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Expression of high- and low-affinity epidermal growth factor receptors in human hepatoma cell lines

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Data are presented from a comparative research on expression of epidermal growth factor (EGF) receptors and response to EGF of six independently established cell lines derived from human hepatoma. These lines differ in terms of the degree of differentiation, presence of hepatitis B virus (HBV) DNA copies in integrated form and expression of HBV genes. Our results indicate differential expression of membrane EGF receptors and differential response to EGF under serum- and hormone-free culture conditions. Furthermore, a significant difference in affinity could be detected between EGF receptors of the two highly dedifferentiated cell lines (HA22T/VGH and Li7A) whose replication is inhibited by EGF concentrations capable of stimulating more differentiated phenotypes.

Epidermal growth factor receptor; Cell replication; (Human hepatoma cell)

1. INTRODUCTION

Human hepatocellular carcinoma (HCC)-derived cell lines are characterized by the maintenance of the degree of cellular differentiation in culture [1]; furthermore, some are able to grow under completely serum- and hormone-free culture conditions [2,3]. This independence from growth factor requirements raises the question of whether products of activated cellular oncogenes (c-oncs) are involved in the process of liver carcinogenesis [4–7] and activation of c-oncs in HCC tissues has recently been demonstrated [8–12].

A further aspect of HCC cells in culture has been reported concerning their response to polypeptide mitogens [13–15], thus indicating that at least in some cases epidermal growth factor (EGF) can play a major role in conditioning the growth characteristics of these cells.

EGF is also known to be necessary in the regenerative process of the liver [16]; furthermore, in recent years, several sources of evidence have implicated EGF in the process of neoplastic transformation [17–19].

For these main reasons, we have used a wide range of HCC cell lines, independently established and showing a different degree of differentiation, in order to study the presence and the integration of HBV DNA sequences, the expression of EGF receptors (EGF-Rs) and the effect of EGF on cell replication.

The principal aim of this research has been to observe whether overexpressed EGF-Rs are involved in the aberrant growth characteristics of HCC-derived cell lines.

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2. MATERIALS AND METHODS

2.1. *Human hepatoma-derived cell lines and cell cultures*

The HCC-derived cell lines studied were cultured in RPMI

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1640 supplemented with 10% fetal calf serum (FCS), glutamine (2 mM) and antibiotics. The PLC/PRF/5 cell line was a gift from Dr Alexander, HA22T/VGH was obtained from Dr Chang, the HCC36 cell line was kindly donated by Dr Chan and the HepG2, Hep3B and Li7A cell lines were a gift from Dr Knowles. The A431 human epidermoid carcinoma cell line was supplied by the European Collection of Animal Cell Cultures (Salisbury, England).

The degree of cellular differentiation of the different HCC cell lines, based on both production of plasma proteins and the levels of major class I histocompatibility antigens induced by interferon γ, has been described elsewhere [1,2,13,14,19–23].

Serum-free cultures were grown using RPMI 1640 supplemented with 10^{-8} M Na_2SeO_3 [12,13] while, for dose-response experiments, 5×10^4 cells were plated onto serum-containing medium which was renewed 24 h later using fresh RPMI 1640 supplemented with selenium as described above. The cells were counted at day 5 using a ZM Coulter Counter (Coulter Electronics, UK).

2.2. *Materials*

EGF from mouse submaxillary glands was supplied by Sigma (St. Louis, MO) and the monoclonal antibody to the A431 EGF-R was supplied by Oncogene Science (Mineola, NY).

HBsAg produced by several HCC cell lines was assayed in the cell cytoplasm using a rabbit anti-HBs antiserum supplied by Behringwerke (Marburg) and again in the crude supernatants using a commercial enzyme immunoassay (Connaught, Willowdale, Canada).

^{125}I-EGF was purchased from Biodata (Milan).

2.3. *Detection of hepatitis B virus DNA sequences*

Southern blot analysis of integrated HBV DNA sequences was performed on cell DNAs extracted using a standard procedure [24]. Hybridization was carried out as in [25] using a complete 3.2 kilobase pair (kb) HBV genome [26].

2.4. *EGF receptors*

^{125}I-EGF binding to HCC cells was assayed using a previously described procedure with minor modifications [14].

Briefly, cells growing in 4-well tissue culture dishes were counted, rinsed using phosphate-buffered saline (PBS) and 0.1 M Hepes buffer containing 0.5 μg/ml ^{125}I-EGF (spec. act. 2.5–3.5 $\times10^8$ cpm/ng) was added.

