The Expression of the Epidermal Growth Factor Receptor on Human Monocytes and Macrophages and its Potential Role in Atherosclerosis

By

Alys Catrin Dreux
BSc (Hons)

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Faculty of Health & Medical Sciences
University of Surrey

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Abstract

The epidermal growth factor receptor (EGFR) and its family of ligands may be associated with the progression of atherosclerosis by mediating the proliferation and migration of endothelial cells and vascular smooth muscle cells. However there is some evidence that EGFR is expressed by cells of the monocytic lineage. This study was undertaken to investigate the expression of the EGFR on monocytes and macrophages and explore its potential function. The expression of the EGFR was studied using the monoclonal antibody ICR62. Flow cytometry revealed that the monocytic THP-1 cell line expressed EGFR on its surface and specific EGFR reactivity was also observed by western blotting analysis. Whilst PMA differentiated THP-1 cells failed to show cell surface expression of the receptor, immunocytochemical analysis revealed the receptor was localized to the nucleus. Human peripheral blood-derived monocytes and monocyte-derived macrophages showed minimal cell surface expression of EGFR. EGFR gene transcription in monocytic THP-1 cells was induced 2.7 fold following treatment with 50nM HB-EGF for 12 hours and this was statistically significant ($P<0.01$). Differentiated THP-1 cells showed a 1.5 fold increase in gene expression following treatment with 50nM TGFα for 12 hours; this increase was also highly significant ($P<0.01$). Gene expression was also significantly increased in both monocytic and differentiated THP-1 cells following 12 hours treatment with 100U/ml IFNγ [3.7 fold ($P<0.01$) and 1.8 fold ($P<0.01$) respectively] and 2.5ng/ml IL-1β [3.6 fold ($P<0.01$) and 1.7 fold ($P<0.05$) respectively)]. Newly isolated human peripheral blood-derived monocytes displayed very low EGFR mRNA expression. However, during maturation in vitro,
Abstract
gene expression peaked following 48 hours in culture and returned to baseline after 96 hours. Exposure of THP-1 cells to HB-EGF (0.1nM) resulted in a 3 fold increase ($P<0.01$) in maximum chemotactic activity. This response was found to be greater than the 2 fold increase seen with the well established monocyte chemokine MCP-1 at 1nM ($P<0.05$). Peripheral blood-derived monocytes had a statistically significant ($P<0.01$) 2.4 fold increase in their chemotactic response compared to unstimulated control cells when exposed to 10nM HB-EGF. This was equivalent to the response seen for 0.1nM MCP-1 with these same cells. Interestingly, as the monocyte matured into an 8 day old macrophage-like cell the chemotactic response to 0.1nM MCP-1 decreased, whereas peak chemotactic activity to HB-EGF occurred at lower concentrations. Incubation of peripheral blood-derived monocytes with the inhibitory monoclonal antibody ICR62 significantly ($P<0.05$) reduced chemotactic activity towards HB-EGF (0.1nM) in a dose dependent manner. Immunohistochemical analysis of human arterial sections revealed EGFR expression within atherosclerotic lesions. Further analysis showed that EGFR expression colocalised with macrophages. It therefore appears that the EGFR is expressed by the THP-1 cell line and human peripheral blood-derived monocytes and may play a role in the pathogenesis of human atherosclerosis.
Acknowledgements

