Epidermal growth factor receptor ligands are chemoattractants for normal human mesothelial cells

U. Palmer*, Z. Liu*, U. Broome+, J. Klominek*

ABSTRACT: Signalling through epidermal growth factor (EGF) receptor leads to several cellular responses including cell division and cell migration. Since EGF receptors are expressed on normal mesothelial cells, this study investigated whether EGF receptor ligands act as chemoattractants on these cells.

The study used Boyden chambers fitted with filters coated with the adhesive matrix proteins fibronectin, laminin, collagen type IV and the nonmatrix adhesive molecule poly-1-lysine, for the migration studies.

Normal mesothelial cells migrated to EGF receptor ligands such as EGF, transforming growth factor (TGF)-α and heparin-binding epidermal growth factor (HB-EGF) at concentrations ranging 0.024–100 ng mL^{-1} (with a peak stimulation at 6.25 ng mL^{-1}), if matrix proteins were present as adhesive substrates. This migration was integrin-dependent, since the same cells failed to migrate in the absence of extracellular matrix molecules or when the Boyden chamber assay was performed in the presence of anti-β1 integrin monoclonal antibodies.

These findings describe for the first time epidermal growth factor receptor ligands acting as chemoattractants on normal mesothelial cells, and that signalling through epidermal growth factor receptors leading to mesothelial cell migration also requires the activation of integrins.

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The mesothelium is a simple squamous epithelium that lines the walls and covers the contents of the pleural, pericardial and peritoneal cavities and provides a slippery nonadhesive surface for the internal organs [1]. Mesothelial cells produce extracellular matrix (ECM) components such as collagen, fibronectin and laminin, which define the architecture and provide support for the cells [2–4].

Cell migration is important in many biological processes such as embryogenesis, angiogenesis, inflammatory reactions and wound repair [5]. These processes are thought to be regulated by interactions with other cells, cytokines and ECM proteins [6]. Several growth factors are also capable of inducing cell migration. For example, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF) stimulate the migration of several mesenchymal cell types [7–9]. Epidermal growth factor (EGF) has been reported to be a chemoattractant for different epithelial cells such as keratinocytes [10], intestinal cells [11], corneal cells [12] and liver epithelial cells [13]. Besides stimulating cell migration, EGF is a growth factor for many types of cells, including normal mesothelial cells [14, 15].

EGF belongs to a family of hormones which include TGF-α, heparin-binding (HB)-EGF, amphiregulin, β-cellulin and several differentially spliced variants of neurotrophin. There are four distinct receptors (called ErbB/HER) that bind hormones belonging to the EGF family. A single ErbB family receptor (e.g. EGFR) can bind several different EGF family hormones (EGF, TGF-α, amphiregulin and β-cellulin), and a single EGF family hormone can activate more than one receptor.

EGF hormones are widely expressed through the body [16–18]. The amounts of EGF receptor ligands in tissues are generally low, but high expression has been shown in nearly all body fluids including pleural fluid and ascitic fluid [16, 19].

In order to investigate whether EGF receptor ligands stimulate cell movement of normal human mesothelial cells, Boyden chamber assays were performed using EGF, TGF-α, HB-EGF, amphiregulin, and β-cellulin as chemoattractants. Furthermore, the study investigated if other growth factors and cytokines were also capable of inducing mesothelial cell migration.

Materials and methods

Reagents

Roswell Park Memorial Institute (RPMI) 1640 culture medium, foetal calf serum (FCS), penicillin, streptomycin and genistein were obtained from Life Technologies Inc. (Paisley, UK); bovine serum albumin (BSA), laminin and collagen type IV were from Sigma (St. Louis, MO, USA); fibronectin from Boehringer Mannheim (Mannheim, Germany). EGF, HB-EGF, TGF-α, amphiregulin, β-cellulin, stem cell factor (SCF), insulin-like growth factor (IGF)-I, IGF-II, granulocyte-macrophage colony-stimulating factor
(GM-CSF), basic fibroblast growth factor (bFGF), acidic (a)FGF, PDGF, interleukin (IL)-8 and regulated on activation, normal T-cell expressed and secreted (RANTES) were purchased from R&D Systems (Abington, UK) whereas IL-6 was from Genzyme (Cambridge, MA, USA).