After incubation, cells were washed using PBS supplemented with 1 mg/ml bovine serum albumin (BSA) (pH 7.0) and lysed by treatment with lysis buffer (0.1 M NaOH, 2% Na_2CO_3 and 1% SDS). Radioactivity was determined using a γ-counter.

All cell lines were incubated at 4, 24 and 37°C; 24°C proved to be the most suitable temperature (not shown). Initially, binding experiments were similarly performed under different pH conditions. Non-specific binding was determined in the presence of a 200-fold excess of unlabeled EGF.

Binding experiments for Scatchard analysis of EGF-receptor affinity were performed using a binding buffer containing increasing concentrations of ^{125}I-EGF (0.1–10 μg/ml). Plates were incubated at 24°C for 3 h in order to approach equilibrium conditions for all cell lines. Finally, cells were rinsed with PBS and prepared for γg-counting as above.

^{125}I-EGF degradation was assayed in the presence of 10% trichloroacetic acid.

Finally, we assayed the effect of a monoclonal antibody (mAb) to the A431 EGF receptor on EGF binding. The treatment with mAb (6.25 μg/ml) for 30 minutes at 37°C was seen to be effective in reducing A431 EGF binding by up to 80% (not shown). We thus used this treatment to study the comparative EGF binding of HCC cells in the presence of this mAb.

3. RESULTS

3.1. *^{125}I-EGF binding to membrane receptors of HCC cells*

Fig.1 shows ^{125}I-EGF binding of the HCC cell lines studied. The HA22T/VGH and PLC/PRF/5 cell lines reached a steady state of binding within 60 min, whereas 80–120 min incubation with labeled EGF was required for other cell lines. Among the cell lines tested, Li7A cells reached the highest level of binding (bound/total = 24.6), although a discrete level of non-specific binding was observed which increased with time.

Scatchard analysis of EGF-R affinity principally shows the great diversity between the high K_a value of the HA22T/VGH EGF-R (8.4×10^{10}) and that of Li7A EGF-R (undetectably low values).

HCC cell lines were also treated with an mAb to A431 (human epidermoid carcinoma cell line) EGF-R capable of reducing ^{125}I-EGF binding in this cell line by up to 80% when used at 6.25 μg/ml (not shown) (table 1). Under the same experimental conditions, although a marked decrease in ^{125}I-EGF binding to HA22T/VGH cells could be detected, we only observed minimal (3% mAb-dependent inhibition of Li7A binding activity.

3.2. *Effect of EGF on HCC cell replication*

The different cell lines under study exhibited a differential capability of replicating under serum-free conditions (fig.2) and a differential attachment to the solid surface after plating. Seeding efficiencies were evaluated and ranged from lower values for less differentiated phenotypes (HA22T/VGH 54%; Li7A 72%; HCC36 74% to higher efficiencies for more differentiated HCC-cell lines (PLC/PRF/5, HepG2 and Hep3B). This is probably due to the different expression and release of serum factors which are involved in the seeding events.

However, although we observed large differences in terms of cell replication among the cell lines under study, in a 5-day experiment, all cells were able to grow under the described conditions