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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AP1/2</td>
<td>Activator protein 1/2</td>
</tr>
<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>ARIA</td>
<td>Acetylcholine receptor-inducing activity</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BTC</td>
<td>Betacellulin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CCR2</td>
<td>CC-motif chemokine receptor-2</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>dTHP-1</td>
<td>Differentiated THP-1</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Epiregulin</td>
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<tr>
<td>ETF</td>
<td>EGFR-specific transcription factor</td>
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<tr>
<td>FcγR</td>
<td>Fragment crystallisable γ receptor</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate isomer I</td>
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<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>GGF</td>
<td>Glial growth factor</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>Guanine triphosphate</td>
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<tr>
<td>H$_2$O$_2$</td>
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<td>HB-EGF</td>
<td>Heparin-binding epidermal growth factor</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid</td>
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<td>HSPG</td>
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<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<td>IGF-1</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
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<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon stimulated response element</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL receptor</td>
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<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LysoPC</td>
<td>Lysophosphatidylcholine</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>M-CSF</td>
<td>Monocyte colony stimulating factor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>MLN-51</td>
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<td>MOPS</td>
<td>4-morpholine propanesulfonic acid</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MTT</td>
<td>Thiazoly blue tetrazolium bromide / 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>NDF</td>
<td>Neu differentiation factor</td>
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<td>NFκB</td>
<td>Nuclear factor-κB</td>
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<td>Nerve growth factor receptor</td>
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<td>NLSs</td>
<td>Nuclear localisation signals</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>Neuregulin</td>
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<tr>
<td>oxLDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTPase</td>
<td>Protein tyrosine phosphatise</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>QPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T-cell expressed, and secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPE-Cy5</td>
<td>R-phycoerythrin-cyanine5</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenger receptor</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TGFα/β</td>
<td>Transforming growth factor α/β</td>
</tr>
<tr>
<td>Th1/2</td>
<td>T helper cell type 1/2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
</tbody>
</table>
Cardiovascular disease (CVD) is the major cause of death globally. An estimated 17.5 million people died from CVD in 2005, representing 30% of all global deaths. Of these deaths, an estimated 7.6 million were due to coronary heart disease and 5.7 million were due to stroke (The WHO, 2008).

CVD, encompassing coronary heart disease (CHD) and stroke, is also the main cause of mortality in the UK and accounts for 38% of all deaths. In 2003, CHD caused over 38,000 premature deaths in the UK (BHF Statistics Database).

1.1 Atherosclerosis

Atherosclerosis is a multi-factorial disease with a complex aetiology. It is a progressive disease characterised by the accumulation of lipids, cellular components and fibrous material within the large arteries (Libby, 2002; Stary et al., 1994). The initiation of the atherosclerotic process is believed to begin with a protective response to injury of the endothelium and the smooth muscle cells (SMCs) of the artery wall which is usually accompanied by an inflammatory response (Ross, 1993). Over time the artery wall can become thickened and calcified, and loses its elasticity. Although advanced lesions can grow sufficiently large to block the arterial lumen, the most prevalent clinical complication occurs if the plaque ruptures or becomes ulcerated. In this situation, platelets adhere and aggregate creating a
thrombus over the site of the rupture further increasing plaque size (Lusis, 2000). The luminal narrowing which follows can lead to ischemia of the heart, brain or extremities resulting in infarction (Ross, 1999).

Many epidemiological studies have revealed numerous risk factors associated with CVD (Table 1.1). They can in general terms be classed as unmodifiable, for example gender and age, or modifiable, for example smoking and hypertension. Due to the complex nature of atherosclerosis it is difficult to define a one particular factor as the major inducer of the disease (Patel & Kent, 1998). However, elevated levels of serum cholesterol are probably unique in being sufficient to drive plaque development in humans and experimental animals, even in the absence of other known risk factors (Glass & Witztum, 2001). One increasingly prevalent clinical scenario involves the intertwining of the development of atherosclerosis with an underlying insulin resistance and its associated metabolic abnormalities (Plutzky, 2003).
Table 1.1 Risk factors associated with cardiovascular disease (CVD).

