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Heparin inhibits enhancing factor/phospholipase A_2 activity and its binding to the cell surface

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Abstract. Enhancing factor (EF), a mouse intestinal phospholipase A_2 (PLA_2), has been isolated and characterized. EF increases the binding of epidermal growth factor (EGF) to A431 cells almost two-fold by interacting with EGF. EF binds to a 100 kDa cell surface receptor and brings about an increase in the binding of EGF. In the present study we demonstrate that EF is a heparin binding protein and at the time of iodination of EF, the heparin binding site of EF has to be protected. Heparin inhibits the enhancing activity of EF as well as the binding of labelled EF to A431 cells. Inhibition of binding of EF to cells by heparin indicates that heparin binding region forms at least part of the receptor binding domain. These data suggest that the receptor for EF on the cell surface could be a heparin sulphate proteoglycan.

Keywords. Epidermal growth factor; enhancing factor; heparin; heparin sulphate proteoglycans.

1. Introduction

Enhancing factor (EF), a 14 kDa polypeptide isolated from mouse small intestines, increases the binding of epidermal growth factor (EGF) to A431 cells (Deo *et al* 1983; Mulherkar *et al* 1986)· It also brings about phenotypic transformation of normal cells in the presence of EGF and stimulates DNA synthesis in EGF-receptorless cells, NR6 (Mulherkar and Deo 1986)· It has been postulated that EF makes EGF available to the cells via its own receptor which has been identified as a 100 kDa cell surface molecule· Recent studies have shown that EF is an isoform of phospholipase A_2 (PLA_2), type II (Mulherkar *et al* 1993a, b)· PLA_2s are hydrolytic enzymes which generate physiologically active products· They have been implicated in digestion (De Haas *et al* 1968), cell proliferation (Arita *et al* 1991) and smooth muscle contraction (Kanemasa *et al* 1992; Sommers *et al* 1992)· Mammalian secretory PLA2s have been proposed to play a key role in the pathogenesis of inflammation (Vadas *et al* 1993)·

In the present study we show that EF/mouse intestinal PLA_2 is a heparin binding protein· During iodination of EF, the heparin binding site of EF has to be protected· Heparin inhibits the increased binding of labelled EGF (enhancing activity) in the radio-receptor assay· Heparin also inhibits the binding of labelled EF to A431 cells· From these studies we postulate that a heparin binding region forms at least a part of the receptor binding domain· The receptor for EF on the cell surface could be a heparin sulphate proteoglycan·

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2. Materials and methods

2·1 *Materials*

Crude intestinal extract and HPLC-purified EF proteins were obtained from Balb/c mice intestines as described earlier (Mulherkar *et al* 1986)· Epidermal growth factor (EGF), N.N-bis[2-hydroxyethyl]-2-aminoethane sulphonic acid (BES), heparin agarose, heparin (porcine intestinal, specific activity 176 USP/mg), and other fine chemicals were purchased from Sigma (USA)· Na-^{125}I was from Amersham (USA)· A431 cells were grown in DMEM supplemented with 5% fetal calf serum (GIBCO BRL, USA) in a 5% CO_2 atmosphere at 37°C·

2·2 *Heparin affinity chromatography of EF*

The procedure as described by Klagsburn *et al* (1987) was followed· Four mg of acid soluble intestinal proteins (crude extract) were fractionated on a 1 ml heparin-agarose column equilibrated with 0·02M phosphate buffer (pH 7·5). The column was washed with the equilibrating buffer and developed with a 0·2–2 M NaCl step gradient in phosphate buffer· One ml fractions were collected, dialyzed against Milli-Q water, lyophilized and resuspended in 100 µl of water· Twenty µl from each fraction were run on a 17·5% SDS-PAGE and silver stained as described by Blum *et al* (1987)· EF activity was determined by testing an aliquot from each fraction in the radio-receptor assay using A431 cells and labelled EGF as described earlier (Deo *et al* 1983)·

