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Differential response of the human hepatoma-derived cell line HA22T/VGH to polypeptide mitogens

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Several human cell lines derived from primary cancer of the liver are able to grow under serum-free conditions and produce spreading and growth factors which are released into the culture medium. Since this autocrine growth under hormone-free conditions might play a basic role in malignant transformation, we studied the effect on cell replication and the presence of specific membrane receptors of epidermal growth factor (EGF) and insulin on a dedifferentiated human hepatoma cell line, named HA22T/VGH. Our results point to a similar inhibitory effect on cell replication in the presence of both EGF and insulin, in spite of detecting different affinities of binding.

Hepatitis B virus; Insulin receptor; Epidermal growth factor receptor; (Human hepatoma cell line)

1. INTRODUCTION

One of the main characteristics of most human hepatoma-derived cell lines is their ability to grow in vitro under serum-free conditions [1-3]. It has been proposed that these autocrine systems may utilize ectopically produced growth factors in place of serum nitrogens [4]. Additionally, several cell lines from liver cancer were seen to produce spreading and attachment factors which are released into the culture medium; these factors act not only on the growth of the producing cell line, but also on that of other hepatoma cells [5,6].

The autocrine growth might play a basic role in malignant transformation of cells [7] and qualitative or quantitative alterations in a growth factor or in its receptor may be related to the expression of oncogenes [8-10]; these hypotheses have been explored in recent years in human hepatoma cell lines [11].

One of the most promising aspects of the hepatoma transformed phenotype is the actual possibility of investigating the effects of different hormones and growth factors under fully controlled conditions [12].

Here, we present data on a dedifferentiated human hepatoma cell line, named HA22T/VGH [13]. Although expression of integrated hepatitis B virus (HBV) 'S' gene was undetectable, we showed by Southern-blot hybridization at least three HBV DNA copies integrated into the host DNA. In terms of growth, this line presents a significantly different response to the presence of epidermal growth factor (EGF), insulin and human growth hormone (hGH) in a serum-free culture medium. In addition, the presence of specific membrane receptors for EGF and insulin (and their affinity) was investigated.

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2. MATERIALS AND METHODS

2.1. *Cells, cell culture and growth factors*

The HA22T/VGH cell line, derived from a primary hepatocellular carcinoma and received in September 1985, was adapted to grow in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY) and has been passaged serially in our laboratory. The cells were also cultured in RPMI 1640 supplemented with 10^{-8} Na_2SeO_3, in the complete absence of FCS. In some experiments a 1:1 mixture (v/v) of RPMI 1640 and RPMI 1640 conditioned by a 48 h cell growth was used. Generally, Nunc (Roskilde, Denmark) 4 or 24-well tissue culture plates were used.

Cell counts were determined using a hemocytometer; two to four experiments were performed for each aspect.

EGF from mouse submaxillary glands and hGH from human pituitary glands were purchased from Sigma (St. Louis); human insulin was a gift from Novo (Copenhagen, Denmark).

2.2. *Hepatitis B surface antigen (HBsAg) detection*

The presence of HBsAg was investigated in both the cell cytoplasm of HA22T/VGH and the crude supernatants of growing and confluent cell cultures.

Intracellular HBsAg was detected by indirect immunofluorescence using a rabbit anti-HBs antibody (Behringwerke, Marburg), while HBsAg released in culture medium was assayed by employing commercial reagents (Ausria II, Abbott, North Chicago, IL), with reference to a standard curve determined using a purified preparation of antigen. PLC/PRF/5 human hepatoma cells were used as positive control [14].