<table>
<thead>
<tr>
<th>Unmodifiable Factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advancing age</td>
<td>(Aronow, 2006)</td>
</tr>
<tr>
<td>Gender</td>
<td>(Nathan &amp; Chaudhuri, 1997)</td>
</tr>
<tr>
<td>Menopausal Status</td>
<td>(Wenger, 2006)</td>
</tr>
<tr>
<td>Familial hypoalphalipoproteinemia</td>
<td>(Vega &amp; Grundy, 1996)</td>
</tr>
<tr>
<td>Family history</td>
<td>(Watkins &amp; Farrall, 2006)</td>
</tr>
<tr>
<td>Ethnicity/Race</td>
<td>(Forouhi &amp; Sattar, 2006; Watson &amp; Topol, 2004)</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>(Sherer &amp; Shoenfeld, 2006)</td>
</tr>
<tr>
<td>e.g. lupus, rheumatoid arthritis</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Major Modifiable Factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td>(Grundy, 2004)</td>
</tr>
<tr>
<td>Smoking</td>
<td>(Lakier, 1992)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>(Tulenko &amp; Sumner, 2002)</td>
</tr>
<tr>
<td>Increased Lipoprotein(a)</td>
<td>(Seman et al., 1999)</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>(Austin et al., 1998)</td>
</tr>
<tr>
<td>Diabetes/insulin resistance</td>
<td>(Lebovitz, 2006)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>(Kannel, 1996)</td>
</tr>
<tr>
<td>High dietary saturated fat</td>
<td>(Griel &amp; Kris-Etherton, 2006)</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>(Dorr &amp; Volzke, 2005)</td>
</tr>
<tr>
<td>Physical Inactivity</td>
<td>(Wannamethee et al., 2002)</td>
</tr>
<tr>
<td>Raised levels of clotting factors</td>
<td>(Kannel, 2005)</td>
</tr>
<tr>
<td>e.g. fibrinogen</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minor Modifiable Factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low antioxidant status</td>
<td>(Aviram et al., 2005)</td>
</tr>
<tr>
<td>Infection – bacterial or viral</td>
<td>(Lowe, 2001)</td>
</tr>
<tr>
<td>Hyperhomocysteinemia</td>
<td>(Fanapour et al., 1999)</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>(Emberson et al., 2005)</td>
</tr>
<tr>
<td>Certain medication e.g. contraceptive pill</td>
<td>(Farley et al., 1998)</td>
</tr>
<tr>
<td>Mental ill-health inc. psychosocial stress</td>
<td>(Andre-Petersson et al., 2006)</td>
</tr>
<tr>
<td>Obstructive sleep apnea</td>
<td>(Parish &amp; Somers, 2004)</td>
</tr>
</tbody>
</table>
Chapter 1 – Introduction

1.1.1 Pathogenesis

In regions of arterial branching or curvature, blood flow is disturbed and non-laminar. In these areas, the endothelial cells (ECs) lining the arterial wall have polygonal shapes and no particular orientation. This may be associated with an increased permeability to macromolecules such as low density lipoprotein (LDL) and triglycerides (Lusis, 2000; Stary et al., 1992). This process formed the basis of the response-to-injury hypothesis which now contributes to the understanding that endothelial dysfunction is the first step in atherosclerosis (Ross, 1999). Other causes of endothelial dysfunction include elevated and modified LDL, free radicals caused by cigarette smoking, hypertension, diabetes mellitus, elevated plasma homocysteine levels and infectious microorganisms such as *Chlamydia pneumonia* (Ross, 1999).

Due to increased endothelial permeability, LDL enters and becomes trapped within the vessel wall (Mora et al., 1987). Accumulations within the vessel wall have been observed in humans (Morton et al., 1986; Hoff & Gaubatz, 1982) and experimental animal models (Rosenfeld et al., 1990; Haberland et al., 1988; Hoff & Bond, 1982). These observations led to the formation of the response-to-retention hypothesis which stated that sub-endothelial lipid retention within the vessel wall leads to a cascade of responses which results in disease in a previously non-lesional artery (Williams & Tabas, 1998). Once retained, the lipoproteins undergo modifications (Aviram, 1993), i.e. oxidation (Palinski et al., 1989; Yla-Herttuala et al., 1989) and acetylation (Goldstein et al., 1979), which contributes to an inflammatory response (Yla-Herttuala et al., 1994). Both of these processes are also regarded to be important steps in the initiation process of atherosclerosis (Ross, 1999; Witztum & Steinberg, 1991).
It is proposed that cardiovascular risk factors enhance endothelial oxidative stress. Superoxide anions are released locally from ECs, attached monocytes or from macrophages and SMCs contained within the sub-endothelial space (Sattar et al., 1998). Oxidised low density lipoprotein (oxLDL) has been shown to reduce nitric oxide (NO) synthesis and promote its destruction. Oxidative stress thus limits the endothelial cell to produce NO and its ability to promote vasodilation leading to endothelial dysfunction and loss of its anti-atherogenic abilities (Sattar et al., 1998).

