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Application of flow cytometry to study receptor binding potency of FITC and/or biotin conjugated human growth hormone in comparison with radio receptor assay

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ABSTRACT

Radio receptor assay (RRA) is the most sensitive and useful method for studying bioactivity of therapeutic proteins. Some of the disadvantages and limitations of the RRA are the biosafety and biohazardous problems. The aim of this paper was to investigate and compare receptor binding potential of non-radioactive conjugated human growth hormone (hGH) by flow cytometry versus to ^{125}I hGH in binding to hGH receptors. hGH was separately labeled with ^{125}I and/or non-radioactive ligands (FITC and/or biotin). The efficiency of labeling was calculated. Thereafter, the receptor binding potential and fraction of the radio labeled and non-radioactive ligand conjugated hGH were assayed by IM-9 human lymphocyte cell lines and pregnant rabbit liver hepatocytes microsomes. Receptor assay was done in a competitive manner and in immunoneutralizing conditions. The fluorescence was observed by fluorescent microscope and quantified by flow cytometry. Labeling of hGH by biotin in theoric 5 molar ratio (5:1; Biotin: hGH) showed good efficiency of labeling and quantitative data of receptor assays comparable with radio receptor assay with hGH-^{125}I. In a competitive manner, the profile of binding to these receptors indicated change in the affinity of labeled hGH and the affinity of FITC conjugated hGH decreased in comparison with biotin conjugated and ^{125}I labeled hGH. In a competitive manner, the profile of binding to these receptors indicated the affinity of labeled hGH was slightly changed. In general, this flow cytometry based receptor assay is simple to perform and comparable with RRA and provides a safer and reliable alternative to radioactive methods.

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Key words: Biological activity, biotinylation, human growth hormone, FITC conjugation, flow cytometry, radio- receptor Assay.

INTRODUCTION

Biological activity of the therapeutic proteins is important from the aspect of applied genetic engineering and is critical in understanding the accurate expression, fermentation, purification, formulation and stability of these proteins during production system (Bristow et al., 2012; Baneyx and Mujacic, 2004). Interaction between hormones to its receptor on the cell membrane of target cell is assumed to be important in the mechanism of action and bioactivity of recombinant hormones such as human growth hormone (hGH) (Nindl et al., 2008; Pagani et al., 2007).

Thermal, chemical and mechanical stress conditions usually apply to therapeutic recombinant proteins such as hGH during the production, which affect hormone stability, structure and aggregation (Pikal et al., 2008). It is suspected that stresses such as shaking caused high adsorption to the gas-liquid interface, which may facilitate protein denaturation (Velega, 2005). Because of different stability of proteins to specific stress conditions, that is necessary to use, an array of complementary analytical methods especially biological function assays such as receptor binding might be required to evaluate the protein stability and function (Lim et al., 2015; Ranke, 2008).

One of the most used and more sensitive methods usually

used for this purpose in the hormone-receptor binding potency is Radio Receptor Assay (RRA). Radioactive labeling of proteins and other biological polypeptide hormones is commonly achieved by iodination. This method relies on the generation upon oxidation of the $^{125}I^-$ ion of the electrophilic forms of iodine (I^+) which can react with aromatic rings that become labeled radioactive (Gregory and Richard, 1997). Therefore, in using RRA to evaluate the biological activity of polypeptide hormones some concepts such as high oxidizing effect of ^{125}I on protein biosafety and biohazardous aspects and finally short life time of ^{125}I must be considered (Hughes et al., 1982; Mulinacci et al., 2013).

For human Growth Hormone (hGH) studying with ^{125}I labeled hGH demonstrated the existence of specific binding sites for the hormone in cultured human lymphocyte cell lines IM-9 (Lesniak et al., 1974; Smal et al., 1985), rat Nb-2 cells (Ashkenazi et al., 1987) and in microsomal membranes prepared from pregnant rabbit and rat liver hepatocytes (Deezagi et al., 2011; Herington et al., 1976) and pre-adipocytes of epididymal of rats (Robert et al., 1990). In these cells, hGH initiates its actions by binding to specific cell surface receptors belonging to the cytokines/erythropoietin receptor super family (Leung et al., 1987; Cunningham and Wells, 1989).

Understanding the receptor binding properties and profiles of rhGH as the most therapeutic recombinant protein is an important problem in genetic engineering and biotechnology. Radio Receptor Assay (RRA) is the most common method usually used for detection and measurement receptor binding capacity of hGH in research and applied fields.

The aim of this study was to introduce flow cytometry and develop protocols for monitoring the receptor binding potency of hGH in bioprocesses. Flow cytometry have recently been used for the understanding of the bioactivity and receptor binding of Glial cell-line derived neurotrophic factor (GDNF) (Quintino et al., 2013), nanoparticles (Nyland, 2012), for the evaluation of antibody-dependent cell-mediated cytotoxicity (ADCC) in cancer cells (Salinas-Jazmín et al., 2014) and for evaluating antiviral potency of compound in dengue infected peripheral blood mononuclear cells (Fu et al., 2014). With flow cytometry, it is possible to count the number of fluorescence ligand labeled cells in a sample. The results could be evaluated against total cells and the hypothesis is that flow cytometry as a non-radioactive and safe method will provide a faster (at-line) method which also can analyze receptor binding potency as a marker of the bioactivity of hGH. For this purpose, we investigated the receptor binding potential of non-radioactive labeled hGH in comparison to RRA. Potency of conjugated hGH by FITC and/or Biotin was studied using flow cytometry. Potency of radioactive and non-radioactive labeled hGH were assayed by human lymphocyte IM-9 cell lines and pregnant rabbit hepatocytes microsomes. The potency of labeled hGH in binding to hGH receptors of IM-9 cell lines and hepatocyte microsomes were also studied.

