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Trace amounts of enhancing factor/phospholipase A_2 in mouse peritoneal exudate cells

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Abstract. Enhancing factor (EF), a mouse phospholipase A_2 (PLA_2), has been purified from the small intestines, based on its ability to increase the binding of epidermal growth factor in a radioreceptor assay. EF/PLA_2 was found to be localized predominantly in the Paneth cells in the small intestines. Whether mouse intestinal EF/PLA_2 is identical/similar to mouse secretory PLA_2 was to be determined. Phospholipases are known to play a crucial role in the process of inflammation. This paper reports the presence of trace amounts of EF/PLA_2 in the peritoneal exudate cells. Western blot analysis of the acid extracts showed the presence of a 14 kDa immunologically cross-reactive protein. RT-PCR analysis using EF specific primers amplified a ~ 700 bp product which was further confirmed to be EF-specific by nested PCR analysis and sequencing. Presence of EF in the peritoneal exudate cells could be a unique mode of transport of growth factor modulator to the site of injury to aid in regeneration/cell proliferation of damaged tissue.

Keywords. Enhancing factor; phospholipase A_2; inflammation; neutrophil; Paneth cells; EGF.

1. Introduction

Enhancing factor (EF) is a 14 kDa heat and acid stable peptide isolated from the mouse small intestines (Deo *et al* 1983; Mulherkar and Deo 1986)· Immuno-histochemical studies have demonstrated that it is predominantly localized in the Paneth cells of small intestine (Wagle *et al* 1989; Mulherkar *et al* 1991; Desai *et al* 1991) and has been used as a marker for Paneth cells (Bry *et al* 1994)· The full length EF cDNA has been obtained by RAcE-RT-PCR· Based on the predicted amino acid sequence EF has been shown to belong to group-II phospholipase A_2 (PLA_2) (Mulherkar *et al* 1993a, b)· EF shares 74% similarity with rat platelet PLA_2 and 66% similarity with human synovial fluid PLA_2· The PLA_2 activity of EF has been confirmed in a blood agar plate assay as well as assay with micellar substrate [^{14}C] phosphatidyl choline and deoxycholate (Mul herkar *et al* 1993b)·

Inflammation is a localized or systemic protective response elicited by injury to cells or tissues· Secretory, non-pancreatic phospholipase A_2 ($sPLA_2$) has been implicated as one of the important pathogenic agents in both local and systemic inflammatory conditions (Walsh *et al* 1983)· Phospholipases are naturally occurring enzymes of lipid metabolism which selectively remove the fatty acids and the phosphobase from the phospholipid· An extractable Ca^{2+} dependent PLA_2 localized to the granules of human neutrophils has been reported by Rosenthal *et al* (1995)· The present study was

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undertaken to investigate if mouse neutrophils synthesize EF/PLA_2 and whether it is the same as the mouse intestinal EF· Mouse peritoneal exudate cells, a rich source for neutrophils, was used for this study·

2.Materials and methods

2·1 *Animals*

Balb/c mice (2-3 months old) used in this study, were obtained from the Animal House, Cancer Research Institute, Mumbai·

2·2 *Induction of ascites*

Starch (2 ml of 2%) was injected i·p· into Balb/c mice and the peritoneal exudate cells collected at different time points by injecting 5 ml sterile phosphate buffered saline· Cells collected from ~ 50 animals were pooled· An aliquot of the cells were used for Giemsa staining, blood agar plate assay (PLA2 assay) and the remaining for extraction of acid stable proteins·

2·3 *Giemsa staining of exudate cells and blood agar plate assay*

The exudate cells were cytospun onto glass slides, fixed in methanol for 10 min, stained with Giemsa stain for 15 min and observed under the microscope· Blood agar plates were made by mixing fresh human red blood cells with agarose prepared in barbitone buffer pH 7 as described by Habermann and Hardt (1972)· Wells were punched after the agar was set· Samples (either whole exudate cells, acid extracts or purified fractions) were loaded along with appropriate controls (snake venom PLA_2 as well as intestinal EF were used as positive controls and PBS as negative control)· The plates were observed after incubation at 37°C for 14–16 h· Clear zone around the well indicated PLA_2 activity·