Once dysfunctional, ECs begin to produce a number of chemotactic and pro-inflammatory molecules including monocyte chemotactic protein-1 (MCP-1) (Navab et al., 1991) and macrophage colony stimulating factor (M-CSF) (Rajavashisth et al., 1990). T lymphocytes and monocytes are subsequently attracted to the area and adhere in clusters to the endothelial cell surface. After rolling over the endothelium, the monocytes and T lymphocytes are tethered to the endothelium via specific adhesion molecules e.g. vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and P & E Selectin (Glass & Witztum, 2001; Lusis, 2000). Under the influence of chemoattractants, such as MCP-1, oxLDL and transforming growth factor β (TGFβ) released by the endothelium, adherent leukocytes and possibly SMCs, the leukocytes penetrate the endothelial cell junctions and accumulate in the subendothelial space (Ross, 1993). High density lipoprotein (HDL) has been shown to inhibit this migration by inhibiting lipoprotein oxidation (Navab et al., 1991).

Beneath the surface of the endothelium the monocytes differentiate into tissue macrophages. Under the influence of M-CSF the macrophages proliferate and begin
to express the scavenger receptors SR-A, SR-B, CD36 and CD68 (Libby, 2002). These receptors mediate the uptake and accumulation of oxLDL which results in the formation of foam cells and increased release of inflammatory mediators such as tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-8 and M-CSF. This in turn leads to the increased transcription of the LDL-receptor gene which results in enhanced binding of LDL to the endothelium and SMCs. The monocyte is possibly primed for this outcome by its strong association with extracellular matrix components such as collagen I as it enters the intima. Together with the T lymphocytes in the intima, the foam cells form the first recognisable lesion of atherosclerosis, the fatty streak (Osterud & Bjorklid, 2003).

The transition from the fatty streak to the more complex lesion is characterised by the immigration of SMCs from the media, through the internal elastic lamina and into the developing intima (Glass & Witztum, 2001). The medial SMCs are attracted into the intima by chemotactic factors such as platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). Here, the SMCs produce extracellular matrix which forms the fibrous cap overlaying the lipid-rich core and with this the lesion is classified as advanced (Plutzky, 2003; Stary et al., 1995; Ross, 1993). With time, the macrophage foam cells die, contributing their lipid-filled contents to the necrotic core of the lesion. This is thought to result from cell-cell interactions and the local cytokine environment within the arterial wall, involving the actions of pro- and anti-apoptotic proteins that include death receptors, proto-oncogenes and tumour suppressor genes (Glass & Witztum, 2001). As a consequence, the lesion expands, initially towards the adventitia through vascular remodelling until a critical point is reached, after which it begins to expand outwards, encroaching into the luminal
space. At this point the plaque may become stable and subsequently pose no further clinical threat. The necrotic core remains small and is separated from the lumen by a thick fibrous cap containing SMCs and type I and III collagen fibres. However, if the lesion continues to undergo further insult, the migration of new mononuclear cells from the blood may occur. These then accumulate at the shoulder region of the plaque and lead to the destabilisation of the plaque (Plutzky, 2003; Lusis, 2000).

