td>9.0</td>
<td>20.7</td>
</tr>
<tr>
<td>F-hGH</td>
<td>29.7</td>
<td>10.2</td>
<td>19.5</td>
</tr>
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</table>

Untreated hGH or F-hGH (5 µg/ml) were used to compete with [125I]hGH. The values were calculated as percentage of binding from the total counts in the assay tubes.
hGH was demonstrated to localize predominantly to the plasma membrane of the cultured human lymphocytes. Study of the binding with F-hGH when incubated at different temperatures showed that with incubation of 90 min at 4°C the fluorescent hormone appeared diffusely distributed on the cell surface (Fig. 1); when further incubated at 37°C for another 30 min the labelled hormone-receptor complexes were seen to cluster, with the formation of a single cap on one pole of the cell (Fig. 2). Similar caps were observed when the lymphocytes were incubated with F-hGH for 30 min at 37°C, without preincubation at 4°C (Fig. 3).

The binding of [125I]hGH at various concentrations to cultured human lymphocytes showed that binding was proportional to the concentration of [125I]hGH in the incubation mixture until saturation of the receptors was achieved (Fig. 4). Binding
of $[^{125}I]hGH$ to cultured lymphocytes was of less intensity compared to human liver receptors and depicted in Table 1. As seen in Fig. 5, which shows the time course of association of $[^{125}I]hGH$ incubated at 37°C with the lymphocytes and the amount of radioactivity non-accessible following treatment with acetic acid which gives an estimate of the internalized hormone, there was continuous internalization of the hormone receptor complexes. At 105–120 min of incubation binding reached an apparent steady-state which was maintained up to the 180 min tested.

**Discussion**

The data obtained in this study further delineate the sequence of events of binding and internalization of hGH in cultured human lymphocytes. The specificity of binding of $[^{125}I]hGH$ and F-hGH was proven and the two analogues competed with each other for its specific receptor in human liver microsomes. The binding of hGH to the IM-9 cultured human lymphocytes was similar to that found with human circulating lymphocytes in previous investigations (Eshet et al. 1975; Stewart et al. 1983) although the ligand receptor binding was of lesser intensity than to human liver microsomes (Fig. 4 and Table 1).

Barazzone et al. (1980), who used autoradiography and a different set of incubation conditions to
study binding of $[^{125}\text{I} ]$hCG on IM-9 human lymphocytes, found that at $15^\circ$C there was localization whereas after 2 min of incubation at $30^\circ$ and $37^\circ$C there began a progressive internalization of the labelled ligand, with binding reaching an apparent steady state by $30-60$ min and maintained up to at least $180$ min. Upon direct visualization of the fluorescent hGH on the plasma membrane, the

Fig. 5.
Time course of association of $[^{125}\text{I} ]$hGH at $37^\circ$C with IM-9 cultured human lymphocytes (A) and the amount of radioactivity non-accessible to acetic acid at this temperature (B).
first such study to be carried out with growth hormone, we found that at 4°C hGH bound to the diffusely distributed receptors with the receptor complexes remaining in this condition for at least 90 min, whereas raising of the temperature to 37°C led to aggregation of the receptor complexes to form patches on the cell membrane. This was similar to the findings reported by Schlessinger et al. (1978, 1980) for insulin. In our study we further found that with binding of \(^{125}\text{I}\)hGH at 37°C there was continuous internalization of the receptor complexes. These findings are compatible with the theory formulated by a number of authors that most if not all polypeptide hormones and growth factors bind to specific sites on the cell surface and that subsequently the labelled hormones are specifically translocated into the cells, presumably by receptor-mediated endocytosis (Terris & Steiner 1976; Carpenter & Cohen 1976; Goldfine et al. 1977; Schlessinger et al. 1978).

The biological role of hGH receptor clustering and internalization is not known. For other hormone-receptor complexes it has been shown that hormone-induced aggregation of specific membrane receptors constitute an important step in the initiation of its biological signal (Anderson et al. 1976). It is possible that similar pathways regulate the action and fate of the hGH receptor complex. It is hoped that this investigation will further our understanding of the action of hGH and its receptor regulation.

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References


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