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# THE BINDING OF HB-EGF TO TUMOUR CELLS IS BLOCKED BY MABS WHICH ACT AS EGF AND TGFα ANTAGONISTS

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**Summary:** Heparin binding EGF (HB-EGF), a newly discovered member of the EGF family of mitogens, binds to the EGF receptor (EGFR) and to heparan sulfate proteoglycans on the cell surface. Here, we show that the binding of HB-EGF to the EGFR is inhibited by mAbs which prevent the interaction of EGF and TGFα with the receptor. Also, we show that, like EGF and TGFα, treatment with HB-EGF inhibits the growth *in vitro* of tumours (HN5, HSC-4) that overexpress the EGFR. We conclude that mAbs which act as EGF and TGFα antagonists should also be effective therapeutic agents for blocking the growth of EGFR overexpressing tumours induced by HB-EGF. 

The human EGF receptor is a 170kD transmembrane glycoprotein with tyrosine kinase activity that transmits the biological and biochemical effects of the EGF family (1-6). The member of EGF family of ligands which includes EGF, TGFα, amphiregulin, HB-EGF, and betacellulin have been grouped together on the basis of their structural and/or biological similarity (1-6). The structural similarity is the presence of of an "EGF unit" in the molecule, a structure containing a sequence of 45-50 amino acids with three disulphide bridges (7). In addition, the mature form of these ligands are cleaved from a larger membrane bound precursor and recent studies have suggested that these ligands may therefore be present in a number of biologically active forms in human tissues (8-10).

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**Abbreviations:** EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; MAB, monoclonal antibody; EGFR, epidermal growth factor receptor.

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HB-EGF is a novel member the EGF family of mitogens that was originally isolated as a 20kDa secreted protein product of a macrophage like cell line U-937 (5). HB-EGF has been shown to inhibit the binding of EGF to the EGF receptor on A431 cells and to stimulate the growth of smooth muscle cells (SMC), fibroblasts, and keratinocytes but not endothelial cells (5, 11-13). Recent studies have shown that HB-EGF is present in many tissues including, lung, skeletal muscle, brain and heart and is synthesized by monocytes, and macrophages, smooth muscle cells, keratinocytes, and T helper cells (5,14-18).

It has become evident, from the examination of human tumour biopsies and cell lines, that overexpression of the EGF receptor is often accompanied by the production of one or more of its ligands (EGF, TGFα, amphiregulin, HB-EGF), suggesting that autocrine or juxtacrine loops may be responsible for growth of tumours of this type (2,10, 19). We have been investigating the potential for use in diagnosis and therapy of a series of rat monoclonal antibodies that have been rasied against five distinct epitopes on the external domain of the human EGF receptor (19-22). To be effective as therapeutic reagents, it is important that these mAbs should be able to block the interaction with the EGF receptor of any member of the EGF family of ligands.

In this paper we have investigated the effect of mAbs ICR62 and ICR64, which react with to two distinct epitopes on the EGF receptor, on the binding of $^{125}$I-HB-EGF to the EGFR on a human bladder carcinoma cell line. Our results show that these mAbs, which block EGF and TGFα binding to the EGFR, can also inhibit the binding of HB-EGF to these receptors. We show also for first time that HB-EGF, like EGF and TGF α, inhibits the growth of tumours overexpressing the EGF receptor in culture.

## MATERIALS AND METHODS

**Cell Culture and Monoclonal Antibodies.** The human head and neck tumour cell lines HSC-4 (kindly provided by Professor Toshio Turoki, Tokyo University, Japan) and HN5, and bladder carcinoma cell line (EJ) were cultured routinely in Dulbecoo's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and the antibiotics penicillin, streptomycin and neomycin essentially as described before (20). MAb ICR62 and ICR64, which bind to two distinct epitopes on the extracellular domain of EGF receptor, were raised against the receptor on the human breast carcinoma cell line MDA-MB 468 and purified as described previously (19,20).