MATERIALS AND METHODS

$Na^{125}I$ (Amersham, UK), Iodobeads (N-Chloro Benzene Sulfonamide) from Pierce Company (Pierce, Canada), Chloramine T (sigma, MA, USA), Sepharose Q-100 (Pharmacia, Sweden), FITC, Biotin, Biotin-NHS (N-Hydroxy succinamade) and Avidin (Calbiochem Inc. Germany), Fetal Calf Serum, RPMI-1640 and DMEM culture medium (Gibco BRL, UK), tissue culture flasks and other cell culture facilities (Nunc Inc, Denmark), human Growth Hormone (Novo, Switzerland and Sigma, MA, USA) hGH ELISA kit (Elegance, Canada), hGH RIA kit (Nicholas institute, Canada) and Biotin labeling kits (Roche, Germany) were used in this study. Other chemicals were provided from Merck (Merck, Dermasted, Germany).

Methods

Iodination of hGH

rhGH was iodinated with ^{125}I using Iodo beads and Chloramine- T separately. Iodination by Iodo beads was done according to the procedure manual of the manufacture (Pierce Company). In brief, two beads were added to a solution containing 10 µg of hGH and 1 mCi of $Na^{125}I$ in a 0.1 M phosphate buffer pH=6.5 for 10 min at 25ºC. The beads were removed and iodination stopped. ^{125}I-hGH fractions were separated using sepharose Q-100 column chromatography.

Chloramine-T was used for iodination by the method described by Gregory and Richard (1997). Briefly, 10 µg of rhGH and 1 mCi of $Na^{125}I$ and 2 mg/ml of Chloramines-T were mixed in the phosphate buffer (0.1M, pH=7.2) for 2 min at 25ºC. Iodination was stopped by adding Sodium Metabisulfite (2 mg/ml). ^{125}IhGH fraction was separated by Q-100 column chromatography. In both methods, 10 µl of each fraction from sepharose Q-100 column chromatography were diluted in phosphate buffer containing 5 mg/ml of Bovine Serum Albumin (BSA) and was precipitated with Tri-Chloro Acetic Acid (12%) on ice. After centrifugation the radioactivity of pellets were counted by γ-coulter counter (Wallack LKB, Sweden). The efficiency and specific activity of labeling were calculated using Equation 1 given as:

$$\text{Specific activity (µCi/µg)} = \frac{\text{CPM of labeled hormone}}{\text{Total CPM}} \times \frac{\text{Total } ^{125}\text{I used (µCi)}}{\text{µg of hGH used}}$$

FITC Conjugation of hGH

hGH was conjugated with Fluorescent Isothiocyanate

(FITC) according to the method described by Sakal et al. (1991). Briefly, hGH was mixed by FITC 1.8 to 36 µg of FITC/ 1.0 mg of hGH (molar ratio of FITC: hGH about 0.5 to 10) in the carbonate buffer (0.2 M pH=9.6) for 24 h at 4ºC. Labeled FITC-hGH was separated using sepharose Q-100 column chromatography. In a few tests, labeled FITC-hGH fraction was separated by HPLC (Beckman Gold HPLC, USA). The absorbance's of each fraction was separately measured at 495 and/or 280 nm, respectively. FITC/Protein (F/P) molar ratio of FITC conjugated hGH was calculated as highlighted in Equation 2:

$$\text{F/P molar ratio} = \frac{\text{FITC used (µg)}}{\text{MW of FITC}} \div \frac{\text{hGH used (mg)}}{\text{MW of hGH (mg)}}$$

MW of FITC=389 Da and hGH=22/000 Da.

Biotinylation of hGH

Two methods were used for biotinylation: the first was based on the method of Storm et al. (1996) and second on the procedure manual of biotinylation kit of Roche. Briefly, biotin was dissolved in DMSO and was mixed with hGH; 75 to 1250 µg of biotin/ one mg of hGH (in a molar ratio about 6.75 to 110) in Sodium Bicarbonate buffer (1.0 M, pH=8.5) at 4ºC for 4 h. Biotinylated hGH was separated by dialysis and column chromatography. Biotinylation efficiency and the amount of biotin molecules coupled to hGH were determined using colorimetric method as described by Green (1970). In this method, when biotin displaced the 4-Hydroxy Azobenzen-2´-Carboxilic Acid (HABA) dye from avidin it resulted in a change and decrease in optical density at 500 nm and this is the base of the calculation of biotinylation. The calibration curve was done by adding 2 µl increments of biotin in PBS (0.5 mM, pH 7.4) to 0.9 ml avidin-HABA mixture (0.4 mg/ml avidin, 0.3 mM HABA in PBS) as standard. Thereafter, biotinylated hGH samples were added in 5 µl increments to a 0.9 ml aliquot of the avidin-HABA solution. The average number of biotin molecules per hGH molecules (B/P Ratio) were calculated by dividing moles of biotin to moles of hGH (MW of Biotin=244 Da and hGH=22/000 Da). The biotinylation efficiency was calculated by dividing the B/P molar ratio of the labeled hGH determined in the HABA assay by the theoretical B/P molar ratio of the reaction mixture.

IM-9 cells receptor assay

Human lymphocyte cell line IM-9 (CCL159) (ATCC, USA) was cultured in RPMI-1640 containing 10% of fetal calf serum (FCS), 120 mg/L penicillin and 200 mg/L streptomycin. Cells were subcultured twice every week. On log phase of growth, RRA was done based on the method described by Jesse (1976) briefly, 24 h before the assay cells were cultured in culture medium containing HEPES buffer (100 mM, pH 7.0). On the day of assay, cells were washed twice with assay buffer (100 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 15 mM Sodium Acetate, 10 mM Glucose, 1 mM EDTA, 10 mg/L BSA and pH=7.0). Thereafter, 10^7 cells were incubated with constant concentration of labeled ^{125}I-hGH and increasing concentration of non-labeled hormone for 2 h at 22 to 25ºC. The cells were thereafter washed with cold assay buffer thrice. Finally, radioactivity of the pellets were counted using γ-coulter counter.

