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ORIGINAL ARTICLE

Glutamate is the chemotaxis-inducing factor in placental extracts

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Abstract Human placental extracts are known to help wound healing. 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 glutamate at an optimum concentration of 8 μM induced phenotypic neutrophil chemotaxis, as seen in the time lapse and transwell assays. Glutamate was also found to induce chemokinesis of the neutrophil, though the stimulation of chemotaxis was more pronounced. The glutamate induced chemotaxis was accompanied by polarization of the actin cytoskeleton, and by polymerization of F-actin. These data indicate that glutamate has a strong chemotactic functionality in the neutrophil, which could be of interest both therapeutically and in further investigation of the molecular basis of chemotaxis.

Keywords Chemoattractant · Migration · Polarization · Neurotransmitter

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Abbreviations

F-actin	Filamentous actin
GPCR	G-protein coupling receptor
HBSS	Hanks balanced salt solution
OPA	*O*-phthalaldehyde
MCF	Mean cellular fluorescence
Plx	Human placental extract

Introduction

Human placental extract (Plx) is known to have wound healing capacity (Wu et al. 2003). In fact, the use of this extract in healing wounds finds mention in Chinese folklore. However, possible molecular mechanism(s) in the activity of the placental extract on wound healing still remain elusive. It is known that neutrophils migrate to the wound site within minutes following an injury. Their principal job is to clear up the invading bacteria by phagocytosis. However, neutrophils are also the source of pro-inflammatory cytokines, which activate the local fibroblasts and keratinocytes (Martin 1997). Thus the neutrophil migration could be one of the critical steps in a normal wound healing process.

Neutrophils have the ability to migrate directionally when exposed to gradients of chemoattractants. This chemotactic response is initiated by binding of chemoattractants to the cognate cell surface receptors. This activates a wide range of signal transduction cascades, leading to cell polarization and culminating in the phenotypic migration (Parent 2004). The acquisition of polarity is accompanied by a dramatic redistribution of the cytoskeletal components, enriching F-actin and numerous actin-binding proteins at the leading edge of the cell (Bagorda et al. 2006).

The present study examined, as an index of wound healing activity, some of the possible factors of a human placental extract that can cause the neutrophil chemotaxis. We identified glutamate in the extract as the key heat-stable factor inducing chemotaxis of the neutrophil.

Materials and methods

Preparation of the human placental extract

Freshly delivered placentas collected from local hospitals were screened for HIV, HAV and HBV viruses. The screened placentas were denuded of the outer fatty membrane and umbilical chord, and washed thoroughly by sterile water. The tissue was then minced in a grinder. The minced tissue was mixed with ten volumes of water in a sterile flask and kept in a water bath at 90°C for 45 min. After cooling, the solution was filtered through Whatman filter grade 1 and the filtrate was again autoclaved as above, to yield the final placental extract (Plx).

Size fractionation of the migration promoting factor in the human placental extract

The placental extract (Plx) was fractionated on a Superdex 200-10/300GL column with 10 × 300-mm bed dimensions and 24-ml bed volume (Amersham Bioscience, Uppsala, Sweden). The column was equilibrated and eluted by sterile water. The elution positions were calibrated using Bio-Rad gel filtration standard marker for the molecular weight range of 1.3–670 kDa. Fivefold concentrated Plx (200 µl) was run through the column with 24 ml of autoclaved water (bed volume), and 1-ml fractions were collected. Upon being diluted as necessary, each fraction was assayed for the ability to induce the migration of human neutrophils.

HPLC for free amino acids of the human placental extract

The separation used a strongly acidic cation exchange resin, styrene-diviny1 benzene copolymer with sulfonic acid (ISC-07/S1504, sodium type, Shimadzu, Tokyo, Japan).The system employed a post-column derivatisation of amino acids with *o*-phthalaldehyde (OPA) which was detected by a fluorescence detector (model, RF-10AXL, Shimadzu). The elution employed three mobile phases in a gradient fashion. Phase A consisted of sodium citrate, ethanol and perchloric acid (active sodium ion concentration 0.2 N) phase B contained sodium citrate and sodium hydroxide(active sodium ion concentration 0.6 N) and phase C was sodium hydroxide (active sodium ion concentration 0.2 N).The sodium ion was used to exchange $^{+}NH_3$ (of the amino acid). This separation is based on a personal communication from Dr. J. Csapo (Faculty of Animal Science, Institute of Chemistry, University of Kaposvar, Kaposvar, Hungary; email csapo@mail.atk.u-kaposvar.hu).