2.3. *Southern-blot analysis*

Southern analysis of *Hin*dIII-digested cell DNA was performed according to [15]. Briefly, the filter was hybridized, after Southern transfer and prehybridization, with nick-translated DNA of a plasmid containing head-to-tail dimer of complete 3.2. kb HBV genomes (HBsAg subtype ayw) in-

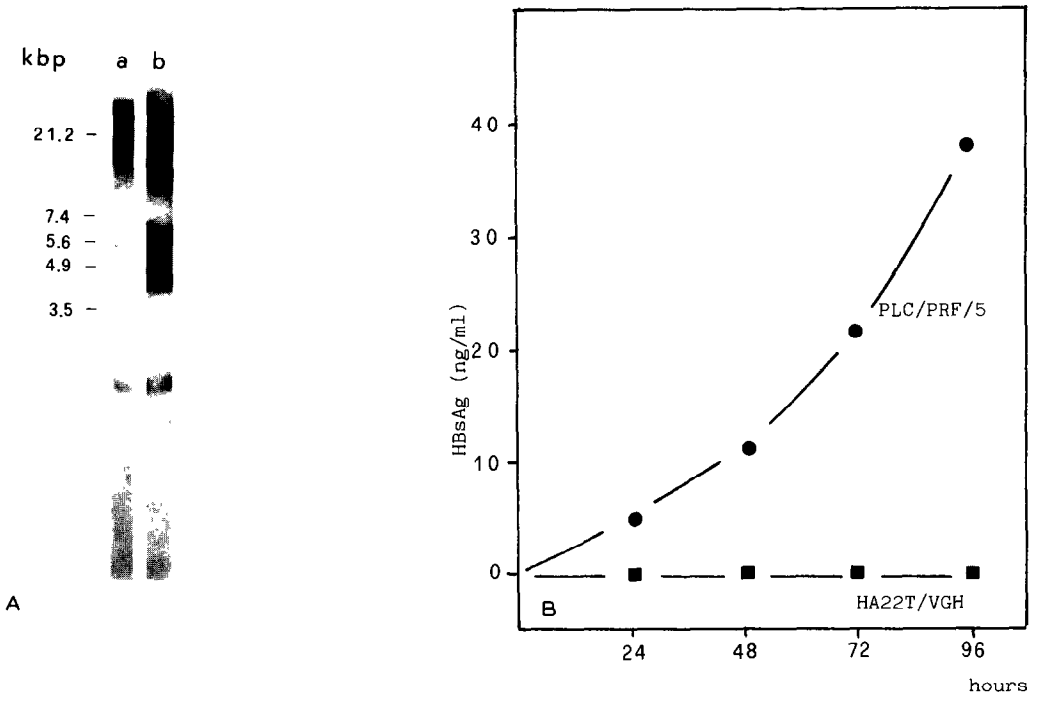

Fig.1. HBV DNA and HBsAg production by HA22T/VGH cells. (A) Southen-blot analysis of the *Hin*dIII-digested DNA (a, HA22T/VGH; b, PLC/PRF/5); three bands of HBV DNA specific sequences of 23, 17 and 1.8 kb in size have been detected. (B). HBsAg production by growing PLC/PRF/5 and HA22T/VGH cells. 5×10^5 cells/25 cm^2 flask were plated at time 0; amounts of antigen were detected at intervals of 24 h for 4 days.

serted into the *Eco*RI restriction site of the pBR plasmid [16–18]. After hybridization, the unincorporated radioactivity was washed out, and the filter dried and exposed to X-ray film with intensifying screens for 10 days at −80°C.

2.4. *Hormone binding*

To measure ^{125}I-EGF binding a previously described procedure with minor modification was applied [19]. Cells were cultured on 4- or 24-well tissue culture dishes at a density of 1–2 × 10^5 cells/well; the culture medium was discarded and cells were rinsed three times using Hank's balanced salt solution. 0.1 M Hepes buffer containing 0.5 μg/ml ^{125}I-EGF (2.4–3.5 × 10^5 cpm/μg) was used; the cells were incubated for different periods and incubation terminated by washing the cells six times with PBS supplemented with 1 mg/ml bovine serum albumin (BSA), pH 7.0, at 4°C. Radioactivity was determined in a γ-counter after treatment with 0.5 ml/well lysis buffer (0.1 M NaOH, 2% $NaCO_3$, 1% SDS). Incubation was performed at 4, 24 and 37°C; 24°C was found to be the most suitable temperature (not shown).