Preparation of rabbit liver microsomes and receptor assay

Rabbit (Newzland) hepatocyte microsomes were prepared as described previously by Deezagi et al. (2011). Rabbit liver was chopped and homogenized in 5 volume of sucrose (0.3 M) for 1 min. The homogenate was filtrated through cheese cloth and centrifuged at 1500 g for 20 min. The supernatant was centrifuged at 15000 g for 20 min again and finally the supernatant of the second stage centrifuged at 100,000 g for 90 min by Ultracentrifuge 90 (Beckman, USA). The pellets (microsomes) were resuspended in Tris-HCl buffer (Tris-HCl 25 mM, $CaCl_2$ 10mM and pH 7.6). In all of steps the total protein content was assayed using Bradford method. Microsome samples were dispensed in reaction assay tubes (2 mg/ml) and freeze dried (Chrest Epson, Germany) and stored at –20°C until receptor assay. RRA of hGH by rabbit microsomes was done considering the aforementioned condition earlier described for IM-9 cells. In brief, on the day of assay lyophilized microsomes were dissolved in HEPES receptor assay buffer. In a competition manner, 500 µl of rabbit hepatocyte microsoms (2 mg/ml) was incubated with constant concentration of radio- labeled hGH (5 to 50 ng/ml) and increasing concentration of non-labeled hormone (0 to 10 µg/ml) for 2 h at 30ºC with shaking (100 rpm). The mixtures were then washed with cold assay buffer thrice (12000 rpm for 20 min). Finally, radioactivity of the pellets was counted by gamma counter. The total binding and specific binding were calculated as follows:

$$\%\ \text{of total binding} = \frac{\text{CPM of test}}{\text{Total count}} \times 100$$

$$\%\ \text{of specific binding} = \frac{\text{CPM of test} - \text{CPM of non-specific binding}}{\text{Total count}} \times 100$$

Receptor assay of biotinylated and/or FITC conjugated hGH by flow cytometery

IM-9 cells were subcultured in RPMI-1640 medium with HEPES (100 mM pH 7.0), 24 h before the assay. 10^5 cells

were washed thrice with assay buffer and incubated with different concentration of FITC-hGH and biotinylated hGH in assay buffer for 60 min at 4°C. Biotinylated samples were incubated with 100 µl of avidin-FITC solution (30 µg /ml) for 60 min at 4°C. Thereafer, the cells were washed thrice with PBS/BSA solution (100 mM/, 10 mg/ml pH 7.4). Subsequently, some parts of the cells were observed using fluorescent microscope (Ziese Axioviolet, Oberkochen, Germany). Quantitative analysis of the fluorescence intensity was assayed by fluorescent activated cell sorting (FACS) (Becton Dickinson, NJ. USA) as outlined in the procedure manual of the system.

In competitive assay, the IM-9 cells were incubated with constant concentration of FITC and/or biotinylated hGH and increasing concentration of non-conjugated hGH (up to 100 fold molar excess of non-conjugated hGH to labeled hGH). After incubation (as the same conditions earlier mentioned), the presence of fluorescence was analyzed.

Immunoneutralizing tests

For neutralizing tests, anti-hGH polyclonal antibodies were produced in rabbit in our laboratory. IgG fractions were purified by ammonium sulphate precipitation and protein-A affinity chromatography. In receptor assays (using radioactive and non-radioactive hGH), anti hGH IgGs (50 µg/ml) was incubated with hGH in assay buffer at 22°C for 30 min. Thereafter, the cells and/or microsomes were added to reaction tubes. The incubation conditions, binding analysis methods and calculations were same as explained in the receptor assay.

Measurement of hGH

In all of the experiments the used concentrations of hGH were measured using commercial ELISA (Elagance) and/or RIA kits (Nicholas Institute) according to the procedure manuals of the kits. The total protein concentrations were measured using Bradford method.

Statistical analysis

Each experiment was performed at least thrice for all data with each carried out in duplicated sequences. Data were analyzed using a One-Way Analysis of variance (ANOVA).Values were given as the mean ± 1 Standard Deviation (SD) and biological variables were compared using the students'T-test. By convention, α-level of $p<0.05$ was considered to be statistically significant. Finally, the correlations between samples were calculated statistically.

RESULTS

In the initial step of this work, it was necessary to understand the spectrum of GH receptor binding property to the used receptors. For this purpose, the reliability of iodination and the binding assay of hGH were optimized by the standard RRA. hGH was iodinated for more than 10 times with Iodo beads and chloramines T methods. The specific activity of iodinated hGH was calculated as earlier described using Equation 1. The efficiency of iodination and specific activity of iodinated hGH were different in each iodination process but in all of the RRA and other experiments, iodinated hGH with specific activity between the range of 40 to 80 µCi/µg were used. Binding of labeled hGH to human lymphocyte IM-9 cell lines and hepatocytes microsomes was time and temperature dependent. The temperatures 4, 22 and 30°C for 2 and 24 h incubation times were tested. Incubation of IM-9 cells with different cell density for 2 h at 30°C showed total binding up to 25% (Figure 1a). The binding of ^{125}I-hGH to cells was directly proportional to the cell numbers up to 10^7 cell/ml. Figure 1b show the total binding of ^{125}I-hGH to hGH receptors from rabbit hepatocytes microsomes. The total binding increased up to 30% for 2 h and 40% for 24 h incubation at 30°C.

In non-radioactive receptor assay, FITC and/or Biotin were conjugated to hGH with different theoric Ligand/Protein (L/P) molar ratios. Accurate L/P molar ratio of conjugated hGH was calculated as earlier mentioned. Tables 1 and 2 depict results of the calculated FITC/hGH (F/P) and Biotin/hGH (B/P) molar ratios and efficiency of the labeling. In conjugation of hGH by FITC with theoric F/P molar ratio from 0.5 to 10, the experimental and calculated results were 0.22 up to 1.4 and optimal conjugation was 1.4 with efficiency of conjugation of about 14% (Table 1). In biotinylation, Table 2 shows (B/P molar ratio from 6.75 up to 110 Biotin/hGH) ratio of the experimental and calculated results of B/P ratio 4.4 up to 46. Figure 2 shows the binding and histochemical localization of FITC and Biotin labeled hGH to IM-9 cells. The intensity of staining was dependent on the L/P molar ratio of labeling. In F/P molar ratio of 5 and B/P molar ratio about 20 of the cells showed high intensity and sharp fluorescence (Figure 2a). The intensity of staining varied between cells and was most intense on the cell membranes and/or fluorescent spots in some cells. Fluorescent staining of hepatocyte microsomes by labeled hGH showed clumping and aggregated spot fluorescence (Figure 2b). Incubation of these cells with constant concentration of labeled hGH and/or by increasing concentration of non-labeled hGH (50 fold) resulted in the elimination of fluorescence in comparison by control cells.