Although advanced lesions can lead to ischemia due to the progressive narrowing of the lumen, acute cardiovascular events that result in myocardial infarction, stroke and in some cases death, are generally thought to result from plaque rupture and thrombosis (Glass & Witztum, 2001). The forces that maintain the stability of the fibrous cap on an advanced lesion are in a fine balance between the synthesis of matrix materials and their degradation. The products of inflammatory cells are likely to influence both processes. For example, macrophages are known to secrete matrix metalloproteinases (MMP-1, MMP-3 and MMP-9) in response to several mediators secreted from T lymphocytes and monocytes e.g. TNFα, and IL-1β. T cells also produce interferon γ (IFNγ) which inhibits matrix production by SMCs (Lusis, 2000). Apoptosis can also play an important role in plaque rupture. SMC death can be harmful because it may weaken the fibrous cap through a decrease in the production of collagen fibres. However, apoptosis of macrophages and T lymphocytes can be beneficial as removal of inflammatory cells from the plaque could attenuate the inflammatory response and decrease the production of MMPs. Unfortunately, apoptosis of these cells can also lead to an increased inflammatory response and a decrease in the uptake of other apoptotic bodies leading further to the formation of an acellular necrotic core (Tabas, 2005; Martinet & Kockx, 2004;
Martinet & Kockx, 2001; Libby et al., 1996). Due to the nature of cells involved in these processes most plaque ruptures occur within the macrophage-rich shoulder region of the lesion (Glass & Witztum, 2001).

The stability of atherosclerotic lesions may also be influenced by calcification and neovascularisation. The process of calcification is regulated by cytokines and oxysterols acting on pericyte-like cells. These cells secrete a matrix scaffold which subsequently becomes calcified, akin to bone formation. Plaques may also become vascularised during the process of remodelling with large numbers of capillaries and venule-like channels maybe as a result of bFGF and/or other angiogenic molecules. These vessels may act as conduits for further entry of inflammatory cells (Lusis, 2000; Ross, 1993).
1.2 The Monocytic Lineage

It is clear from the evidence in the previous section that monocytes and macrophages are present during all stages of atherogenesis and play a vital role in the development and stability of the plaque.

1.2.1 The Monocyte

From its production in the bone marrow, under the influence of various cytokines including M-CSF, the monocyte circulates within the blood both as a defence cell and as a precursor to the tissue macrophage. In response to pathogens, monocytes and macrophages play a key role in cell-mediated immunity. Cross-linking of these receptors by immobilized immunoglobulin (IgG) or immune complexes results in the production of cytokines, i.e. MCP-1, IL-8, TNFα and M-CSF, and reactive oxygen species (ROS) (Baran et al., 2004).

The recruitment and translocation of monocytes to and within the intima is an important determinant of plaque development (Lessner et al., 2002). Many chemokines are thought to be involved in this process. MCP-1, considered the principal cytokine in attracting monocytes to the intima has been found to be abundantly expressed in human lesions and has been extensively investigated within in vivo models (Ikeda et al., 2002; Reape & Groot, 1999; Han et al., 1998). Its effects are mediated through the CC-motif chemokine receptor-2 (CCR2) which is expressed on circulating monocytes. Other chemokines include macrophage inflammatory protein-1 alpha (MIP-1α), MIP-1β and regulated on activation, normal T-cell-expressed and secreted (RANTES). Their common receptor, CCR5, is
expressed during monocyte differentiation into macrophages. As CCR2 expression has been found to be decreased during monocyte maturation (Phillips et al., 2005), it has been speculated that these chemoattractants play their main role in the migration of macrophages already established within the main lesion (Osterud & Bjorklid, 2003; Reape & Groot, 1999; Cross et al., 1997; Uguccioni et al., 1995). Lysophosphatidylcholine (lysoPC), formed by the oxidation of LDL particles, is also a potent chemoattractant (Quinn et al., 1988) as are oxLDL, TGFβ and the colony stimulating factors (CSFs) (Ross, 1993).