**Radioiodination of EGF, TGFα, and HB-EGF.** Human recombinant EGF (AUSTRAL Biologicals, San Ramon, California), TGFα (Collaborative Research, Mass), and HB-EGF (carrier free, R&D System, Oxon, UK) were labelled, using the iodogen method, with Iodine-125 (Na$^{125}$I, Amersham International ) to a specific activity of 10μci/μg as described previuosly (20). All tubes and the column used during labelling with HB-EGF were pre-washed throughly with PBS containing 0.5% BSA in order to minimize the nonspecific binding of HB-EGF.

**Competitive Radioimmunoassays (RIA).** EJ cells were grown to near confluency in 96-well plates and then washed twice with DMEM containing 2%FCS. Duplicate samples (50$\mu$l) of doubling dilutions of monoclonal antibodies, HB-EGF, or TGF$\alpha$ in DMEM containing 2%FCS were mixed with an equal volume of $^{125}$I-TGF$\alpha$ or $^{125}$I-HB-EGF to give a total activity of $5.5x10^4$ cpm/well. Control wells containing diluent alone were also set up in the same way. Aliquots of 90$\mu$l of each mixture were then transferred to monolayers of EJ cells. After incubation for one hour on ice the cells were washed three times, then lysed in 1M NaoH containing 1% sarkosyl and the bound radioactivity was determined in a Hydragamma spectrometer (Oakfield Instrument Ltd, Oxford). In some experiments, the cells were washed twice before use, with 200$\mu$l of PBS/2M NaCl to prevent the subsequent interaction of HB-EGF with cell surface HSPG (10).

**Cell Growth Studies.** A colourimetic assay was used to monitor effects on cell growth (20). Briefly, about $5x10^3$ cells in 100$\mu$l of DMEM containing 2%FCS were seeded into each well of a 96-well plate. After 4 hours incubation at 37°C, 100$\mu$l aliquots of dilutions of HB-EGF, TGF$\alpha$, or EGF were added in triplicate to the wells and the cultures were incubated at 37°C. Controls were also set up that contained medium alone. When the cells in wells containing medium alone were almost confluent, all cells were fixed with 0.25% gluteraldehyde, washed in water, air dried and stained with 0.05% methylene blue. The absorbance of each well was measured at 620 nM in a Titerteck Multiscan. To determine the initial number of cells present at the start of each experiment, an additional plate was set up and the cells were fixed after 4hr incubation at 37°C. Growth as percentage of control was determined using the formula: % Growth = (B-A:C-A) x 100, where A = A620 at the start of incubation, B= A620 after treatment with ligands, and C= A620 after incubation in medium alone (16).

## RESULTS

We have shown previously that mAbs ICR62 and ICR64 inhibit the binding of both EGF and TGF$\alpha$ to the receptor on a wide range of EGFR expressing tumours (16). Therefore, we investigated first whether these mAbs were also capable of inhibiting the binding of HB-EGF to the EGF receptor.

The results in figure 1 show that at concentrations above 3nM mAbs ICR62 and ICR64 reduced the binding of both $^{125}$I-HB-EGF and $^{125}$I-TGF$\alpha$ to EJ cells, but the mAbs were less effective in inhibiting the binding of HB-EGF. For example at 200nM, mAbs ICR62 and ICR64 inhibited the binding of $^{125}$I-TGF$\alpha$ to EJ cells by 88.4% and 84.3%(Fig. 1A) and the binding of $^{125}$I-HB-EGF by 66.9% and 74.8% respectively (Fig. 1B). However we found that the total amount of $^{125}$I-HB-EGF (6700cpm) bound to EJ cells in the absence of the mAbs was substantially higher than that of $^{125}$I-TGF$\alpha$ (2261cpm) (See Fig. 1A&1B). Since HB-EGF has been shown to have