The chromatographic conditions were as follows: 0–17 min, 100% of solvent A; 17–40 min, 84% of solvent A 16% of solvent B; 40–50 min, 40% of solvent A, 60% of solvent B; 50–60 min, 100% of solvent B; 60–65 min, 100% of solvent C; 65–90 min, 100% of solvent A. Twenty standard amino acids were run individually to calibrate the retention times. This calibration was followed by chromatography of Plx to determine the amino acid profile of the extract.

Preparation of human neutrophils

Human neutrophils were prepared by the procedure of (Badwey et al. 1982) from freshly drawn blood of healthy volunteers. Briefly, the purification included sedimentation of leukocytes from whole heparinized blood after addition of 6% dextran (containing 0.9% NaCl) (Amersham Biosciences, Uppsala, Sweden), followed by centrifugation through Ficoll-Paque, (Amersham Biosciences, Uppsala, Sweden), and finally hypotonic lysis of the contaminating erythrocytes. The cells were resuspended in Krebs–Ringer HEPES buffer (118 mM NaCl, 4.8 mM KCl, 25 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.5 mM glucose). The purity of the neutrophils was checked microscopically. Blood was collected from healthy volunteers in accord with the guidelines of the Committee on Ethics in Experimentation, University of Calcutta.

Chemotaxis and chemokinesis assay

Chemotaxis assays were performed using transwell plates (Corning, NY, USA) as described by Dominique Filippi et al. (2004). Glutamate or carrier (water) diluted in HBSS (Hanks balanced salt solution, containing Ca^{2+} and Mg^{2+}) (Hyclone,South Logan, UT) were placed in the lower chamber (for examination of chemotaxis), or in both chambers (to follow chemokinesis). The neutrophils in HBSS were loaded in the upper wells, at 4×10^5 cells per well. The upper and lower wells were separated by a 3-µm pore size polycarbonate filter, which passes the neutrophils, but not the macrophages. The plate was incubated at 37°C for 2 h. After recovery, the upper chamber side of the filter was wiped to remove the nonmigrated cells, and the filter was fixed and stained with the Giemsa stain. The migrated cells on the reverse side of the filter were counted under an inverted microscope.

The assay of F-actin

The flow-cytometric quantitation of the relative amount of filamentous actin per neutrophil was done as in Roberts et al. (1999). Neutrophils (aliquots of 10^6 cells in HBSS) were fixed after the specified time of incubation by the addition of 10 vol of PBS containing 4.6% paraformaldehyde. The fixed cells were permeabilised with 0.1% Triton X-100 in PBS for 5 min, washed, and then incubated with 100 nM Alexa 488 phalloidin for 30 min. After that, the cells were washed three times with PBS and analyzed on a BD Science FACS Calibur. 10,000 neutrophil events were counted and the results are expressed as mean cellular fluorescence (MCF).

Immunoflorescence

Immunofluorescence of the neutrophils was used to determine the polarization of the actin cytoskeleton following the procedure of Dominique Filippi et al. (2004). Neutrophils (3×10^5 cells) were plated on glass cover slips coated with fibronectin (0.1–1 mg ml). The cells were then stimulated by a uniform concentration of glutamate or carrier(water) for a specified time, fixed with 3.7% paraformaldehyde for 20 min, followed by permeabilisation with 0.1% Triton X-100. The cells were then stained with 10 units/ml Alexa-488 phalloidin for 20 min, and washed three times with PBS. Finally, images were captured at 63× magnification in a confocal microscope.

Time lapse video microscopy

The pH of the glutamate solution used for the above experiments was 6.0, the same as the pH of the placental extract.