2·3 *Effect of heparin on enhancing activity*

Radio-receptor assay was carried out as described earlier (Deo *et al* 1983)· EF (5 µg) was added to 10^4 A431 cells per well in the presence or absence of 10 µg heparin per well· Enhancement was calculated over and above binding of [^{125}I] - E G F to A431 cells in the absence of any additive, which was taken as 100% binding· Specific binding in each case was determined by deducting the non-specific binding obtained in the presence of 100-fold excess cold EGF·

2·4 *Labelling of EF*

Five µg of HPLC purified EF was added to 0 1 ml suspension of heparin-agarose beads. The volume was made to 1 ml with 0·02 M sodium phosphate buffer (SPB), pH 7·5, and allowed to shake overnight at 4°C· The beads were washed with SPB to remove unbound protein, resuspended in 250 µl of the same buffer and added to iodogen coated glass vials along with 500 µCi of [125_I] - N a for 2 min at RT. Unbound radioactivity was removed by giving 3 washes with SPB. Bound labelled EF was eluted by adding 250 µl of 2 M NaCl to the pellet followed by vortexing centrifuging· The elution was repeated 5 to 6 times and the supernatant collected in separate tubes· Maximum counts were seen in first 4 tubes which were pooled and stored after addition of BSA. Specific activity was around 1750 cpm/fmol.

2·5 *Effect of heparin on EF binding*

[^{125}I] - E F (50,000 cpm) was added to fixed A431 cells and kept at RT for 1 h. Binding of the radiolabeled EF was studied in the presence of serially diluted concentrations of heparin ranging from 0·0195IU to 10IU per well· The radio-receptor assay was carried out as described earlier for [^{125}I]-EGF. Non-specific binding, in the presence of 1000-fold excess cold EF, was deducted from every reading to give specific binding·

3. Results and discussion

We have reported earlier that EF, a modulator of EGF, binds to a cell surface receptor and upregulates the number of EGF receptors on A431 cells· The molecular weight of the receptor for EF on A431 cells was determined to be ~ 100 kDa by immunoprecipitation studies (Mulherkar and Deo 1986)· Recently our cross-linking studies with the labelled EF have confirmed that the molecular weight of the EF receptor is ~ 100 kDa (data not shown)· The nature of the EF receptor is under investigation·

3·1 *EF binds heparin with a high affinity*

Since EF is an isoform of PLA_2 (Mulherkar *et al* 1993a, b) and PLA_2 are known to bind heparin (Diccianni *et al* 1990), we investigated whether EF binds heparin· Acid soluble proteins from intestines of Balb/c mice were subjected to heparin affinity chromatography. On SDS-PAGE, the 0·6 M–2 M eluates resolved as 14 kDa bands (figure 1). When tested in the radio-receptor assay, maximum enhancing activity (87%) was observed in

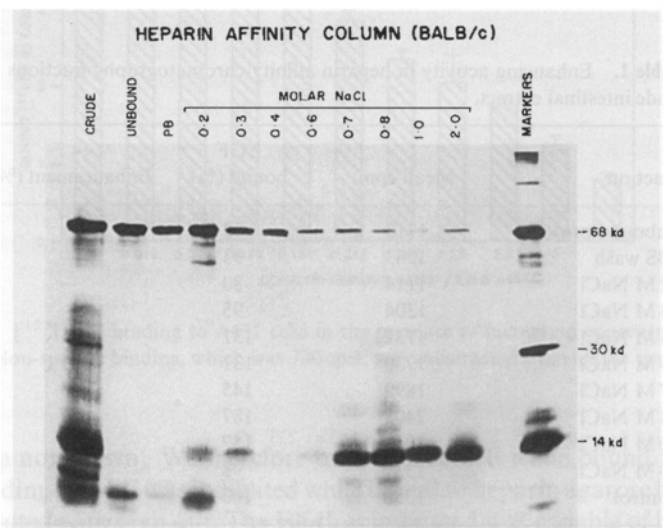

Figure 1. Fractions eluting from heparin affinity column were subjected to SDS-PAGE (17·5% gel) and the proteins visualized by silver staining· 20 µg acid soluble intestinal proteins (crude extract) were run for comparison (PB-PBS wash).

the 0·8 M fraction (table 1). Fractions eluting at 0·4 M and 0·6 M NaCl showed marginal activity. This indicates that EF binds heparin and can be eluted with high salt concentrations. Other group II PLA_2s are also known to bind heparin with high affinity. Human synovial fluid PLA_2 elutes from a heparin affinity column at 0·5 M NaCl while rat platelet PLA_2 activity elutes at 0·6 M -l·1 M KCl (Hara *et al* 1991).