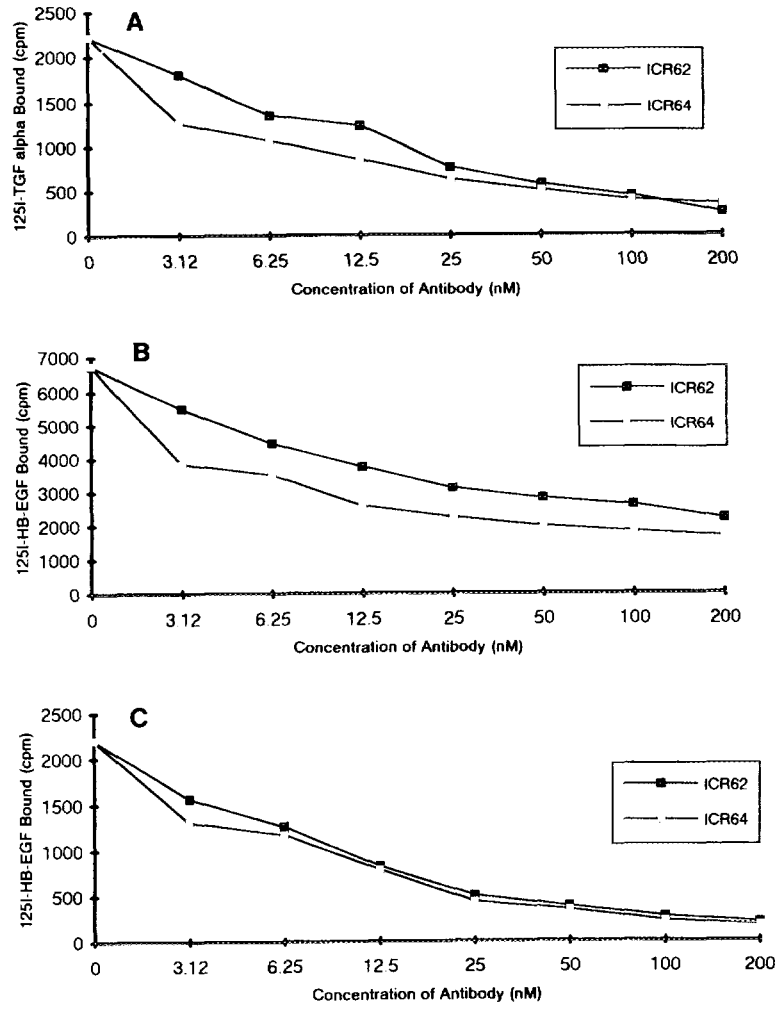


**Figure 1.** Competitive RIAs showing the effect of EGFR mAbs on the binding of $^{125}$I-TGFα (A) or $^{125}$I-HB-EGF (B,C) to the EJ cells. Cells were washed twice with DMEM/2%FCS(A,B) or PBS/2M NaCl (C) before use.

two binding loci on cell surfaces namely heparan sulfate proteoglycan (HSPG) and the EGFR, the increased level of HB-EGF binding to EJ cells could have been due to its interaction with cell surface HSPG. To decrease the interaction between HB-EGF and the cell surface HSPG cells were washed twice with PBS/2M NaCl, prior to addition of $^{125}$I-HB-EGF (10). This resulted in a substantial decrease in the total amount of $^{125}$I-HB-EGF bound to EJ cells to a level similar to that of $^{125}$I-TGFα (Figs. 1A,B and Table 1). Furthermore, in this situtation mAbs ICR62 and ICR64 were more effective in blocking the binding of $^{125}$I-HB-EGF to EJ cells (Fig. 1C, Table 1) and the binding of $^{125}$I-HB-EGF to the EGF receptor was inhibited by 90.7% and 92% in the presence of 200nM of ICR62 or ICR64 respectively (Table 1). On the other hand,

Table 1. Inhibition of binding of 125I-labelled ligands to EJ cells by treatment with mAbs to the EGFR or unlabelled ligands

| Treatment | $^{125}$I-HB-EGF Bound (cpm) | | $^{125}$I-TGFα Bound (cpm) | |
|---|---|---|---|---|
| | Washed with DMEM/2%FCS | Washed with PBS/2M NaCl | Washed with DMEM/2%FCS | Washed with PBS/2M NaCl |
| ICR62(200nM) | 2215 | 203 | 251 | 270 |
| ICR64(200nM) | 1684 | 176 | 348 | 254 |
| Medium | 6700 | 2200 | 2261 | 1837 |
| HB-EGF (100nM) | 1201 | 391 | 411 | 456 |
| TGFα (100nM) | 2383 | 323 | 351 | 279 |
| Medium | 7288 | 2200 | 2444 | 1867 |

Cells were washed twice with either DMEM/2%FCS or PBS/2M NaCl before addition of radiolabelled ligands and/or competitors .

washing of EJ cells with PBS/2M NaCl, led to only a small decrease in the binding of radiolabelled TGFα to these cells (Table 1).