Time lapse video microscopy was performed according to Servant et al. (2000) to detect the real-time changes on the surface of living neutrophils upon addition of glutamate. The cells were imaged after point source stimulation from a micropipette with an opening of 0.5 μm (Eppendorf Femtotips). The micropipette was back-loaded with 8-μM glutamate or with carrier (water). The micropipette was positioned as necessary, and a chemotactic gradient was generated with passive diffusion from the tip. Images were captured every 10 seconds at 100× magnification.

Results

The placental extract causes neutrophil migration

Neutrophil migration to the wound site could be the rate-determining step in a normal wound healing process (Martin 1997). We therefore tried to find out whether Plx causes neutrophil migration, as an indicator of the wound healing capability. Transwell assays were performed to determine the neutrophil migration. The dose dependence was bell-shaped, with a sharp increase followed by a decrease in migration (Fig. 1). The optimum dose for neutrophil migration was found to be 20 μl of Plx.

Identification of the migration promoting factor of the placental extract

To find out the factor(s) of Plx, which cause(s) human neutrophil migration, a gel filtration was performed (Fig. 2a). After 20-, 100- and 200-fold dilutions, individual fractions were analyzed for migration in transwell assays. Only fractions 12 and 13 tested positive for migration promoting activity (Fig. 2b). As the active fractions were towards the end of elution (fraction 12 and 13), coincident with vitamin B12 (Mr 1350), the active compounds obviously were small molecules. Dialysis with benzoylated tubing (2-kDa exclusion limit) completely abrogated the migration promoting activity of fractions 12 and 13 (Fig. 2c), which confirmed the small molecular size of the active material. HPLC for free nucleotide/nucleoside of the active fractions did not give much conclusive clue (data not shown). For further classification of the small molecule present in those two fractions, all fractions were tested with ninhydrin (0.1%), which is known to react only with free amino groups. Only fractions 12 and 13 were ninhydrin positive (data not shown). The migration promoting factor(s) present in those two fractions therefore must be amino acids or very short peptides. Characterization of these two fractions by HPLC revealed glutamic acid as the predominant component, with retention time of 20.21 min (Fig. 2d), present at a concentration of 240 μM in Plx. When subjected to gel filtration under identical conditions,