Non-specific binding was determined in the presence of a 200-fold excess of unlabeled EGF and was less than 3% in all experiments.

1–600 μg/ml of unlabeled EGF was added to the binding buffer for the competition assay. ^{125}I-EGF degradation during binding experiments was studied by precipitation in the presence of 10% trichloroacetic acid.

Confluent monolayers of cells were rinsed and incubated with 0.1 M Hepes supplemented with 0.04 mCi/ml ^{125}I-insulin for insulin binding. 24°C was determined to be the most suitable incubation temperature. Non-specific binding was determined in the presence of unlabeled insulin (1 mg/ml) and the competition curve established by adding unlabeled insulin (1–1000 ng/ml) to the binding buffer. ^{125}I-insulin degradation was defined by using trichloroacetic acid precipitation.

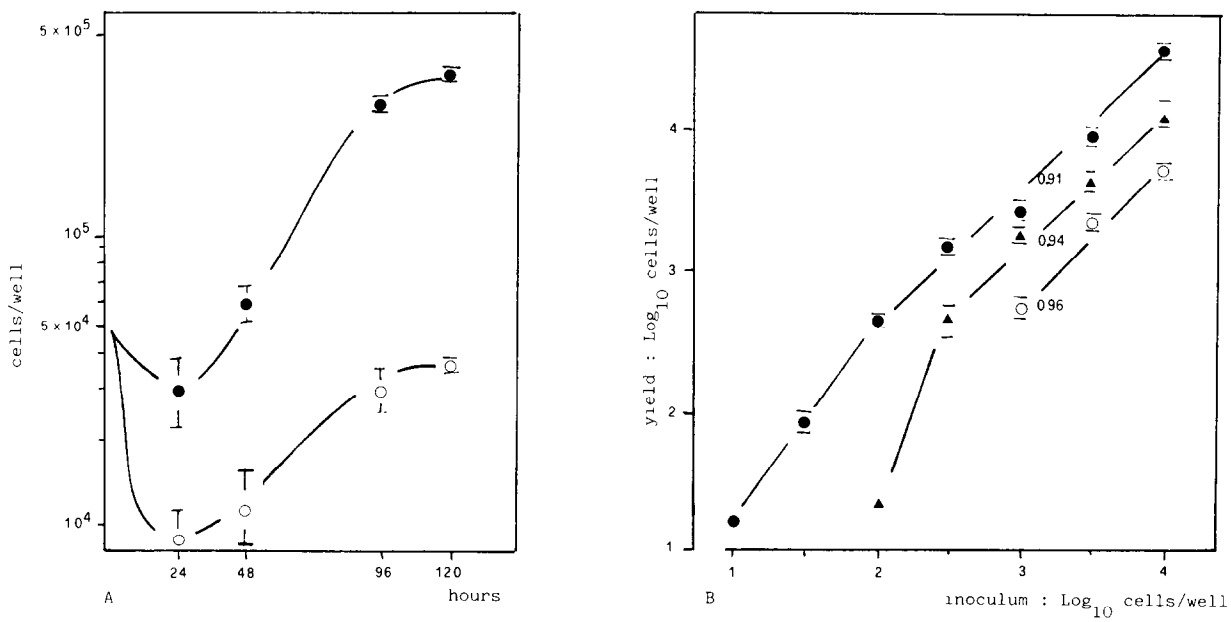

Fig.2. Growth of HA22T/VGH cells under serum-free conditions. (A) Growth curves of cells cultured in RPMI 1640 supplemented with 10% FCS (●) and RPMI 1640 supplemented with 10^{-8} M Na_2SeO_3 (○). Cells were plated at a density of 5×10^4 cells/well and counted at intervals of 24 h. Each point is the mean ± SE of four determinations. (B) Relationship between viable HA22T/VGH cell yield and cell inoculum in medium supplemented with 10% FCS (●), 10^{-8} M Na_2SeO_3 (○) or in a conditioned medium (see section 2) (▲). 150–15 000 and 300–30 000 viable cells were seeded in 0.2 ml of each medium in triplicate samples (96-well tissue culture dishes). Cells were counted after growth for 4 days. Straight lines were fitted by regression using three points (inocula from 1000 to 10 000). The value beside each straight line indicates the slope. Each point is the mean ± SE.