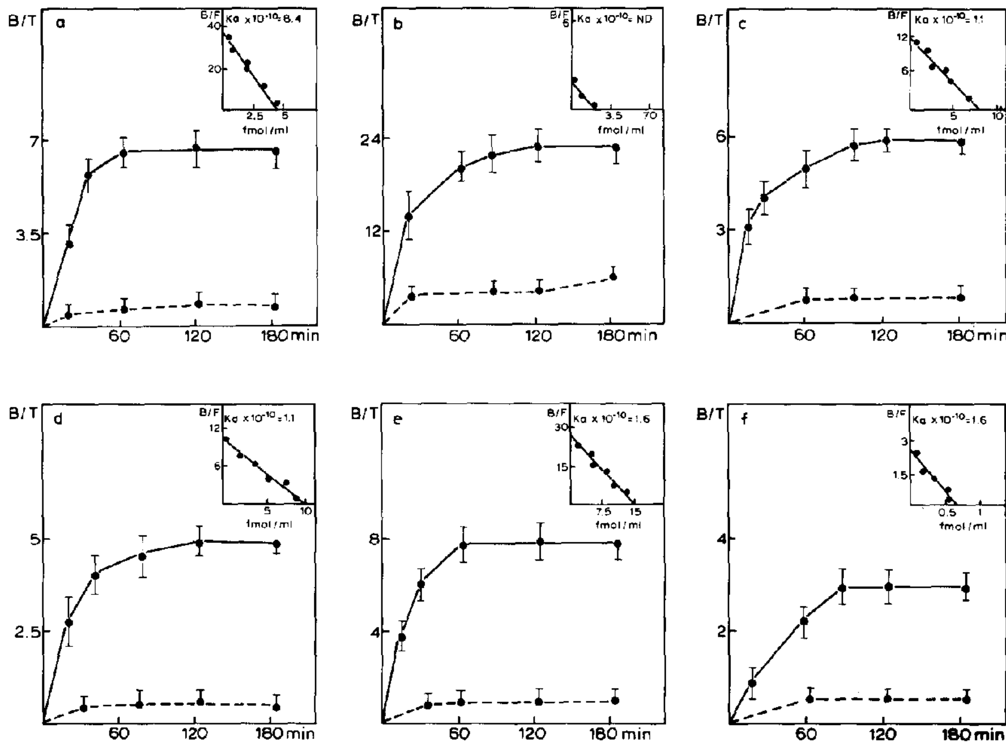

Fig.1. ^{125}I-EGF binding to HCC cell lines (*B/T*, bound/total; incubation period in min). The dashed line represents non-specific binding. Scatchard plots of EGF-receptor affinities are enclosed for each cell line (*B/F*, bound/free). Numbers represent the K_a values calculated ($\times 10^{10}$); this could not be evaluated for Li7A cells. a, HA22T/VGH; b, Li7A; c, HCC36; d, Hep3B; e, PLC/PRF/5; f, HepG2.

showing at least one doubling, with reference to the actual initial input of attached cells.

Only two HCC-derived cell lines (HA22T/VGH and Li7A) were inhibited in terms of cell replication by increasing concentrations of EGF in dose-response experiments (fig.2). All other cell lines except one showed a small mitogenic response to EGF within different ranges, the exception being HepG2 (a well differentiated HCC cell line), whose cell replication proved to be stimulated up to

Table 1

Comparative analysis of six HCC-derived cell lines in culture

Cell line	Degree of differentiation	HBV DNA (copy no.)	HBsAg expression	EGF-receptor affinity K_a ($\times 10^{10}$)	EGF-receptor affinity mAb
HA22T/VGH	low	+ (3)	–	8.4	55
Li7A	low	–	–	ND	3
HCC36	medium	+ (4)	–	1.1	54
PLC/PRF/5	high	+ (7)	+	1.6	47
Hep3B	very high	+ (2)	+	1.13	54
HepG2	very high	–	–	1.6	66

See section 2. Affinity of EGF-receptors (expressed as $K_a \times 10^{10}$) was determined by Scatchard analysis (ND, undetectable under our experimental conditions). The K_a value of the A431 human epidermoid carcinoma cell line EGF-R resulted to be 3.6×10^{10}. mAb: inhibition, expressed as per cent vs controls, of ^{125}I-EGF binding observed after incubation of cells (30 min at 37°C) with a monoclonal antibody to the A431 EGF-R