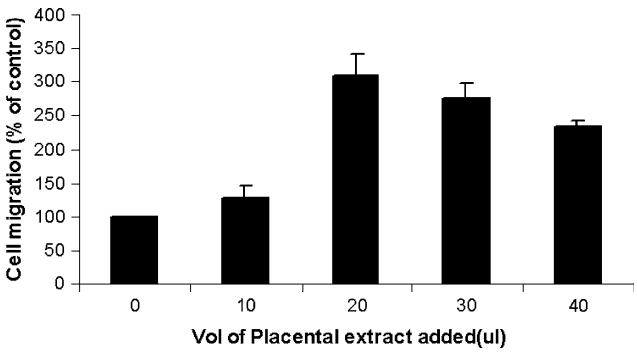
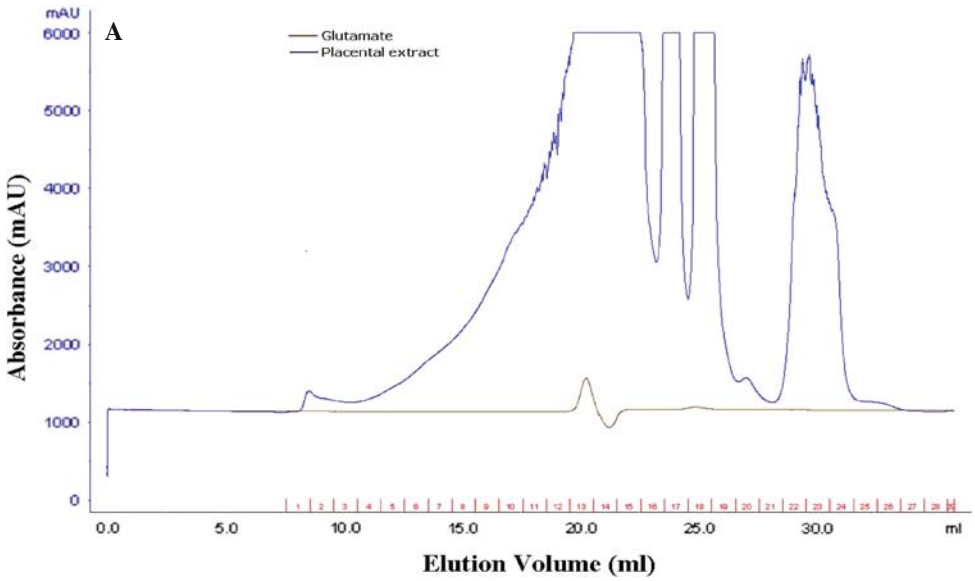
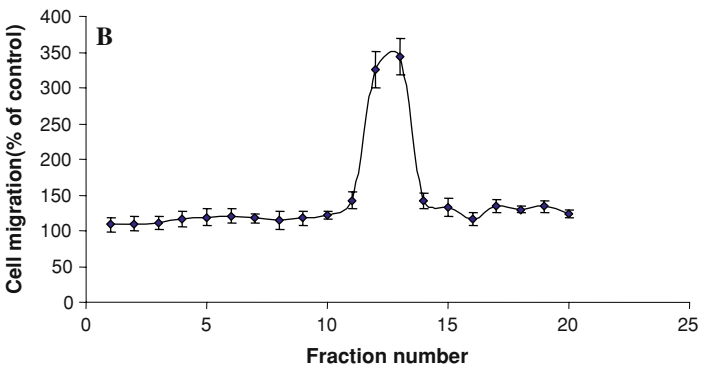
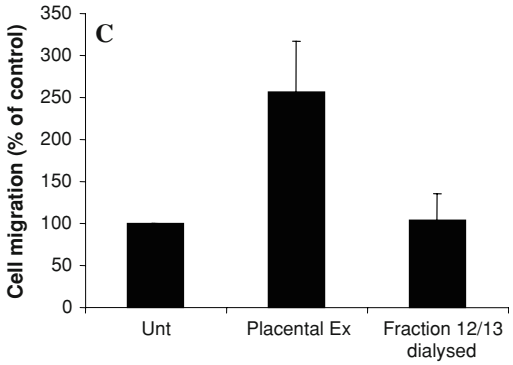
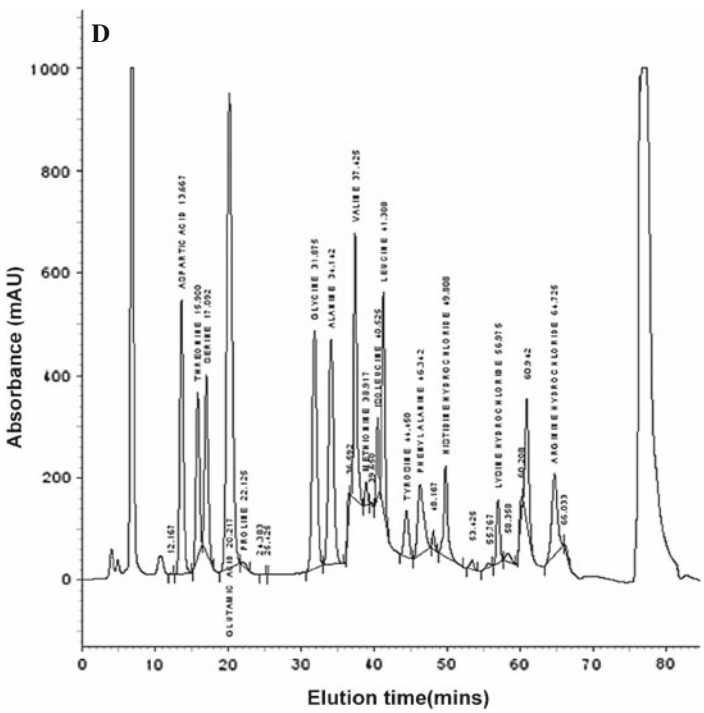


Fig. 1 Effect of the placental extract on the chemotaxis of neutrophils. Neutrophils were left untreated, or were treated with 10–40 μl of the placental extract for 2 h at 37°C. Data represents mean of three separate experiments