For quantitative analysis of fluorescence intensity, the cells were assayed by Fluorescent Activating Cell Sorting (FACS). Figure 3 shows the cytograms of FACS. Cytograms and quantitative results of FACS, such as means, geomeans and median of channels for relative fluorescent intensity were shown in Figure 3 and collected in Table 3.

Receptor binding of non-radioactive and radioactive labeled hGH was also studied. Figure 4 shows competitive

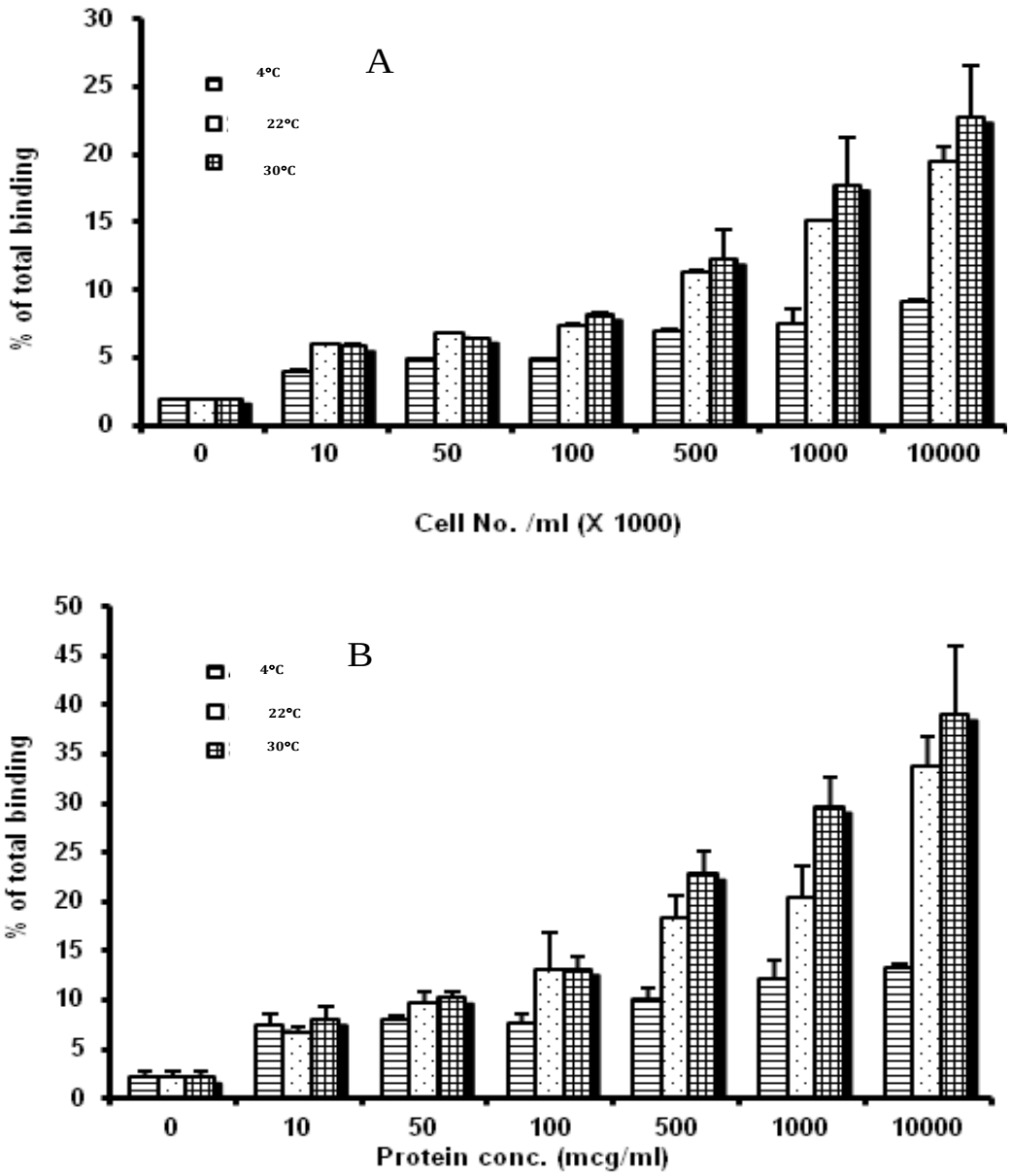


Figure 1: Analysis of the profile and optimum conditions of the binding of labeled hGH to human lymphocyte IM-9 cells and/or hepatocytes microsomes. The temperatures 4, 22 and 30°C for 2 and 24 h incubation times were tested as described in materials and methods. Incubation of increasing cells numbers up to 10^7 of IM-9 cells (total cell number was determined using a hemocytometer) at 4, 22 and 30°C for 2 and 24 h (a). The total binding of ^{125}I-hGH to hGH receptors from increasing concentration (up to 10 mg/ml) of rabbit hepatocytes microsomes at 4, 22 and 30°C for 2 and 24 h. The results are mean ± S.E.M. for three separate experiments.

Table 1: Theoretical and calculated experimental ligand/hGH molar ratio (FITC/hGH ratio). MW of FITC=389 Da and hGH=22/000 Da, MW of Biotin=244 Da and hGH=22/000 Da.

µg of FITC/ 1.0 mg of hGH	Theoric F/P ratio	Experimental F/P ratio	% of labeling efficiency
1.8	0.5	0.22	44
3.6	1.0	0.33	33
5.4	1.5	0.48	32
18.0	5.0	1.1	22
36.0	10.0	1.4	14

Table 2: Theoretical and calculated experimental ligand/hGH molar ratio. Biotin/hGH Ratio. MW of FITC=389 Da and hGH=22/000 Da, MW of Biotin=244 Da and hGH=22/000 Da.