2·4 *Acid extraction and fractionation of exudate cell proteins*

The protocol for EF extraction as described by Deo *et al* (1983) was followed to extract acid soluble proteins from the exudate cells· The acid soluble proteins were purified on a Biogel P-100 column as described (Mulherkar *et al* 1986) and the fractions tested in ELISA (as described below)· The positive fractions were pooled and further purified on a RP-HPLC column (Waters), as described earlier (Mulherkar *et al* 1986)· The eluate was monitored at 280 nm, all the peaks collected separately, lyophilized and tested in various assays described below·

2·5 *ELISA and Western blot analysis*

For ELISA,.200 ng of EF protein in PBS was coated overnight at 4°C in 96 well plates. The wells were blocked with 1% skimmed milk in PBS for 2h at 37°C· Primary

antibody (antiserum raised against EF, described in Wagle *et al* 1989) was added at a dilution of 1:5000 followed by second antibody (goat anti rabbit antibody tagged to horse radish perioxide enzyme) at a dilution of 1:500· Intermittent washes were given with PBS containing 5% skimmed milk and 1% Tween-20· The final colour was developed by adding OPD as the substrate prepared in citrate phosphate buffer and the reaction stopped with 2N H2SO4· The plate was read at 492/690 nm on an ELISA reader (Flow Laboratories)·

For Western blot analysis, proteins were separated on a 17·5% SDS-PAGE gel and transferred on to a nitrocellulose membrane in a transblot apparatus (BioRad)· The blot was developed using antibodies against EF at a dilution of 1:5000 and ECL kit (Amersham) as per the manufacturer's instructions·

2·6 *RT-PCR and Southern blot analysis*

Total RNA was extracted from the peritoneal exudate cells by the method of Chomcyznski and Sacchi (1987) and subjected to first strand cDNA synthesis using oligo(dT) as a primer and AMV reverse transcriptase (Amersham)· PCR amplification was carried out using Taq DNA polymerase (Amersham) for 30 cycles at 95°C-1', 55°C-2', 72°C-3' during the last cycle, extension at 72°C was caried out for 10'· Primers used were EFH5' sense (5' CCCAAGCTTGGGCTGACAGCATGAAGGTCC3') and EFX3' antisense (5' GCTCTAGAGCCCAGGACTCTCTTAGGTAGG 3')· For nested PCR, primers EFH5' sense and EF244-277 antisense (5' TCTGGTTTGCAGAACAGG 3') were used· The expected 315 bp product was obtained· The plasmid pUC-EFcomp carrying the full length EF cDNA was used as a control for comparison of the amplified products·Theamplified DNA was subjected to Southern blotting using EF cDNA as a probe·

3. Results

Exudate cells collected at 6, 16, 24, 48, 72 and 96 h post starch injection were stained with Giemsa· There was a change in the cell types from the predominant neutrophils in the early/acute stages to predominant monocytes in the late/chronic stages of inflammation (data not shown)· Cells collected at 16–18 h showed that more than 90% of the cells were neutrophils· These cells were disrupted by sonication and tested in blood agar plate assay for PLA_2 activity (figure 1)· The exudate cells (figure 1,10^6 cells in wells Nos 2 and 4; and 10^5 cells in well No· 3) showed PLA_2 activity as seen by the clear zones around the wells· Snake venom PLA_2, a highly purified protein (50 ng), was used as the positive control (figure 1, well No· 1) and showed high activity· PBS served as the negative control (figure 1, well No· 5)· Partially purified intestinal EF showed a much greater activity compared to exudate cells (data not shown)·

