Characterization of the migration-promoting factor of Placentrex
5.1. Introduction.

Human Placental extract (Plx) has been well documented as having wound healing capacity since a long time (Wu et al, 2003). However, the exact molecular mechanism of placental extract on wound healing still remains elusive. It is known that neutrophils are the first cells which migrate to the wound site within minutes of injury. Their prime job is to clear up the invading bacteria by virtue of their phagocytic property. Further, neutrophils are also the source of pro-inflammatory cytokines, which serve as signal to activate the local fibroblasts and keratinocytes (Martin, 1997). So, neutrophil migration is perhaps one of the most critical steps for a normal wound healing process.

Neutrophils have the ability to migrate directionally when exposed to gradient of chemoattractant. This chemotactic response is initiated when chemoattractants bind cell surface cognate receptors and activate a wide range of signal transduction cascade, leading to cellular polarization and culminating in phenotypic migration (Parent, 2004). The acquisition of polarity is accompanied by a dramatic redistribution of cytoskeleton component, where F-actin and numerous actin binding proteins are enriched at the leading edge of the cell (Bagorda et al, 2006).

In the present study, we set out to find out the factor(s) of human placental extract which cause(s) neutrophil chemotaxis, enroute wound healing. We report the identification of glutamate as the key heat stable neutrophil chemotaxis inducing factor of human placental extract.
5.2. Results.

5.2.1. Plx causes human neutrophil migration.

Placental extract is known to cause wound healing for a long time (Wu et al., 2003). Neutrophil migration to the wound site is perhaps the rate-determining step for a normal wound healing process (Martin, 1997). So, we tried to find out whether Plx causes neutrophil migration, enroute wound healing process. To determine neutrophil migration, transwell assays were performed. A dose dependent bell shaped curve revealed a sharp-increase followed by a decrease in migration, with increasing dose of Plx. The optimum dose for neutrophil migration was found to be 20μl of Plx.

![Graph](image)

Fig. 5.1. Effect of Placental extract on the chemotaxis of Neutrophils. Neutrophils were left untreated or treated with 10 to 40μl of placental extract for 2 hours at 37°C. Data represents mean of 3 independent experiments.
5.2.2. Identification of the migration promoting factor of Plx.

To find out the factor(s) of Plx, which cause(s) human neutrophil migration, a gel filtration chromatography was performed.

**Fig 5.2.1. Size fractionation of the migration promoting factor of placental extract.** A 200µl sample of placental extract was fractionated on a superdex 200-10/300GL gel filtration column as described in methods. The UV absorbance at 210 nm, representing the absorption maxima of glutamate was shown with the position of glutamate (2mg/ml), along with the chromatogram.

Individual fractions (after 20, 100 and 200 fold dilutions) of the gel filtration were analyzed for migration in transwell assays. Only two fractions (12 and 13) tested positive for migration promoting activity.
Fig 5.2.2. Migration profile of the fractions collected from the Gel filtration of placental extract. Each fraction (with further dilutions) was assayed for cell migration on human neutrophils. The chemotaxis assay was performed for 2 hours at 37°C. Data represents mean of 3 independent experiments.

As the active fractions were towards the end of elution (fraction 12 and 13) that coincided with Vitamin B12 (Mr 1350), we speculated it to be a small molecule. Dialysis with benzoylated tubing (2kDa exclusion limit) completely abrogated the migration promoting activity of fractions (12 and 13) that reconfirmed the small nature of the active fractions.

Fig. 5.2.3. Small nature of the migration promoting factor of placental extract. The migration promoting fractions (fraction 12 and 13) collected from gel filtration of placental extract were dialysed overnight against water with benzoylated tubing (sigma) and assayed for cell migration on neutrophils. The chemotaxis assay was performed for 2 hours at 37°C. Data represents mean of 3 independent experiments.
HPLC for free nucleotide/nucleoside of the active fractions with Cosmosil C18 column (Shimadzu) did not give much conclusive clue. For further classification of the small molecules present in those two fractions, all the fractions were tested with Ninhydrin (0.1%), which is known to react only with free amino groups. Once again, only fractions 12 and 13 tested to be ninhydrin positive (data not shown). So, the migration promoting factor, present in those two fractions falls in the category of amino acids/very small peptides. Further characterizations of the amino acids of those two fractions by HPLC (as described in method section 3.25) revealed the presence of glutamic acid to be the predominant amino acid, present at a concentration of 280\mu M in Plx.