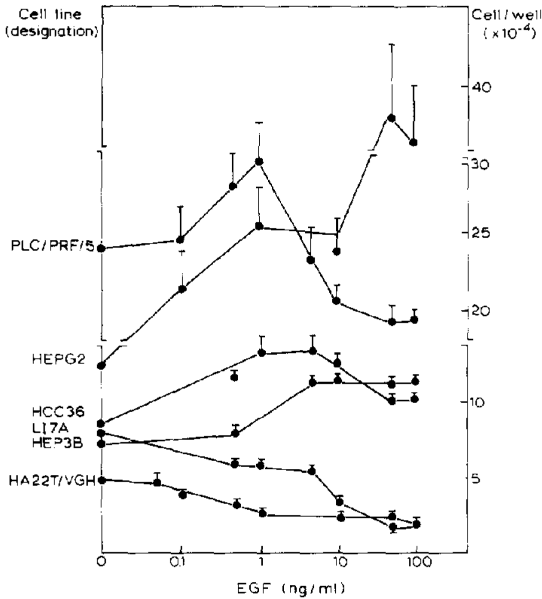

Fig.2. Dose-response curves for EGF of serum-free cultured HCC cell lines. 5×10^4 cells were plated in a medium containing 10% FCS; culture medium was discarded at day 1 and fully serum- and hormone-free, selenium-supplemented RPMI 1640 containing different amounts of EGF (0.05–100 ng/ml) was added. Seeding efficiencies were calculated for each cell line 12 h after plating: HA22T/VGH, 54%; Li7A, 72%; HCC36, 74%; Hep3B, 90%; PLC/PRF/5 and Li7A, above 95%. Cells were counted at day 5. Each point represents the mean of four different replicates. Two to three experiments were performed for each aspect and all standard errors were less than 10%. a, HA22T/VGH; b, Li7A; c, HCC36; d, Hep3B; e, PLC/PRF/5; f, HepG2.

3-times vs controls using EGF concentrations above 10 ng/ml.

3.3. *Southern blot analysis of HBV DNA and expression of the HBV S gene*

*Hin*dIII-digested cell DNAs (fig.3) showed the presence of integrated HBV DNA sequences in four out of six HCC cell lines (lanes a,b,d,e). However, only two HBV DNA-positive cell lines (PLC/PRF/5 and Hep3B) were seen to produce HBsAg and release it into the culture medium (table 1).

4. DISCUSION

We have reported the differential expression of EGF-Rs in six independently established HCC cell lines and found that an inhibitory response to EGF

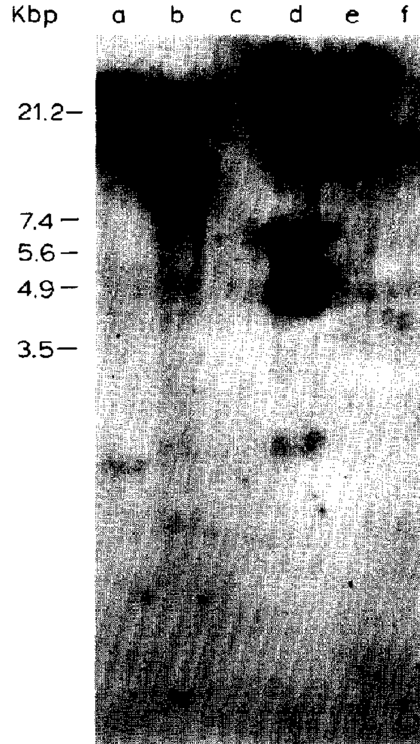

Fig.3. Integrated HBV DNA sequences showed by Southern blot analysis of *Hin*dIII-digested cell DNAs. a, HA22T/VGH; b, HCC36; c, Li7A; d, PLC/PRF/5; e, Hep3B; f, HepG2.

in terms of cell replication occurs only in two highly dedifferentiated cell lines (Li7A and HA22T/VGH) exhibiting quite different EGF-R affinity. These data are at variance with those of others which suggest that either EGF-R expression is decreased in hepatoma cells [27], or that abnormal expression of the EGF-R of HCC-cells is not among the factors responsible for the aberrant cell proliferation [15].

EGF has been observed to inhibit growth in vitro of a variety of carcinoma-derived cell lines [28–31], most of which exhibit high levels of high-affinity EGF receptors as a consequence of gene amplification. It has been proposed that this EGF-linked inhibition of cell replication may be due to excessive kinase stimulation, which depletes cellular energy stores [32].

To our knowledge, no cell line expressing a high level of low-affinity EGF-Rs has been observed to be inhibited by EGF in cell proliferation. The Li7A cell line has exhibited a particularly high level of

^{125}I-EGF binding, dependent mainly on overexpressed low-affinity EGF-Rs and only minimally on high-affinity EGF-Rs, thus being a possible tool for testing EGF-linked inhibition of cell replication. On the other hand, HA22T/VGH EGF-Rs are represented by a single class of receptors with a particularly high level of affinity.

Since the EGF-R is the product of the c-erb-B proto-oncogene [33], which is amplified in many cells of cancer origin [34] and its ligand TGF (an EGF-family protein) is also expressed in many cancer cells [35], production of EGF receptors may be closely related to mechanisms of oncogenesis.

The present data taken together show that HCC-cell lines may exhibit overexpression of EGF-Rs at least in some cases. This may be responsible for the modified characteristics of cell replication and it opens up the question of whether activation and/or amplification of the c-erb-B proto-oncogene occurs, thus supporting the possible role of this mechanism in the onset and development of liver cancer [36].

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