3.2 *Heparin inhibits EF activity*

In the radio-receptor assay, using A431 cells, EF brings about a two-fold increase in the binding of labelled EGF (Deo *et al* 1983; Mulherkar *et al* 1986)· The enhancing activity (EF + EGF, figure 2) is completely abrogated in the presence of heparin (figure 2). Heparin does not interfere with the binding of EGF to the cell surface (~ 1000 cpm of bound EGF without/ with heparin, figure 2). Heparin also inhibits the phospholipase activity of EF (data not shown). Heparin has been implicated in the regulation of PLA_2 activities (Dua and Cho 1994). Hayakawa *et al* (1988) have reported that heparin binds tightly to human secretory group II PLA_2 and regulates its binding to heparin like molecules on the cell surface (Murakami *et al* 1993). Inhibition of binding of type II PLA_2 to HUVEC cells, in the presence of excess heparin, has been attributed to the binding site being a heparan sulphate proteoglycans (Murakami *et al* 1993). In the present study, heparin could be binding to EF and sequestering all the EF molecules; or heparin could be binding to EF and masking the binding site of EF for its membrane receptor·

3·3 *Heparin protects receptor binding site during iodination of the EF molecule*

Ealier attempts to label EF using the conventional methods had been unsuccessful. Although the molecule was labelled (as was seen by SDS-PAGE) it lost its binding

Table 1. Enhancing activity in heparin afinity chromatography fractions of crude intestinal extract·

Fraction	Mean cpm	EGF bound (%)	Enhancement (%)
Unbound proteins	1113	82	—
PBS wash	1283	93	—
0·2 M NaCl	1114	80	—
0·3 M NaCl	1304	95	—
0·4 M NaCl	1732	131	31
0·6 M NaCl	1730	131	31
0·7 M NaCl	1899	145	45
0·8 M NaCl	2408	187	87
1·0 M NaCl	2043	157	57
2·0 M NaCl	1337	98	—
Control	1356	100	—

4mg of acid soluble intestinal proteins from Balb/c mice were eluted stepwise from 1 ml heparin agarose column by a 0·2 to 2·0 M NaCl gradient· The eluates were tested for enhancing activity in a radio-receptor assay using A431 cells and [^{125}I]-EGF.

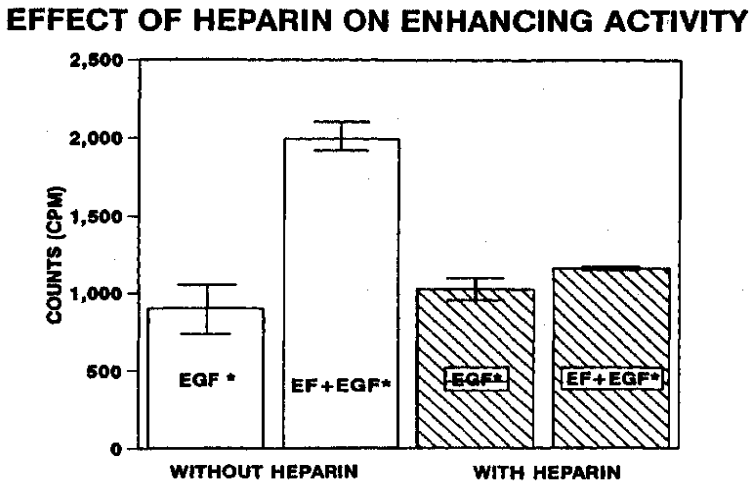

Figure 2. [^{125}I] -EGF binding to A431 cells in the presence/absence of 10 µg heparin, incubated with/without 5 µg EF· Non-specific binding, which was 120 cpm, was subtracted from all the readings·