3. RESULTS

3.1. *HBV DNA integration and HBV gene expression*

HBsAg expression of HA22T/VGH was undetectable under routine culture conditions in both the cell cytoplasm (not shown) and culture medium, whereas the PLC/PRF/5 cell line produces and releases large amounts of antigen (fig.1B). In spite of these negative results, we observed after *Hin*dIII digestion three bands of 23, 17 and 1.8 kb size specific for HBV DNA by Southern hybridization, using a complete HBV DNA probe (fig.1A).

3.2. *Cell growth under serum-free conditions and in the presence of hormones*

The human hepatoma cell line HA22T/VGH is able grow in fully defined serum-free, mitogen-free medium in the presence of the trace element selenium (as Na_2SeO_3). Under these culture conditions, an initial loss of adhering cells was detected (fig.2A), but cell replication with a doubling time of 30 h (rather than 20 h for cells cultured in the presence of 10% FCS) was observed. The cell input was also related to cell release under three different culture conditions; regression analysis of data (fig.2B) shows that better attachment and growth can be achieved by culturing cells in a conditioned medium, rather than in fresh RPMI 1640. The regression curve for cells cultured by using a conditioned medium shows increased cell replication even at low densities, when compared with cells cultured in fresh medium.

For dose-response experiments, cells were plated and maintained for 24 h in a medium supplemented with 10% FCS, in order to avoid the initial loss of adhering cells which was observed