Total acid-soluble proteins, obtained from exudate cells collected from 215 animals injected with starch, were subjected to fractionation on a Biogel P-100 column (figure 2)· Alternate fractions were tested in ELISA· Fractions 19–25 showing maximum immunological cross reactivity (figure 2), were pooled, lyophilized, reconstituted in distilled water and designated as IEP-P100-A· This pool was further fractionated on HPLC column· The peak fractions eluting from the column, monitored at 280 nm, were lyophilized, run on a SDS-PAGE gel and subjected to Western blot

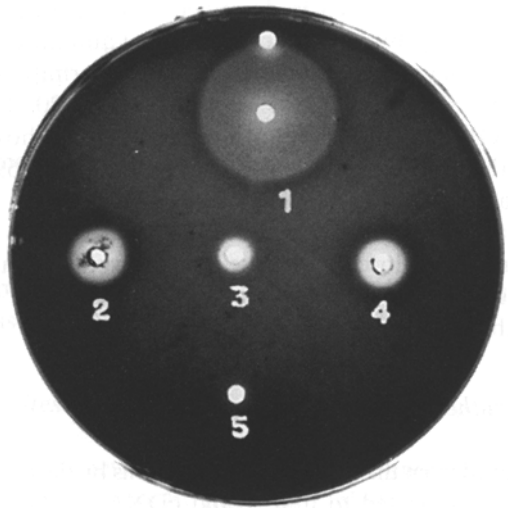

Figure 1· Blood agar plate assay showing PLA_2 activity of exudate cells· 10_6 cells were loaded in well Nos 2 and 4, and 10_5 cells in well No· 3· Well No· 1 was loaded with 50 ng of snake venom PLA_2 (Sigma) and well No· 5 with PBS (negative control)·

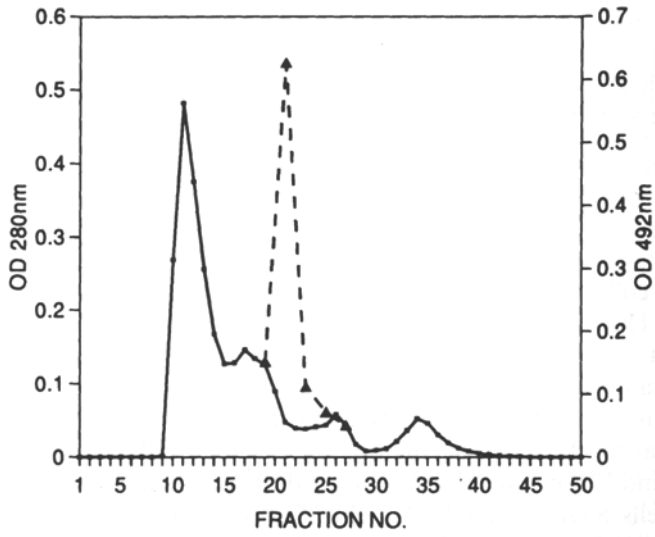

Figure 2. Biogel P-100 profile of the acid soluble proteins extracted from exudate cells· Acid soluble proteins extracted from exudate cells were separated on Biogel P-100 column monitored at 280 nm· The dotted line denotes the fractions showing mmunological cross-reactivity in ELISA read at 492 nm·

analysis (figure 3)· Lane 1 shows intestinal EF-P100 (17·5 µg, the amount loaded was too much in excess), lanes 2, 3, 4 contain fractions of IEP-P100-A pool eluting at different time points from the HPLC column, the autoradiogram showed a 14 kDa band in the HPLC fraction eluting between 26'-32' (in lane 4) corresponding to the