Next, we investigated the effect unlabelled HB-EGF or TGFα on the binding of $^{125}$I-HB-EGF to the EGF receptor on EJ cells. The results presented in Fig. 2 and table 1 show that the binding of $^{125}$I-HB-EGF to EJ cells was inhibited more effectively by unlabelled HB-EGF than by TGFα. However, if EJ cells were washed with PBS/2M NaCl prior to addition of $^{125}$I-HB-EGF, then TGFα was as effective as HB-EGF in blocking the binding of radiolabelled HB-EGF to EJ cells (table 1). Also, we observed that HB-EGF inhibited the binding of $^{125}$I-TGFα to EJ cells (Table 1).

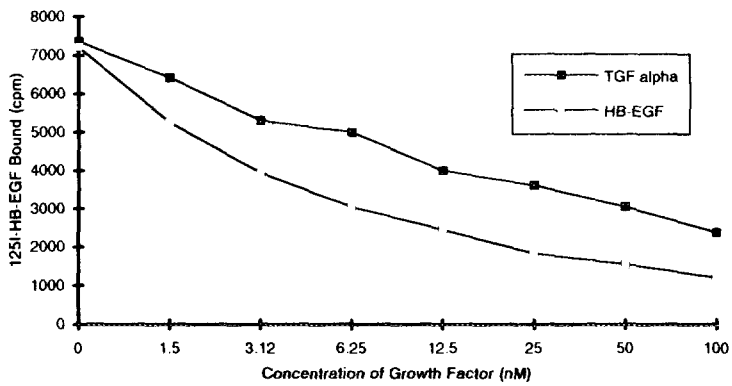


**Figure 2.** Competitive RIA showing the effect of unlabelled HB-EGF or TGFα on the binding of $^{125}$I-HB-EGF to EJ cells. The cells were washed twice with DEM/2%FCS before use.