µg of Biotin/ 1.0 mg of hGH	Theoric B/P ratio	Experimental F/P ratio	% of labeling efficiency
75	6.75	4.4	72
225	20.0	11.6	61
750	67.5	26.6	44
1250	110	46	43

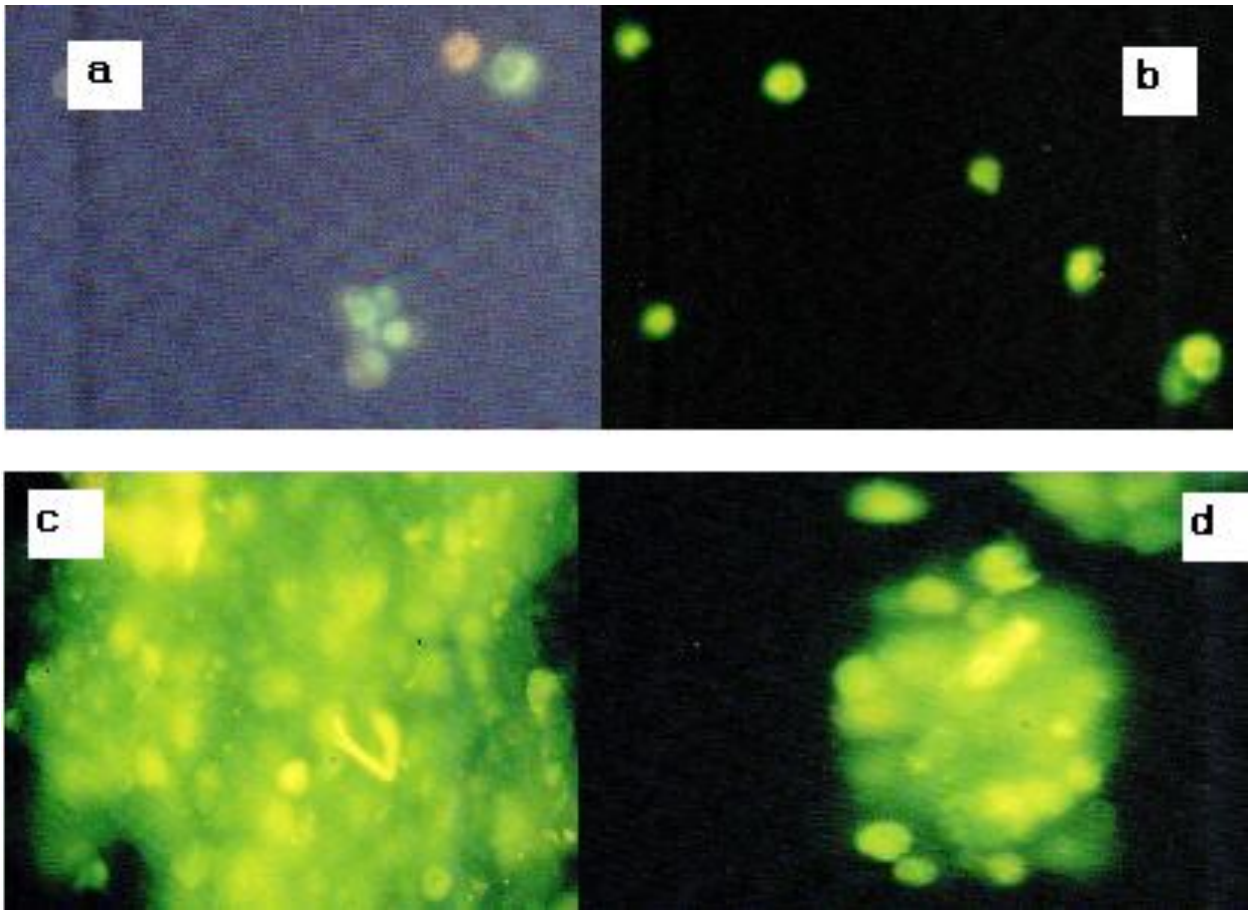
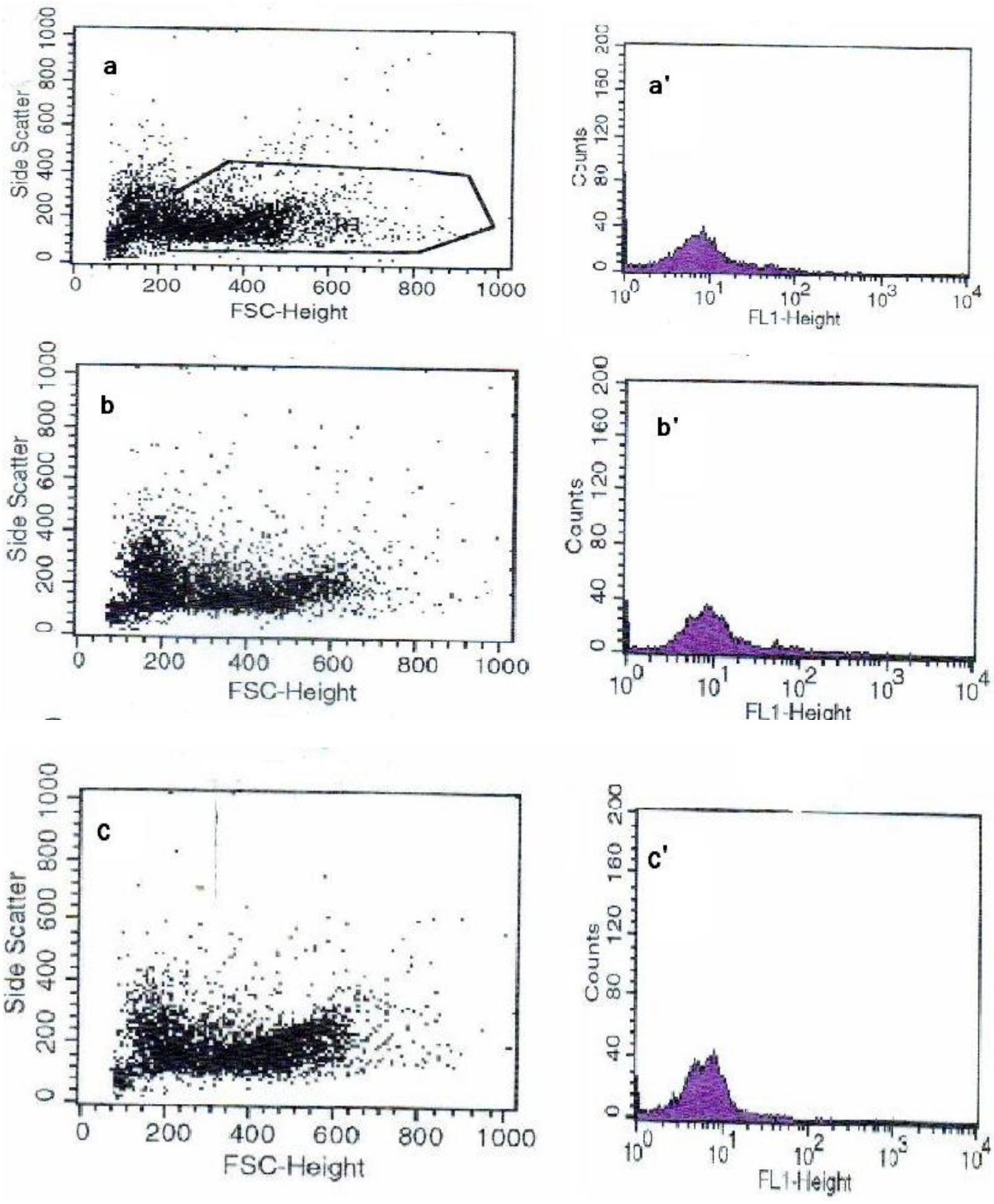