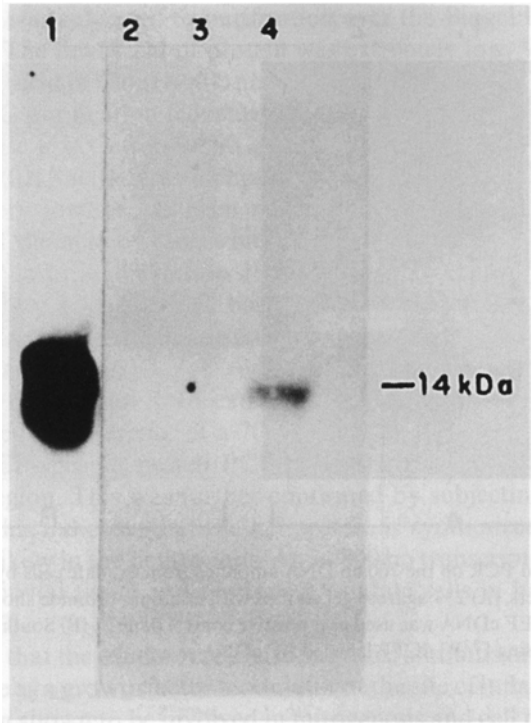

Figure 3. Western blot analysis of HPLC fractions of exudate cell acid soluble proteins· Proteins were separated on a 17·5% SDS-PAGE and subjected to Western blot analysis· Lane 1 shows intestinal EF-P100 (17·5 µg) as a positive control, lanes 2 and 3 contain the HPLC protein peak fractions eluting at 21 min (2·3 µg) 25 min (2·3 µg) respectively and lane 4 shows 14 kDa band in exudate protein HPLC fraction eluting between 26–32 min (4·2 µg)·

control intestinal EF-P100 band in lane 1· The other HPLC fractions in lanes 2 and 3, did not show any cross-reactivity.

Total RNA (5 µg), extracted from exudate cells was subjected to RT-PCR using EF specific primers (EFH5' and EFX3') pUC-EFcomp DNA, containing the full length cDNA, was used as a positive control· RT-PCR resulted in a ~ 700 bp band which corresponded with the positive control (data not shown)· The possibility of non-specific amplification was ruled out by doing nested PCR using internal primers EFH5' and EF244-227 antisense (figure 4A) and subjecting the same to Southern blot analysis (figure 4B)· The gel shows amplification of the expected ~315bp. The amplified product was subjected to direct sequencing and was found to be identical to that of intestinal EF (data not shown)·

4. Discussion

Secretory phospholipase A_2 plays a crucial role in inflammation and in the cascade of events following inflammation (Pruzanski and Vadas 1991)· The first biological response to injury or inflammation is the massive influx of polymorphonuclear

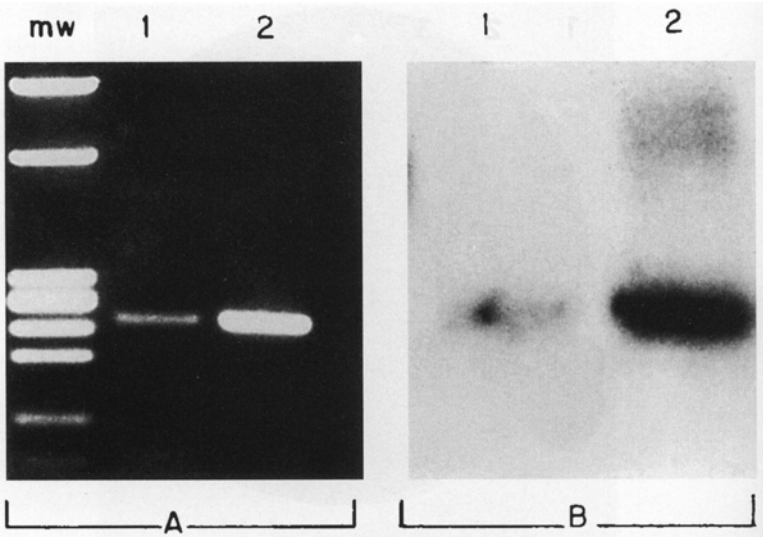

Figure 4. Nested PCR on the 700 bp DNA amplified from exudate cells by RT-PCR using EF specific primers, **(A)** 2% agarose gel stained with ethidium bromide showing a ~ 315 bp product (lane 1)· EF cDNA was used as a positive control (lane 2)· **(B)** Southern blot analysis of the same gel using [^{32}P] dCTP labelled EF cDNA as a probe·