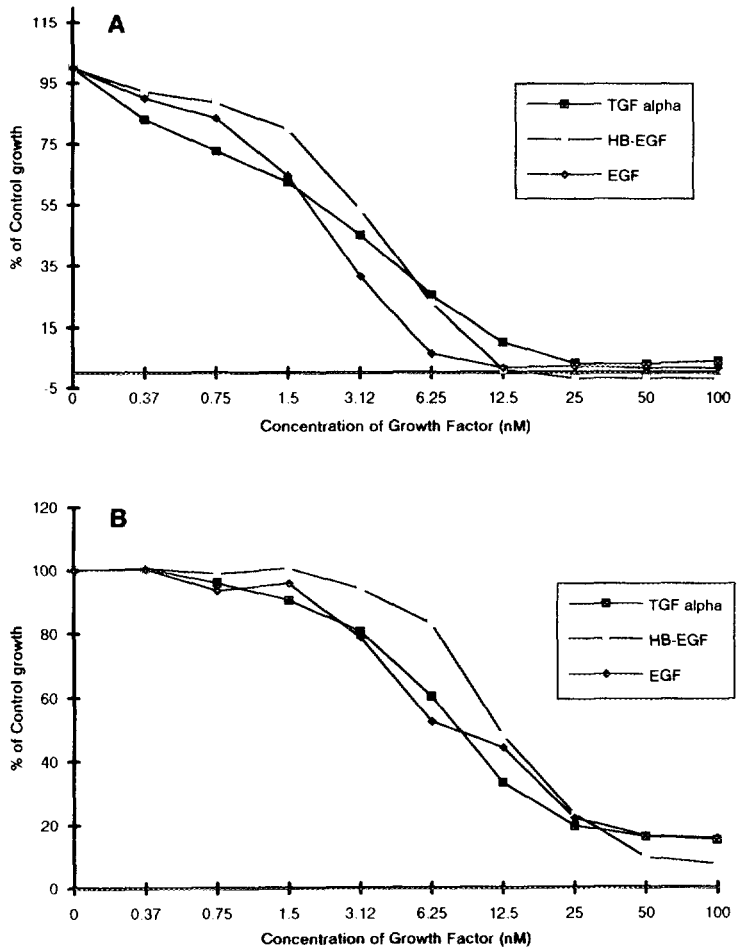


**Figure 3**. Effect of treatment with TGFα, HB-EGF or EGF on the growth in vitro of the head and neck tumour cells lines HN5 (A) or HSC-4 (B). The number of cells were determined using a colourometric assay after incubation for 5 days (HN5) or 7 days (HSC-4).

Finally, we have compared the effect of treatment with EGF, TGFα or HB-EGF on the growth *in vitro* of two head and neck tumour cell lines which overexpress the EGFR namely; HN5 [$1.4 \times 10^7$EGFR/cell, (23)] and HSC-4 [$2.1 \times 10^6$ EGFR/cell, (24)] overexpressing the EGF receptor. At concentrations above 25nM, EGF, TGFα and HB-EGF completely inhibited the growth of HN5 cells (Fig. 3A). Proliferation of HSC-4 was also inhibited by all three ligands, although growth was not completely prevented. Treating with 100nM of HB-EGF, EGF or TGFα was found to inhibit the growth of HSC-4 cells by 92.5%, 84.3% and 84.8% respectively (Fig. 3B).

## DISCUSSION

Despite the structural and biological similarity between the EGF family of ligands, HB-EGF differs from EGF and TGFα in a number of ways. For example, while the mature form of HB-EGF has been shown to have 41% homology at the C- terminal region with other members of the EGF family, it contains up to 86 amino acids which is substantially larger than EGF (53 amino acids) or TGFα (50 amino acids) (5,12). HB-EGF has also been shown to be a far more potent mitogen for SMCs than EGF (5). Moreover, unlike EGF and TGFα, HB-EGF binds tightly to immobilized heparin or cell surface heparan sulfate proteoglycan (HSPG) requiring 1.0- 1.2 M NaCl for elution (5,11). The ability of HB-EGF to bind cell surface HSPG has been postulated to be responsible for the greater potentency of HB-EGF in stimulating the proliferation of SMC cells compared to EGF (5,13,25). The cell surface associated form of HB-EGF has been shown to act as a receptor for diphtheria toxin (10,26)

Here, we have shown that mAbs ICR62 and ICR64, which block the interaction of EGF and TGFα with the EGF receptor on a wide range of tumours, can also inhibit the binding of HB-EGF to the EGFR (Fig.1 (20)). However, in this investigation, substantially more radiolabelled HB-EGF bound to EJ cells than either $^{125}$I-TGFα (Fig. 1A&1B, Table 1) or $^{125}$I-EGF (data not shown). This increase in binding of HB-EGF to EJ cells was most likely due to the ability of this ligand to bind to cell surface HSPG in addition to the EGFR (5, 27-28). Indeed, washing of EJ cells with PBS/2M NaCl, prior to addition of radiolabelled HB-EGF, decreased the total amount of radiolabelled HB-EGF bound to EJ cells by 67% ( i.e to a level similar to that found with radiolabelled TGFα, (Table 1)). Furthermore, EGF at doses that were mitogenic for a variety of human cells including fibroblasts, keratinocytes and certain tumour cells has been shown to inhibit the growth of a number of human tumour cell lines which overexpress the EGF receptor (29-33). Although, HB-EGF has been shown to be mitogenic for BALBc-3T3 fibroblasts, keratinocytes, SMC and human pancraetic tumour cells but not for endothelial cells (5,11-13,34), the results presented here show that HB-EGF, like EGF and TGFα, also inhibits the growth of tumours that overexpress the EGF receptor.

Finally, on the basis of our results we conclude that mAbs ICR62 and ICR64, which act as EGF and TGFα antagonists, should also be effective in inhibiting the HB-EGF induced growth of tumours which overexpress the EGF receptor.

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