Antibodies

Monoclonal antibodies used in this study were: anti-EGF receptor monoclonal antibody (clone 225, immunoglobulin (IgG1) (Oncogene Research Products, Cambridge, MA, USA), anti-CD44 monoclonal antibody (clone 2C5, IgG1) (R&D Systems), anti-keratin (clone AE1/AE3, IgG1) and anti-vimentin antibodies (clone V9, IgG1x) (Dakopatts a/s, Glostrup, Denmark), anti-intercellular adhesion molecule (ICAM)-1 (clone 84H10, IgG1), antivasculär adhesion molecule (VCAM)-1 (clone 1G11, IgG1) (Immunotech SA, Marseille, France) and anti-integrin β1 monoclonal antibody (clone P4C10, IgG1) (Gibco BRL, Gaithersburg, MD, USA).

Cells

Normal mesothelial cells were obtained from the ascitic fluid of two patients with alcoholic liver cirrhosis undergoing palliative procedures. Five litres of ascitic fluid were centrifuged, and the cells obtained further centrifuged on a Lymphoprep (Nycomed, Oslo, Norway) gradient. Cells were recovered from the interface and washed in phosphate buffered saline (PBS), suspended and cultured in RPMI 1640 containing 10% FCS. Mesothelial cells were used between first and second passages. Normal human mesothelial cells transformed by transfection with an simian vacuolating virus 40 (SV40) large T-antigen construct (MeT-5A) [20] were purchased from the American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Mesothelial cells were maintained in a humidified incubator in a 95% air 5% CO2 atmosphere at 37°C. Cells were maintained present during the assay. After 5 h of incubation, the filters were fixed in methanol and stained with Giemsa. The number of cells that had migrated to the lower side of the filters was determined and the counts were averaged.

Flow cytometry

In order to characterize the expression of ICAM-1, VCAM-1 and CD44 on normal human mesothelial cells, flow cytometry was performed using a FACS trac analyzer (Becton Dickinson) after indirect immunofluorescent staining of the cells. The cells were stained with 1 μg of antibody diluted in 50 μL PBS. As a negative control, fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse IgG were used. FITC-conjugated F(ab')2 fragments of rabbit anti-mouse IgG were used for detection.

Immunoblot analysis

Confluent monolayers of mesothelial cells were washed in a buffer containing 0.02 M NaH2PO4, 0.15 M NaCl (pH 7.4) and lysed in a lysis buffer containing 50 mM tris-hydroxymethyl-amino methane (Tris), 5 mM ethylenedia-
Results

Characterization of normal human mesothelial cells

Mesothelial cell cultures were characterized on the basis of coexpression of cytokeratins and vimentin as previously described [23] (data not shown). In addition, mesothelial cells were analysed by flow cytometry using anti-ICAM-1, anti-VCAM-1 and anti-CD44 monoclonal antibodies [24-26]. As shown in figure 1, normal human mesothelial cells obtained from the ascitic fluid of patients without malignant disease expressed ICAM-1, VCAM-1 and CD44.

Whole-cell homogenates of mesothelial cell cultures were separated on SDS-PAGE gels, electroblotted onto nitrocellulose filters and probed with the anti-EGF receptor monoclonal antibody. Depicted in figure 1c are results of Western blots showing the presence of a 180 kDa band in the mesothelial cells. The molecular weight of this band corresponds to a mature form of the EGF receptor.

Epidermal growth factor receptor ligands simulate migration of normal human mesothelial cells

EGF is a growth factor for many types of cells including mesothelial cells [14, 16]. In addition to mitogenesis, EGF has also been reported to stimulate chemotactic migration.

Fig. 1. Characterization of intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1 and CD44 expression on normal mesothelial cells from the ascitic fluid of patient 1 (a) and patient 2 (b) without malignant disease. Mesothelial cells were labelled with anti-ICAM-1, anti-VCAM-1 and anti-CD44 monoclonal antibodies and subjected to fluorescein-activated cell sorter analysis as described in the Materials and methods. RCN: relative cell number. c) Equal amounts of mesothelial cell homogenates from the two patients were loaded onto each lane and resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (4-20%). Proteins were transferred to nitrocellulose sheets and probed with anti-epidermal growth factor receptor antibodies. Bound antibodies were visualized using an enhanced chemiluminescence kit. The cell homogenates from the two patients were compared against normal human mesothelial cells containing a large T-cell construct (MeT-5A).