Figure 2: Direct fluorescence analysis of IM-9 cells and hepatocytes microsomes after interaction by FITC and/or Biotinylated hGH. IM-9 cells were subculture in RPMI-1640 medium with HEPES, 24 hrs before the assay. 10^5 cells were washed 3 times with assay buffer and were incubated with different concentration of FITC-hGH and biotinylated hGH in assay buffer for 60 min at 4°C. Biotinylated samples were incubated with 100 µL of avidin-FITC solution (30 µg /ml) for 60 min at 4°C. After that, the cells were washed 3 times with PBS/BSA solution. Subsequently some part of the cells was observed by fluorescent microscope (400 ×). IM-9 cells were interacted by Biotin-hGH (a) and by FITC-hGH (b). Rabbit Hepatocyte microsomes were interacted by Biotin-hGH (c) and by FITC-hGH(d).

binding of labeled hGH to IM-9 cells and/or rabbit hepatocyte microsomes. The total binding was about 10 to 15.5% with specific binding up to 5.5% for IM-9 cells and total binding between 16 to 25% with up to 9% specific binding for microsomes. As the results show, conjugation of FITC to hGH caused a shift in slightly binding to the right and biotinylation of hGh did not have a significant effect on the hGH receptor binding in comparison with ^{125}I-hGH in microsomes.

In immunoneutralizing tests of hGH with anti-hGH polyclonal antibodies, the fluorescence of IM-9 cells was eliminated in the presence of 50 µg/ml of Abs (data not shown). These results indicate specific binding and confirmed the specifity of this manner.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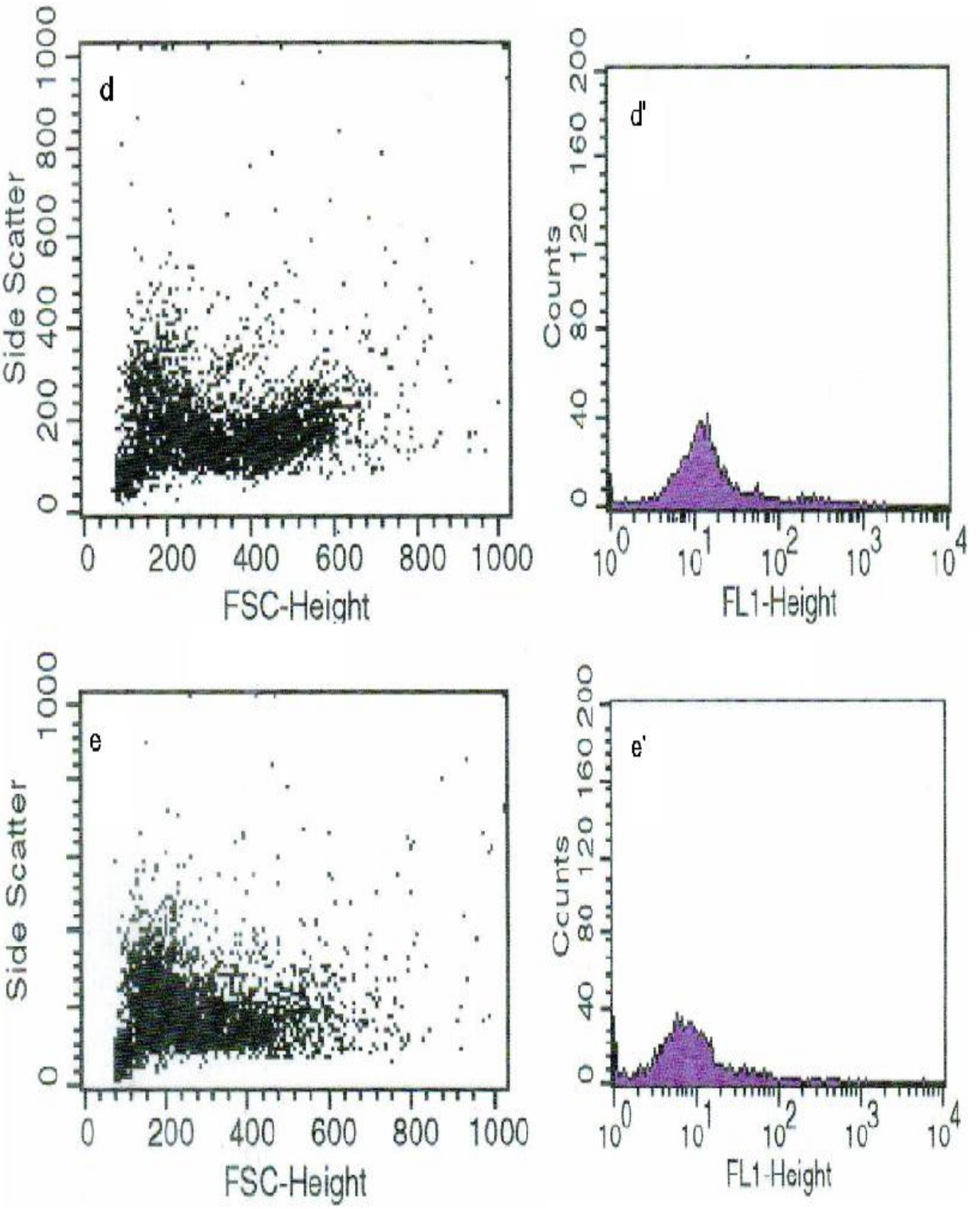