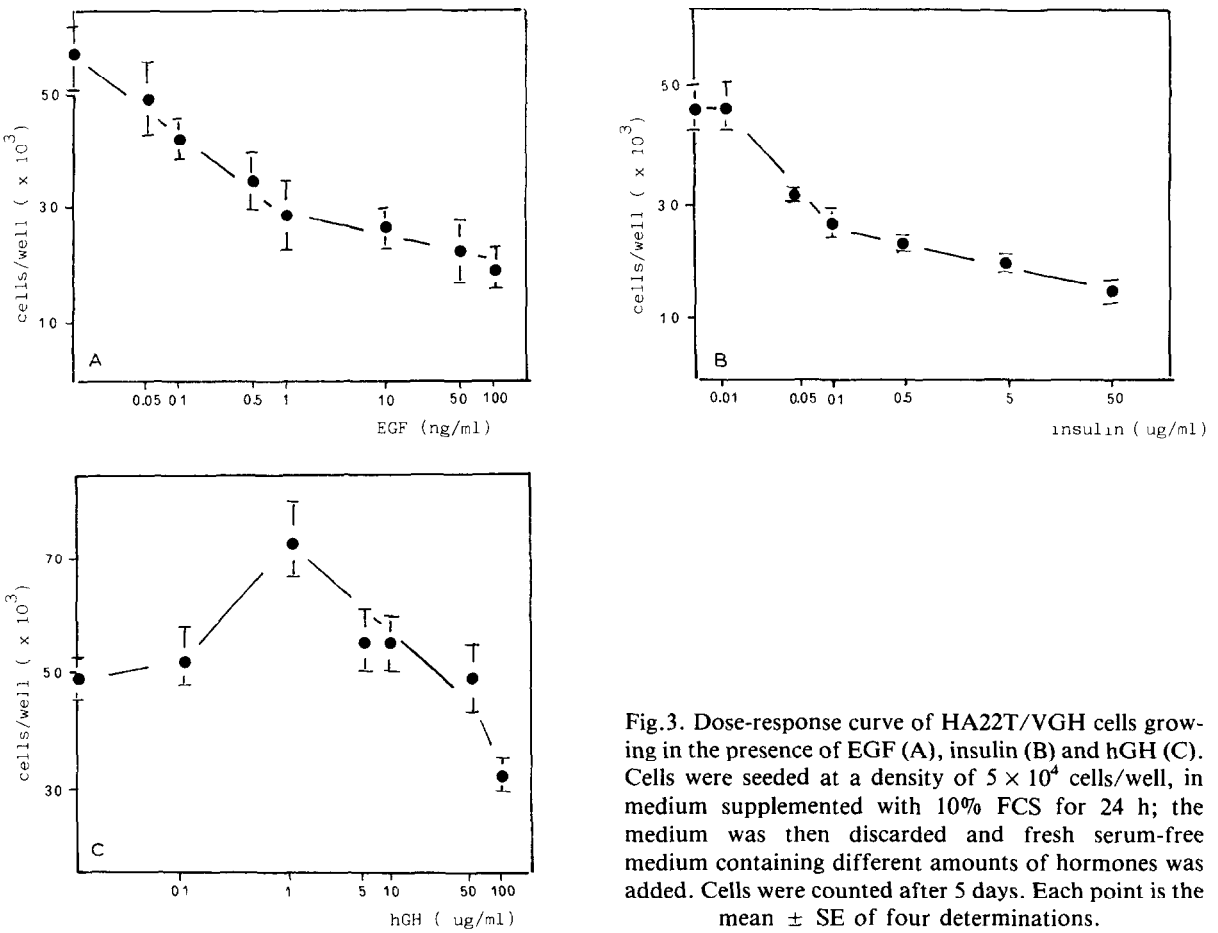

Fig.3. Dose-response curve of HA22T/VGH cells growing in the presence of EGF (A), insulin (B) and hGH (C). Cells were seeded at a density of 5×10^4 cells/well, in medium supplemented with 10% FCS for 24 h; the medium was then discarded and fresh serum-free medium containing different amounts of hormones was added. Cells were counted after 5 days. Each point is the mean ± SE of four determinations.

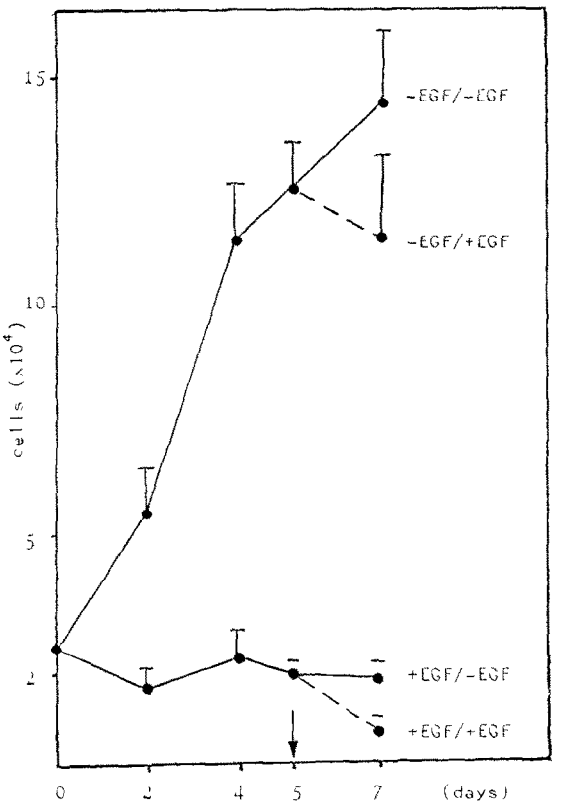

Fig.4. Reversal of EGF inhibition of HA22T/VGH cell growth. 4×10^4 cells/well were plated in RPMI 1640 medium supplemented with 10% FCS and, after repeated washing 24 h later, the medium was changed to RPMI 1640 supplemented with 10^{-8} M Na_2SeO_3 in the absence or presence of 10 μg/ml EGF (day 0). Each experimental group was subdivided on day 5 (arrow) into two groups with the substitutions indicated. Values are means ± SE ($n = 4$).

under fully serum-free conditions. 24 h after plating, cells were washed three times and serum-free medium containing different amounts of EGF, insulin and hGH was added. The results (fig. 3A,C) show that hGH induces slight stimulation within a narrow range, whereas strong dose-dependent inhibition of cell replication was detected in the presence of both EGF and insulin. The EGF-mediated inhibitory effect on cell replication seems to be dependent on the continuous presence of the growth factor in the culture medium as shown in fig.4, because cell replication is reduced in the presence of EGF, while cells grow normally in its absence.