Fig. 2. Chemotactic migration of mesothelial cells from a) patient 1, b) patient 2 and c) the large T-antigen construct cell line (MeT-5A) in response to different concentrations of recombinant human epidermal growth factor (rhEGF). Lower wells of Boyden chambers were filled with the indicated concentrations of rhEGF diluted in serum-free Roswell Park Memorial Institute medium supplemented with 1 mg·mL⁻¹ bovine serum albumin. The filters were coated on both sides with 10 mg·mL⁻¹ of fibronectin (F), laminin (L) or collagen (C) type IV. The number of migrated cells is the mean±SD of triplicates for each data point. HPF: high power field, magnification × 400.
The motile behaviour of mesothelial cells is rarely studied. There are no studies describing the role of growth factors or cytokines in the migration of mesothelial cells. To address the question, of whether EGF stimulates the migration of mesothelial cells, Boyden chamber assays were performed using filters coated with different ECM molecules with recombinant human EGF used as a chemoattractant. Firstly, whether EGF stimulated chemotaxis, i.e. whether the mesothelial cells responded to a gradient of EGF across the filter was examined. As shown in figure 2, normal human mesothelial cells and the mesothelial cell line MeT-5A migrated to increasing concentrations of EGF. The peak motile response was seen at 6.2 ng·mL⁻¹, with fewer cells migrating to higher EGF concentrations. The cells migrated equally well on filters coated with fibronectin, laminin or collagen type IV. No migration occurred in the absence of matrix proteins, that is when filters were uncoated, coated with control non-adhesive BSA protein or coated with non-matrix adhesive substrate PLL (fig. 3). In addition, preincubation of mesothelial cells with anti-integrin β₁ monoclonal antibodies resulted in nearly complete inhibition of migration on fibronectin, laminin and collagen type IV (fig. 3), thus indicating that the engagement of integrins is also required for the migration of mesothelial cells to EGF.

In order to study whether EGF also has chemokinetic properties, i.e. if cells are capable of migrating in the absence of a factor gradient (random motility), checker-
Inhibition of epidermal growth factor-induced mesothelial cell migration by anti-epidermal growth factor-receptor antibody and genistein

In order to confirm the involvement of the EGF receptor in EGF-mediated migration, mesothelial cells were preincubated with blocking anti-EGF receptor monoclonal antibody. As shown in figure 7, anti-EGF-receptor antibody inhibited the migration towards EGF by 60–95%. In order to investigate whether tyrosine phosphorylation was necessary for the motile response of mesothelial cells to EGF, the cells were preincubated with a protein tyrosine kinase inhibitor, genistein [29], and Boyden chamber assays performed with EGF as the chemoattractant (fig. 7). Preincubation of mesothelial cells with 10 µg·mL⁻¹ of genistein inhibited migration to EGF without affecting cell viability as determined by trypan blue exclusion (data not shown) over the 5 h assay period.

Discussion

In addition to mitogenesis, some growth factors can also induce cell migration. A good example of such a molecule is EGF which has been described both as a growth factor and as a motility factor. Previous studies have described the mitogenic properties of EGF on normal human mesothelial cells [14, 15]. However, the possible role of EGF as a motility factor in these cells has not been investigated. In the present work, evidence that normal human mesothelial cells migrate to increasing concentrations of EGF and EGF-related molecules, such as TGF-α and HB-EGF is provided. The results also show that mesothelial cell migration to EGF depends on adhesive interactions with the ECM molecules fibronectin, laminin and collagen type IV.