Figure 3: Flow cytometric histograms and quantitative analysis of the fluorescence intensity of IM-cells incubated with 0.5 up to 10 FITC/hGH molar ratio and/or biotinylated hGH. IM-9 cells were subcultured in RPMI-1640 medium with HEPES, 24 h before the assay. 10^5 cells were washed thrice with assay buffer and were incubated with different concentration of FITC-hGH and biotinylated hGH in assay buffer for 60 min at 4°C. Biotinylated samples were incubated with 100 µl of avidin-FITC solution (30 µg /ml) for 60 min at 4°C. After that, the cells were washed thrice with PBS/BSA solution and followed by flow cytometry. The results shown are one of three representative experiments performed. The dot plot cytogram and histograms of experimental F/P molar ratio of: a, a' for 0.22; b, b' for 0.48 and c, c' for 1.4. d, d and e, e' represent the experimental molar ratio of 11.6 and 46 respectively.

Table 3: Flow cytometry analysis data from interaction of conjugated hGH with IM-9 cells. Means, geomeans, median and % of coefficient variation were summarized in table.

F/P molar ratio and FACS Data	Mean of channel	Geo-means	% of coefficient variation
0.22	13.8	7.6	9.11
0.33	16.7	9.7	2.61
0.48	7.3	6.1	1.54
1.10	29.0	13.9	2.60
1.40	14.1	7.9	6.23

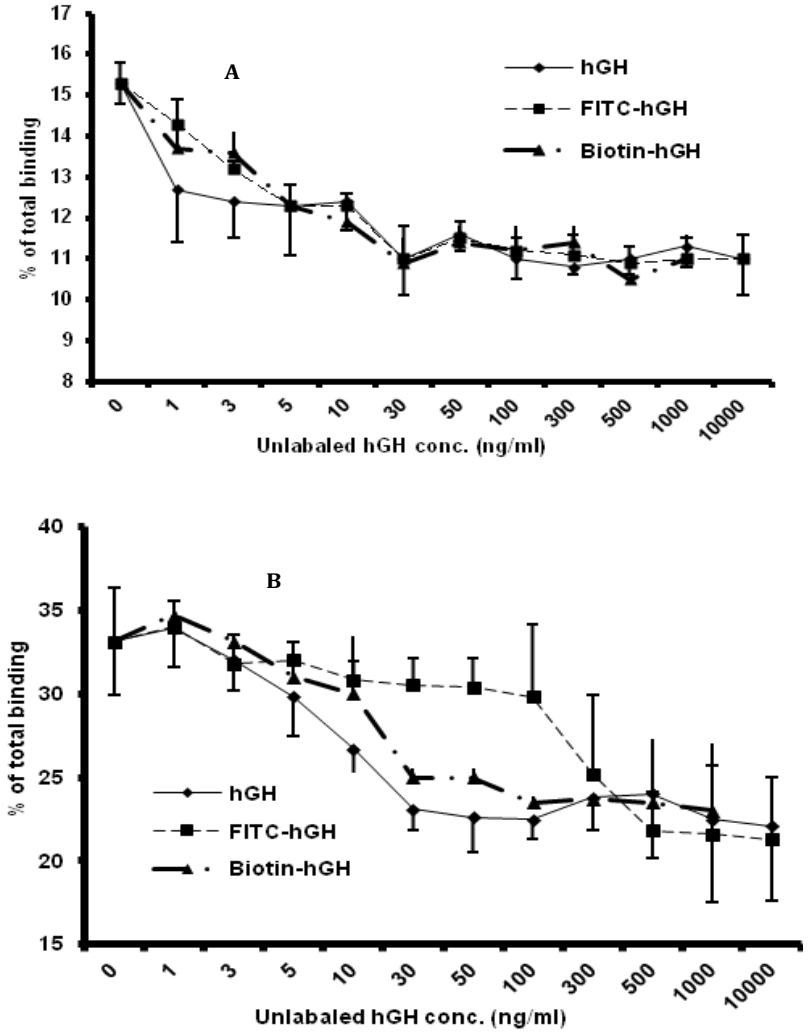

Figure 4: Competitive receptor binding of non-radioactive and radioactive labeled hGH. to IM-9 cells and/or rabbit hepatocyte microsomes. a) 24 h before the assay IM-9 cells were cultured in culture medium, which contain HEPES buffer. In the day of assay, cells were washed twice with assay buffer After that 10^7 cells were incubated with constant concentration of labeled ^{125}I-hGH (5 to 50 ng/ml) and increasing concentration of non-labeled and/or ligand labeled hormone (up to 10,000 ng/ml) for 2 h at 22 to 25ºC. Thereafter, cells were washed with cold assay buffer thrice and radioactivity of the pellets counted by γ-coulter counter. b) In the day of assay lyophilized microsomes were dissolved in HEPES receptor HEPES assay after that radio receptor assay was done in a competition manner. 500 µl of rabbit hepatocyte microsoms (2 mg/ml) was incubated with constant concentration of radio- labeled hGH (50 ng/ml) and increasing concentration of non-labeled hormone (up to 10 µg/ml) for 2 h at 30ºC. Thereafter, the mixers were washed with cold assay buffer thrice. The radioactivity of the pellets was counted using gamma counter. The total binding and specific binding were calculated as earlier described. The results are mean ± S.E.M. for three separate experiments.