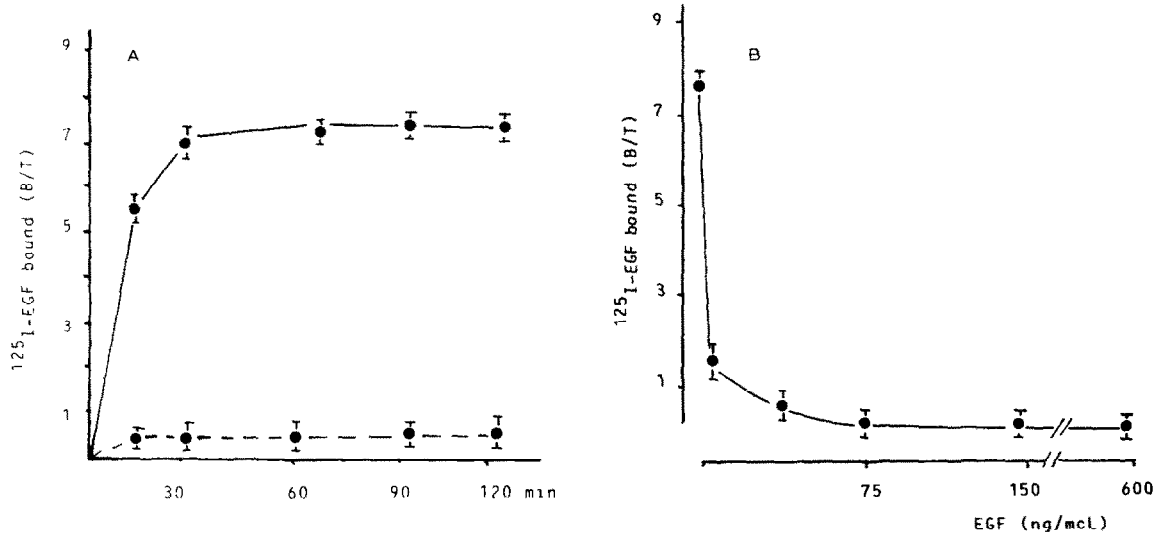

Fig.5. EGF receptors of HA22T/VGH. (A) ^{125}I-EGF bound to cells cultured in 4-well tissue culture dishes incubated at 4°C. Cells were incubated for different periods in the presence of ^{125}I-EGF; incubation was terminated by washing and radioactivity determined after treatment with lysis buffer. Non-specific binding (----) was determined in the presence of a 200-fold excess of unlabeled EGF. (B) Competition assay of unlabeled EGF. 1–600 ng/ml unlabeled EGF was added to the binding buffer (see section 2).