EGF is a known growth factor for many types of cells, including mesothelial cells [14, 15]. Other growth factors capable of inducing cell migration include FGF, which stimulates migration of endothelial cells, PDGF which induces migration of smooth muscle cells, fibroblasts and some haematopoietic cells, TGF-β which acts as a chemoattractant in normal fibroblasts, and insulin and insulin-like growth factor I which stimulate the migration of normal human bronchial cells [30, 31]. EGFs exert chemotactic activities on keratinocytes and several epithelial cell types [10, 12, 30]. However, there are no studies that describe whether growth factors or cytokines affect the migration of normal mesothelial cells.
molecules are also known to bind to the ErbB-3 and ErbB-act through EGF or the ErbB-1 receptor. However, both mechanism of action important, since it can provide clues as to the possible motility using checkerboard analysis. This method established that epidermal growth factor receptor ligands play a role in the repair of mesothelial injury. Recombinant human EGF stimulated the migration of normal human mesothelial cells and the virus transformed mesothelial cell line MeT-5A in a dose-dependent manner. The bimodal migratory response has been previously observed in Boyden chamber experiments involving matrix components [32]. In the current study, the inhibition of migration at high EGF concentrations is probably caused by a stronger adhesion to matrix components. Furthermore, EGF-stimulated mesothelial cell migration was inhibited when the cells were preincubated with anti-EGF receptor monoclonal antibodies. There are currently four known receptors that bind molecules belonging to the EGF family of molecules [33]. This study confirms that EGF and other motility-inducing EGF receptors ligands (data not shown) act through EGF receptor in normal human mesothelial cells.

The absence of a motile response to β-cellulin and amphiregulin is currently not understood. Both molecules act through EGF or the ErbB-1 receptor. However, both molecules are also known to bind to the ErbB-3 and ErbB-4 receptors [34]. Further studies will help to determine whether the lack of a motile response to β-cellulin and amphiregulin is due to an absence of the appropriate receptors on normal mesothelial cells.

The current study further analysed EGF-stimulated motility using checkerboard analysis. This method establishes whether certain motility-inducing substances induce chemotaxis, chemokinesis or both [27]. This distinction is important, since it can provide clues as to the possible mechanism of action in vivo. In this study, EGF receptor ligands were both chemotactic and chemokinetic for normal mesothelial cells. During pleural injury, EGF receptor ligands present in pleural or ascitic fluid might stimulate the migration of the mesothelial cells necessary for wound closure. Since EGF receptor ligands are chemokinetic, such a migration could occur in the absence of a gradient, i.e. in the presence of uniform concentrations of EGF.

Signalling through the EGF receptor requires the phosphorylation of specific substrates that results in various biological effects [33]. In order to establish whether phosphorylation is necessary for EGF-stimulated mesothelial cell migration, these cells were preincubated with genistein and Boyden chamber assays were performed. Treatment of mesothelial cells with genistein was found to suppress EGF-induced migration. Genistein is a tyrosine kinase inhibitor that has been shown to inhibit several biological phenomena requiring tyrosine phosphorylation, including signalling via many different receptors [35]. It is not considered likely that inhibition of chemotaxis by genistein is a nonspecific toxic effect on mesothelial cells, since the viability of the cells was unaffected after 5 h of incubation with this agent. These experiments led us to the conclusion that inhibition of EGF-stimulated migration in normal mesothelial cells is due to the interruption of a signalling pathway involving EGF receptor-associated protein-tyrosine phosphorylation.

Cell migration is a vital component of many biological processes such as embryogenesis, recirculation of the cells of the immune system, angiogenesis and wound healing [6]. During migration, cells extend pseudopodia, form matrix/cell attachments, assemble and contract the cytoskeleton and finally translocate forwards [36]. Integrins that mediate substratum contacts play an important role in this process in addition to signals provided by motility factors [37, 38]. The current experiments show that interactions with the ECM are necessary for migration of normal mesothelial cells to EGF receptor ligands. In the absence of contact with substratum-bound fibronectin, laminin or collagen type IV or when these matrix interactions were inhibited by anti-β integrin monoclonal antibodies, normal mesothelial cells did not migrate to EGF even if good adhesion was provided by PLL. These observations indicate that normal mesothelial cells migrate to EGF only when cell/matrix interactions also occur. This finding is in agreement with previous observations showing that substratum/motility factor interactions are necessary for the motile response in normal and malignant cells [38, 39].

In conclusion, this study has shown that normal human mesothelial cells migrate to epidermal growth factor receptor ligands and that this migration requires integrin-mediated matrix interactions. The data also invite speculation that epidermal growth factor receptor ligands play a role in the repair of mesothelial injury.

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