DISCUSSION

The first step activity of hGH was initiated by the binding of hGH to its high affinity receptors on target cells. The receptor dimerizes in the presence of hGH. The receptor dimerization is crucial for phosphorylation of the intracellular domains of hGH receptor and for activation of specific signaling pathways required for hGH-induced gene regulation (Brooks and Waters, 2010; Mockridge et al., 1998). Evaluation of bioactivity and profiles of rhGH is an important issue in bio-production. Cell proliferation and Radio-Receptor Assay (RRA) were used for these purposes. Some limitation and disadvantage such as long time table course in cell proliferation assays, biosafety and biohazardous problems of RRA exist in the use of these methods. In the present research, a new flow cytometric non-radioactive receptor assay was introduced to study the binding of hGH to hGH receptors.

One of the useful systems to determine the expression of protein interest and the amount of protein expressed by a single cell on the basis of intensity of fluorescence is flow cytometry. Flow cytometry can be used to study expression of proteins on the surface of cells and the binding of hormone to specific receptor on the cell surface as well as, those localized within the cytoplasm (Butts and Sternberg, 2009). Here, we described flow cytometry system as a valuable method for evaluating the binding of fluorescence conjugated human growth hormone to hGH receptors in IM-9 cells and rabbit hepatocyte microsomes. Our result indicated and provided information on both the binding and bioactivity of hGH, as well as, the intensity of a particular hGH hormone receptor expressed by IM-9 cells. Using fluorescent microscopy methods and quantitative Radio Receptor Assay (RRA) showed that the flow cytometry result is comparable by the RRA result. In order to demonstrate that this technique can be used in a more generalized manner in the study of bioactivity of hGH in binding to hGH receptors, we also included analysis of receptors collected from hepatocyte microsomes and show that this method is exclusively useful for understanding of the biological activity of hGH as a therapeutic hormone, a particular tissue type. The binding of ligands to proteins frequently causes change in their three-dimensional structure. If this structural change has an effect on the environment of an intrinsic or extrinsic fluorophore in the protein, this can result in measurable change in the fluorescence spectrum (Moller and Denicola, 2002).

Given the result of this research by the powerful tool of flow cytometry, showed that the binding of hGH to hGH receptors to a cell surface or microsomes occurred and indicated that IM-9 cells is most relevant and more likely to respond to hGH treatment. In addition to analysis of bioactivity by flow cytometry, because of some indications, limitations and pitfalls in the determination of human growth hormone, IGF-I and their binding proteins were also noticed (Laron et al., 2007).

This technique and findings provides analysis of hGH receptor expression in patient samples and cell lines in future. In the analysis of glucocorticoid receptors expressed by murine thymocytes; Boldizsar reported that flow cytometry gives an indication of the number of receptors expressed by an individual cell, thus, providing a means to quantify receptors on each cell. Advantage of this technique is that expression of proteins of interest can be correlated with the degree of activation, maturation or differentiation of the given cell type (Boldizsar et al., 2006). This method is, however, limited in its inability to specify the location within the cell (nucleus, cytoplasm and mitochondria) of intracellular proteins that are identified.

In development of flow cytometry many factors were controlled based on experience and factors such as FITC labeling, available reagents and instruments. We found that many factors such as assay buffer, labeling agents and times of incubation are critical in determining optimal condition of the assay. Therefore, these conditions should be tightly controlled to prevent failure of the assay.

The results of flow cytometry showed that the binding of ^{125}I-hGH, FITC-conjugated hGH and biotinylated hGH to hGH receptors on IM-9 lymphocytes and microsomes were directly related to the concentration of hGH. The results of FITC and Biotin labeled hGH showed more spot fluorescence in IM-9 cells. Considering of homodimerization mechanism of hGH in binding to hGH receptors (Mockridge et al., 1998), these results suggest that these fluorescence spots were not random phenomena and labeling of hGH does not have effect on the receptor binding property of hGH.

Results from quantitative analysis of intensity of fluorescence in IM-9 cells by FACS show that, IM-9 human lymphoid cell lines have been characterized and are well known to have 3000 to 4000 receptor for growth hormones per cell (Smal et al., 1985). As these cells do not express prolactin receptors they were obvious choice for developing this non-radioactive receptor assay. This subject is most important from the aspect of production and evaluation of the biological activity of this hormone as a therapeutic agent. Possible explanation for this results and this manner include heterogeneity in the IM-9 cell lines used and variation in receptor expression during the cell cycle. This problem is solved with synchronization of the cells.

Finally, these results demonstrate that FITC or Biotin conjugated hGH can be used as a probe for identification of the biological activity and potential of receptor binding property of this recombinant hormone in biotechnology instead of radioactive ligands. This is more important and noticeable from some aspect such as limitation in providing ^{125}I and labeled hormones in some countries, short half life of radioactive Iodine, biosafety and biohazardous problems of radio ligands. Another perfect method of using non-radioactive ligands such as biotin and FITC are biosafety and less biohazardous aspect of these materials in

comparison with radioactive ligands. Besides, the radioactive iodine biohazardous material and short half-life, limitations in providing and preparing of ^{125}I and labeled hormones in some countries must be also considered.

At the moment, more than 50 radiopharmaceuticals are available in Iranian Drug List that most of them are produced and used in the nuclear medicine centers at a regular basis and others available per request. The research and development for more sophisticated radiopharmaceuticals based on biomolecules such as peptides, antibodies and their fragments are ongoing and related clinical evaluations initiated (Jalilian and Beiki, 2016). It must be noted that, for routine applications during process development, other formats of receptor assays are required for quantifying the biological activity in a short time.

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