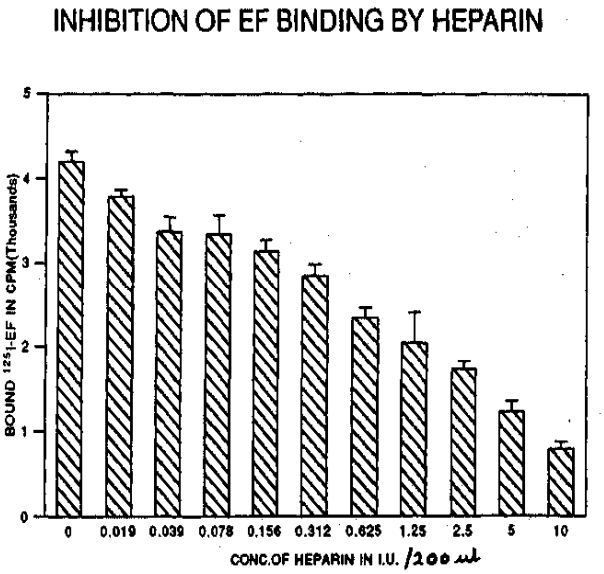

Figure 3. [^{125}I] - E F binding to A431 cells in the presence of increasing concentrations of heparin· Non-specific binding, which was 700 cpm, was substracted from all the readings·

property (data not shown)· We therefore tried to label EF while bound to heparin to protect its binding site· EF was iodinated while bound to heparin agarose beads and the abelled EF eluted with high salt· The EF thus iodinated was capable of binding A431 cells specifically· In the radio-receptor assay heparin was found to block the binding of iodinatedEF to the cells (figure 3)· This indicates that the receptor binding domain of EF is perhaps involved in the heparin binding·

In EF (Mulherkar *et al* 1993b) as well as porcine pancreatic PLA_2 (Hanasaki and Arita 1992) the linear amino acid sequence which satisfies criteria of heparin binding domain (Cardin *et al* 1986) is absent. In anti-thrombin, lipoprotein lipase and FGF-2 heparin binding domains though located on different peptide sequences, are shown to be spatially proximal based on evidence from their crystal structures (Mayo *et al* 1995). It is possible that the heparin binding domain of EF comprises amino acid sequences not adjacent to each other in the polypeptide chain. Thus a conformational change in EF on binding heparin is envisaged· Though the exact nature of the interaction is not known, it does not seem to be a simple non-specific interaction. Kinetic and binding studies have shown that heparin is a potent inhibitor of human $sPLA_2$ and this inhibition is due to a specific interaction of heparin with cationic N-terminal (Dua and Cho 1994). The rat platelet PLA_2 has cationic residues from 54–57 and its heparin binding site is distinct from its catalytic site (Murakami *et al* 1991). EF also has a few cationic residues from 31–37 which could be involved in heparin binding.

Recently two groups have independently reported identification of transmembrane glycoprotein receptors for pancreatic PLA_2 (Ishisaki *et al* 1994) and secretory mammalian PLA_2 (Lambeau *et al* 1994; Ancian *et al* 1995)· Both the receptors have a molecular weight of 180 kDa. Lazdunski and his colleagues have tested EF and shown that EF competes with snake venom PLA_2s-OS1 and OS2, for binding to the PLA_2 receptors on rat brain as well as rabbit muscles (personal communication). Pancreatic $sPLA_2$ as well as snake venom PLA_2s-OS 1 and OS2, which bind to the M-type receptor show a conserved lysine 121 (Lambeau *et al* 1995)· EF also has a lysine at position 121. This indicates that EF could have more than one binding site—the 180 kDa PLA_2 receptor (Lambeau *et al* 1994; Ancian *et al* 1995) and the 100 kDa receptor identified on A431 cells (Mulherkar and Deo 1986). The 100 kDa receptor described earlier (Mulherkar and Deo 1986) could be a H SPG on A431 cell membrane or a PLA_2 receptor with which EF interacts through its heparin binding domain. Lambeau *et al* (1994) have proposed that presence of different PLA_2 receptors in different tissues could explain the tissue specificity of some of the PLA_2 actions. The mouse intestinal EF/PLA_2 bound to HSPGs probably modulates the binding of EGF thus giving the intestinal stem cells a growth advantage.

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