A
mAU
Glutamate
Placental extract
Absorbance (mAU)
Elution Volume (ml)
ml


B
Cell migration(% of control)
Fraction number


C
Cell migration (% of control)
Unt
Placental Ex
Fraction 12/13 dialysed


D
Absorbance (mAU)
Elution time(mins)

◀ **Fig. 2** Characterization of the neutrophil migration factor of the placental extract. All results are means of three independent experiments. **a** Size fractionation of the migration promoting material. A 200-µl sample of the placental extract was fractionated on a Superdex 200-10/300GL gel filtration column (10 × 300-mm bed dimensions and 24-ml bed volume) as described in methods. Fractions were assayed (after serial dilutions of 20-, 100- and 200-fold) for cell migration on neutrophils. The UV absorbance was at 210 nm, representing the absorption maxima of glutamate. A chromatogram of glutamate (2 mg/ml), was shown superimposing along with the placental extract chromatogram. **b** Migration profile of the fractions collected from the gel filtration of the placental extract. Each fraction (with further dilutions) was assayed for cell migration on human neutrophils. The chemotaxis assay was performed for 2 h at 37°C. **c** The migration promoting factor of the placental extract is a small molecule. The migration promoting fractions (fraction 12 and 13) collected from gel filtration of the placental extract were dialysed overnight against water using benzoylated tubing (Sigma) and assayed for cell migration on neutrophils. The chemotaxis assay was performed for 2 h at 37°C. **d** HPLC of placental extract for free amino acid profile. The migration promoting, ninhydrin positive fractions (12 and 13) were separated using cation exchange styrene-diviny1 benzene sulfonic acid column as described in Sect. "Materials and methods". Twenty standard amino acids were run individually to determine the retention time. Thereafter fractions 12 and 13 were run. Proline and cysteine were not detected in the placental extract fractions

glutamic acid (3 mg/ml) also eluted in fractions 12 and 13 (Fig. 2a).

Glutamate causes neutrophil migration

To investigate glutamate's potential as the key migration promoting factor of Plx, the following tests were done. 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 (Fig. 3a). Optimum dose for migration was found to be 8 µM. However, glutamate added in equal concentration to both wells also induced migration (chemokinesis) although this was less than for glutamate addition to lower chamber only (Fig. 3b). Second, F-actin polymerization assays were performed with glutamate (8 µM) over a period of 30 min. F-actin polymerization peaked at 1 min, and then decreased with time (Fig. 3c). Third, time-lapse video microscopy (span 500 s) was performed in a gradient of glutamate (8 µM) generated from a micropipette. Before stimulation by a chemoattractant, neutrophils lack polarity. Between 0 and 120 s of exposure to glutamate, neutrophils begun extending their surface towards the pipette. The process became more pronounced with further exposure (between 120 and 500s). Only the neutrophil surface directed up the chemotactic gradient, ruffling and extending as neutrophils became polarized in the direction of the micropipette (Fig. 3d).

There was also a polarization of the actin cytoskeleton when the neutrophils were stimulated by a fixed concentration of glutamate (8 µM). A majority of glutamate-treated cells (70–75%) showed a polarized asymmetric organization of F- actin producing a pseudopod-like protrusion at one pole of the cell (Fig. 3e).

Discussion

Placental extracts have long been known to be efficient in wound healing (Wu et al. 2003). Neutrophil migration is the most critical step for normal wound healing process (Martin 1997). In fact, previous reports have shown wound healing to be severely delayed with impaired neutrophil migration (Peters et al. 2005). This justifies our choice of neutrophil chemotaxis as the model for studying the wound healing facet of the placental extract. Trophoblastic cells of the placenta express several potent growth factors, including placental growth factor, epidermal growth factor, vascular endothelial growth factor, and transforming growth factor. They also express various matrix metalloproteases like MMP-2, MMP-9, MMP-11, urokinase plasminogen activator, and many peptide messengers, such as somatostatin, endorphin, enkephelin and tumor necrosis factor (Ferretti et al.2007). Most of these factors are potent stimulators of cell migration and proliferation. Placental extract is thus a rich source of these stimulatory molecules.