Fig 5.2.4 HPLC of placental extract for free amino acid profile.
The migration promoting, ninhydrin positive fractions (12 and 13) were separated using cation exchange styrene-divinyl benzene sulfonic acid column as described in methods. Twenty standard amino acids were run individually to know the retention time. Thereafter fractions 12 and 13 were run. There was an absence of amino acids Proline and Cysteine in the placental extract fractions.
5.2.3. *Glutamate causes neutrophil migration*

To investigate glutamate's potential as the key migration promoting factor of Plx, the following tests were performed. First, transwell assays were performed with glutamate. A dose dependent bell shaped curve revealed a gentle increase followed by a decrease in migration, with increasing dose of glutamate. Optimum dose for migration was found to be 8μM.

![Graph showing cell migration](image)

**Fig 5.3.1. Glutamate induces neutrophil migration (Chemotaxis).**

The migration assay was performed either by using untreated cells (4×10⁵ cells/well) or with different doses of glutamate for 2 hours. Glutamate was added in the lower chamber. fMLP (10⁻⁶M) was used as positive control. Data represents mean of 3 independent experiments.

However, glutamate when added in equal concentration to both the wells also induced migration (chemokinesis) although it was less than when added only to lower chamber (Chemotaxis).
Fig 5.3.2. Glutamate induces neutrophil migration (Chemokinesis).

The migration assay was performed either by using untreated cells (4×10^5 cells/well) or with different doses of glutamate for 2 hours. Glutamate was added in equal concentration in both the chambers. Data represents mean of 3 independent experiments.

Second, F-actin polymerization assays with Alexa 488 Phalloidin were performed with glutamate (8μM) in a time dependent fashion. F-actin polymerization peaked at 1 min, thereafter it decreased with time.

Fig 5.3.3. Increased F-actin synthesis by glutamate.

Neutrophils were left untreated or treated with 8μM of glutamate for specified time at 37°C. Cells were fixed at the time points shown by addition of 10 vol of 4.6% paraformaldehyde in PBS. Treatment with carrier (water) did not stimulate F-actin synthesis. Results are expressed as mean cellular fluorescence (MCF) with the...
baseline florescence arbitrarily assigned a value of 100%. Data represents mean of 3 independent experiments.

Third, time lapse video microscopy (span 500 seconds), was performed, in a gradient of glutamate (8μM) generated by a micropipette. Before stimulation by chemoattractant, neutrophils lack polarity. Between 0 sec and 120 sec of exposure to glutamate, supplied through a micropipette, neutrophils begin to extend their surface towards the pipette. The process became more pronounced during the later stages of exposure (between 120 and 500 second). Only the neutrophil surface directed up the chemotactic gradient, ruffles and extends as neutrophils became polarized in the direction of the micropipette.

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<th>Time (in secs.) after Glutamate addition</th>
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Fig 5.3.4 Polarisation of a neutrophil in response to a gradient of glutamate (a-e)
Images of an unpolarised neutrophil responding to a micropipette containing 8μM glutamate (red arrow) at a 0 sec, b 120 sec, c 240 sec, d 360 sec, e 500 sec

There was also a polarization of the actin cytoskeleton when the neutrophils were stimulated by a uniform concentration of glutamate (8μM). Most glutamate treated
cells (70-75%) showed a polarized asymmetric organization of F-actin producing a pseudopod-like protrusion of F-actin at one pole of the cell (leading edge).

Fig 5.3.5 Glutamate induces actin cytoskeleton polarization.
Neutrophils were stimulated for specified time with 8 μM glutamate with a uniform stimulus concentration. Furthermore, they were fixed and stained with alexa-488-phalloidin and finally images were captured in confocal microscope. After stimulation with glutamate neutrophils showed a distinct polarization of the actin cytoskeleton towards one side of the cell.
5.3. Conclusion.

Human placental extract has long been shown to be an efficient wound-healing agent. Rapid migration of neutrophils to the wound site is a prerequisite to the wound healing process. Gel filtration analysis of heat-treated placental extract gave the initial cue to the small nature of the migration-promoting factor of the extract. HPLC analysis of the extract revealed glutamate to be the predominant free amino acid. Our studies show that the amino acid glutamate at an optimum concentration of 8μM induced phenotypic neutrophil chemotaxis, as seen in time lapse microscopies and transwell assays. Glutamate was also found to induce neutrophil chemokinesis, though chemotaxis was more pronounced than chemokinesis. The glutamate induced chemotaxis was accompanied by actin cytoskeleton polarization and F-actin polymerization. These data indicate that glutamate exhibits a very novel chemotactic functionality that opens up new avenues for the investigation of molecular basis of chemotaxis.