leukocytes (PMNL), especially neutrophils, followed by monocytes/macrophages during the chronic stages of inflammation· Neutrophil dependent tissue damage in inflammation is either because of release of active oxygen species such as O_2^- and H_2O_2, or hydrolytic enzymes, such as proteases and lipases· By the action of PLA_2 rachidonic acid (AA) is released from human neutrophils (Walsh *et al* 1983)· Balshinde *et al* (1988) have shown release of AA in the surrounding medium after neutrophil activation·

An acid stable, calcium-dependent PLA_2 activity has been shown to be present in human neutrophil homogenates (Marki and Franson 1986), membrane fractions (Frans on *et al* 1977) and localized to the granules (Rosenthal *et al* 1995)· Mucosal PLA_2 was shown to be increased in active colitis· It has been suggested that increased intestinal permeability may be a primary factor in activation of mucosal PLA_2 in intestinal inflammation (Olaison *et al* 1989)· Thrombin activated platelets and rabbit peritoneal neutrophils exposed to fMLP have shown release of PLA_2 activity· In case of neutrophils, the release of this enzyme coincides with the release of β-glucoronidase and lysozyme, thereby suggesting a lysosomal granule origin (Mayer and Marshall 1993)·

Here we report the presence of trace amounts of EF protein in the inflammatory peritoneal exudate cells of mouse. Acid stable proteins extracted from the exudate cells (predominantly neutrophils) showed the presence of a 14 kDa protein, cross-reacting immunologically with antibodies to EF· EF has been shown to be localized predomi antly in the Paneth cells of small intestine (Wagle *et al* 1989; Mulherkar *et al* 1991; Desai *et al* 1991; Bry *et al* 1994)· Although its precise role in the intestines has not yet been established, we have speculated that it plays a role in modulating cell proliferation in the intestinal crypts·

The crude extract was subjected to purification over the Biogel P-100 column and RP-HPLC column· The final yield of protein was extremely low· Wright *et al* (1990) have reported a final yield of 6 mg (~ 400 nmol) of the 14 kDa PLA_2 starting from 10^{11} PMNCs after HPLC purification (constituting just 9% of the total cellular PLA_2). In a blood agar plate assay after P-100 as well as HPLC purification the protein showed decreased PLA_2 activity as compared to the crude protein· The enhancing activity was also very low· It has been reported in literature that contamination of some sediment of the acid extract, which was lipid extractable, suppresses phospholipase activity (Marki and Franson 1986)· *In vitro* inhibition of PLA_2 activity by *cis*-unsaturated fatty acids has also been shown and thought to be mediated by the formation of an enzymatically inactive enzyme-substrate inhibitor complex (Raghupathi and Franson 1992)·

RT-PCR analysis of the total RNA extracted from exudate cells using EF specific primers demonstrated the presence of a 700 bp transcript· To further verify that the PCR product was EF-specific, nested PCR analysis was carried out using primers flanking a 315 bp region· This was further confirmed by subjecting it to sequencing (data not shown)· This indicates that the EF protein is synthesized in the peritoneal exudate cells, most likely in the neutrophils· An ~ 800 bp transcript has been reported from inflammed synovial tissue and peritoneal exudate cells in bacterial peritonitis (Seilhamer *et al* 1989).

We conclude here that the exudate cells harbour trace amounts of EF/PLA_2 protein· Its physiological role as a growth factor modulator at the site of inflammation is not yet clear· PLA_2 has been shown to be involved in mitogenesis and cell proliferation (Arita *et al* 1991)· Earlier we have shown that EF modulates the action of EGF (Mulherkar and Deo 1986)· Its presence in the peritoneal exudate cells could establish a novel mode of transport of crucial growth factor modulators in repair following injury/ inflammation·

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