The placental extract preparation involves heat extraction (90°C, 45 min,) and terminal sterilization by autoclaving (see Sect. "Materials and methods"). Based on this, the migration promoting key factor(s) was speculated to be heat-stable. The HPLC analysis of the free amino acid profile showed glutamate to be present at a high concentration in the placental extract, as expected (e.g. Philipps et al. 1978).

By transwell assays and time lapse microscopy, we find that glutamate can promote neutrophil chemotaxis. The observation that glutamate promotes the neutrophil chemotaxis is novel, but not unexpected. There are previous reports of glutamate causing chemotaxis in the T-lymphocyte and the monocyte (Malone et al. 1986; Ganor et el 2003; Sarchielli et al. 2007) Glutamate is primarily known for its important neuroexcitatory activity in central nervous system. During development of the nervous system, glutamate regulates proliferation, migration and survival of neuronal progenitors and immature neurons. Thus, NF-κB has been shown to be activated by glutamate in the cerebral granule neuron during synaptic activation (Guerrini et al. 1995). The glutamate signaling has long been assumed to be restricted to the central nervous system and the immune system (Boldyrev et al. 2005), but a growing body of evidence documents that non-neural cells also possess glutamate receptors. This includes bone osteoblasts,

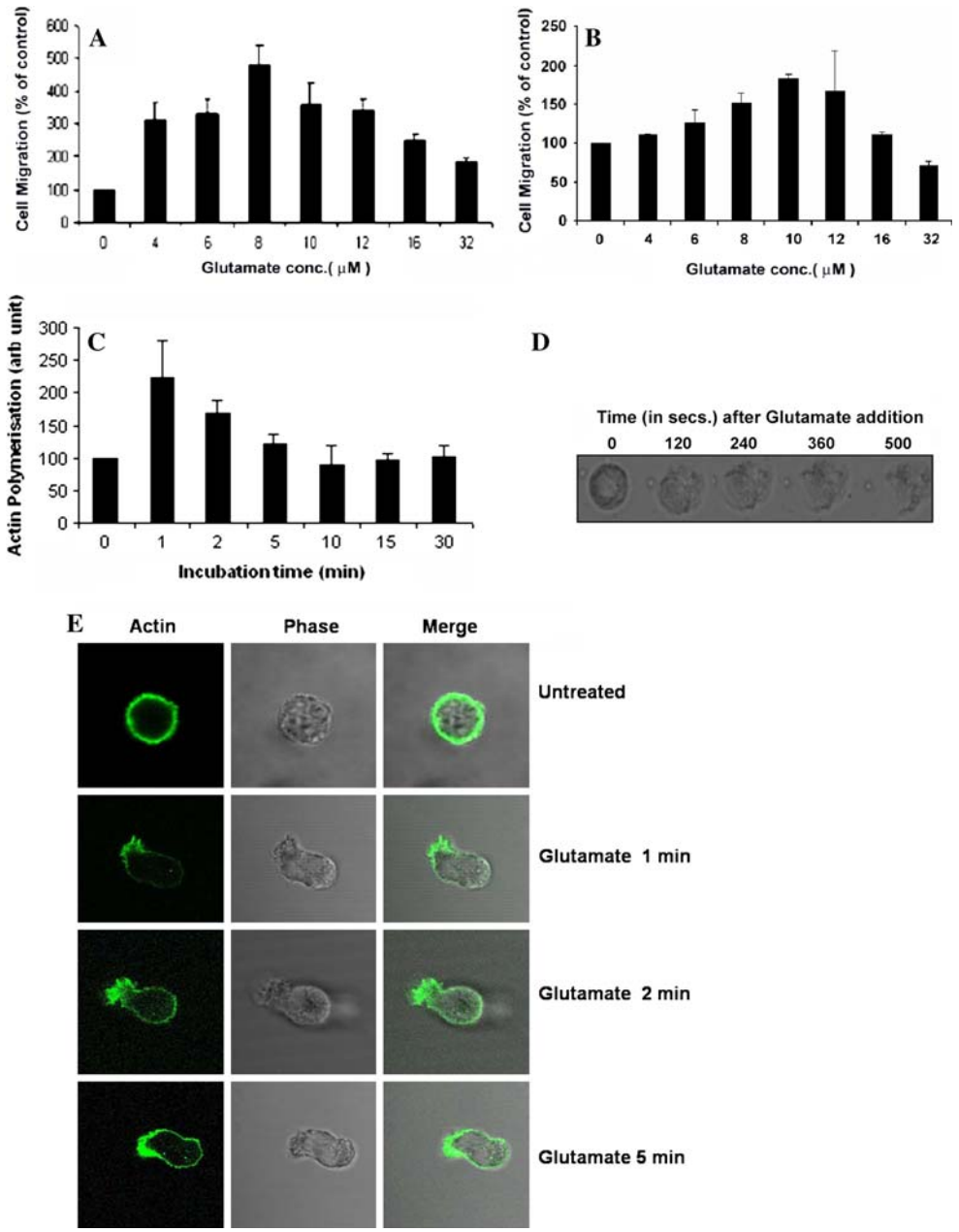


Fig. 3 Glutamate as the neutrophil mingration factor. All data represents mean of three independent experiments. **a** Glutamate induces neutrophil migration (chemotaxis). The migration assay was performed either by using untreated cells (4 × 105 cells/well) or with different doses of glutamate for 2 h. Glutamate was added to the lower chamber. **b** Glutamate induces neutrophil migration (chemokinesis). The migration assay was performed either by using untreated cells (4 × 105 cells/well) or with different doses of glutamate for 2 h. Glutamate was added in equal concentration to both chambers. **c** Glutamate increases the synthesis of F-actin. Neutrophils were left untreated or treated with 8 μM 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 florescence (MCF), with the baseline florescence arbitrarily assigned a value of 100%. **d** Polarization of a neutrophil in response to a gradient of glutamate. (a-e) Images of an unpolarised neutrophil responding to a micropipette containing 8 μM glutamate (red arrow) at a 0 sec, b 120 sec, c 240sec, d 360 sec, e 500 sec. **e** 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-phalloidoin 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