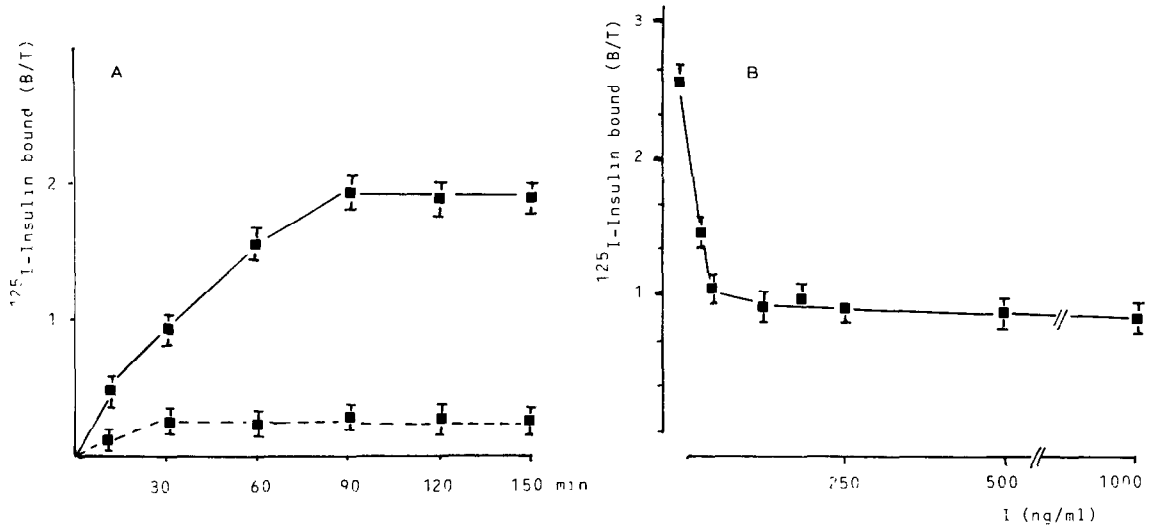

Fig.6. Insulin receptors of HA22T/VGH cells. (A) ^{125}I-insulin bound to cells incubated at 24°C. (- - - -) Non-specific binding determined in the presence of a 200-fold excess of unlabeled insulin. (B) Competition assay of unlabeled insulin. 1-1000 ng/ml unlabeled hormone was added to the binding buffer.

3.3. *Hormone binding to specific membrane receptors*

^{125}I-EGF binding at 24°C reached the maximum level within 30 min and this plateau was maintained for more than 3 h. Non-specific activity was less than 3%. The specificity of the receptor was evaluated by increasing the doses of unlabeled EGF (fig. 5A,B). The highest degree of insulin binding was found to occur within 90 min (b/t 3%) and was maintained for more than 3 h. The competition curve of unlabeled insulin shows the low affinity of the insulin receptor of HA22T/VGH cells (fig. 6A,B).

4. DISCUSSION

EGF enhances DNA synthesis in cell culture [20,21] and, similarly to insulin, increases the uptake of glucose, amino acids and uridine [21-23], indicating that these hormones can regulate a variety of cell functions. In addition, the EGF receptor is a glycosylated protein [24], autophosphorylated through a kinase that is an integral part of the receptor [25]. Similarly, insulin exherts numerous effects on the metabolism of carbohydrates, lipids and amino acids, as well as on membrane transport processes. The insulin receptor is composed of two subunits with kinase activity; insulin binding to its receptor stimulates this activity and the receptor phosphorylates itself as well as other proteins [26]. Both hormones are involved in the regulation of cell growth [27].

Our data show that a dedifferentiated human hepatoma cell line (HA22T/VGH) has both high-affinity EGF receptors and low-affinity insulin receptors; EGF binding appears to be similar to those reported for most cells known to show a mitogenic response to EGF [28]. Nevertheless, similarly to insulin, EGF inhibited cell proliferation.

EGF was recently observed to inhibit the growth of a variety of carcinoma-derived cell lines [29-33]; in addition, it was shown that the growth of a clone from a human hepatoma cell line (named LI7A) is inhibited by the presence of EGF in a serum-free medium [34]. It has been proposed that these inhibitory effects might be due to the presence of a large number of high-affinity membrane receptors, causing excessive kinase stimulation [35].

Taken together, data reported here point to the strong inhibition of cell replication in the presence of two hormones with membrane receptors of different degrees of affinity, whereas only hGH appears to be mitogenic for HA22T/VGH within a narrow range. In our opinion, these cells can be

useful as a model for elucidating mechanisms whereby EGF, in this case similarly to insulin, inhibits the proliferation of cell lines with membrane receptors of different degrees of affinity.

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