keratinocytes, and cells of pancreas, liver, lung, kidney, adrenal and heart (Skerry and Genever 2001). It remains to be determined what role glutamate plays in these various tissues. During inflammatory states, activated neutrophils also release glutamate that regulates the human endothelial barrier function (Collard et al. 2002).

Numerous classical chemoattractants, including the microbial formylated peptides, complement fragments (C3a, C5a), platelet-activating factor (PAF), leukotrienes, and the various chemokines all induce neutrophil migration (Rollins 1997). The small bacterial chemoattractants, however, are not present significantly in the healthy placentae or in sterilized placental extracts, and the large chemokines and other proteinaceous chemotactants should be inactivated by extraction at high temperature (see Sect. "Materials and methods"). Chemotactic effects of the above molecules on their target cells would be mediated by specific heptahelical G-protein coupled receptors (GPCRs) (Murdoch and Finn 2000), which clearly do not respond to glutamate. However, glutamate could activate the metabotropic glutamate GPCRs on leukocytes to enhance the production of chemokines (e.g. Pacheco et al. 2006) and induce chemotaxis in an indirect fashion.

Glutamate, the major excitatory neurotransmitter in the mammalian CNS, acts through both ionotropic (NMDA, AMPA and kainate) receptors and G-protein coupled metabotropic receptors (Watkins and Evans 1981). The absence of any report stating the presence of conventional glutamate receptors (ionotropic or metabotropic) on the surface of neutrophils (IUPHAR receptor data base, http://www.iuphar-db.org/) is surprising, as there are reports of the presence of conventional (ionotropic) glutamate receptors on other leukocytes (Ganor et el 2003; Sarchielli et al. 2007; Malone et al. 1986). Neutrophils are also known to express nicotinic acetylcholine receptors (Benhammou et al. 2000), which play an important role in migration of cells (Tournier et al. 2006). Work is in progress in our laboratories to identify the receptor(s) present on the neutrophil surface that could be involved in the glutamate mediated chemotaxis.

The present study identifies glutamate, a neurotransmitter amino acid, as a key molecule of the placental extract that promotes neutrophil chemotaxis. The finding that glutamate is chemotactic for the neutrophil is also of interest regarding the relationship between neural and immune systems.

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