Once attracted to the endothelium they adhere and roll over the cellular membrane with the aid of VCAM-1, ICAM-1 and the P and E-selectins (Osterud & Bjorklid, 2003). Monocytes migrate through the endothelial barrier at junctional sites, with the assistance of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and CD99 (Schenkel et al., 2002; Muller & Randolph, 1999). Once in the intimal space, monocytes interact with components of the extracellular matrix (ECM), thought to be a prerequisite for the formation of foam cells and modified LDL particles (Ross, 1999). Monocytes also differentiate into tissue macrophages under the influence of M-CSF. This process is thought to depend on many transcription factors including nuclear factor-κB (NFκB), which has been shown to accumulate in the cytoplasm of differentiated macrophages (Osterud & Bjorklid, 2003).

1.2.2 The Macrophage

Macrophages are present in all stages of atherogenesis carrying out roles such as acting as antigen-presenting cells to T-lymphocytes, acting as scavenger cells to remove noxious materials, and as a source of growth-regulatory molecules,
cytokines, chemokines, metalloproteinases and other hydrolytic enzymes. The macrophage is therefore considered to be the principal inflammatory mediator of cells in the atheromatous plaque microenvironment (Ross, 1999).

Sub-populations of macrophages appear to develop in part as a result of the different stimuli that they encounter within a tissue microenvironment. Macrophages can be 'classically activated' in response to a priming signal from IFNγ produced by type-1 T helper (Th1) cells and a secondary signal from lipopolysaccharide (LPS) inducing TNF production through toll-like receptors (TLRs). These activated macrophages migrate to sites of inflammation and act as effector cells in Th1 cellular responses, i.e. cell mediated immunity. They are characterised by an increased expression of interleukins (IL-1, IL-6 and IL-12), NO and chemokines (MIP-1α and MCP-1). These cells are highly microbicidal and proinflammatory. ‘Alternatively activated’ macrophages or type-II activated macrophages mediate type-2 T helper (Th2) cell adaptive immune responses, i.e. antibody production. These macrophages develop following exposure to IL-4 and IL-13 and appear to be involved in tissue repair and immunosuppression and could serve a more regulatory and recovery function. Under these conditions they fail to produce NO but do produce IL-1, IL-10 and high levels of fibronectin. (Mosser, 2003; Ma et al., 2003; Gordon, 2003). Both Th1 and Th2 responses regulate each other; IFNγ inhibits the Th2 pathway and IL-10 inhibits the Th1 pathway (Linton & Fazio, 2003). There are several other factors that can either induce or enhance monocyte/macrophage activation. These are summarised in Table 1.2.
Table 1.2 Agents that induce/enhance monocyte/macrophage activation that have been associated with the development of atherosclerosis (Ma et al., 2003; Osterud & Bjorklid, 2003).

<table>
<thead>
<tr>
<th>Immune Stimulatory Agents</th>
<th>Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria - LPS</td>
<td>IL-1β, IL-6, IL-12, IL-8</td>
</tr>
<tr>
<td>Immune Complexes</td>
<td>TNFα</td>
</tr>
<tr>
<td>Complement Factors (C3a &amp; C5a)</td>
<td>IFNγ</td>
</tr>
<tr>
<td>Lectins</td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Platelet-derived activation products</td>
</tr>
<tr>
<td></td>
<td>P-selectin</td>
</tr>
<tr>
<td></td>
<td>Platelet microparticals</td>
</tr>
<tr>
<td></td>
<td>Platelet factor 4</td>
</tr>
<tr>
<td>Growth Factors</td>
<td>Proteins</td>
</tr>
<tr>
<td>PDGF</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Proteases</td>
</tr>
<tr>
<td></td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Lipids</td>
<td>O₂⁻, ·OH</td>
</tr>
<tr>
<td>Oxidised LDL</td>
<td></td>
</tr>
<tr>
<td>Lipoproteins</td>
<td></td>
</tr>
<tr>
<td>Eicosanoids</td>
<td></td>
</tr>
</tbody>
</table>
IL-10 and TGFβ play an important role in dampening macrophage activation. IL-10 is a broad spectrum inhibitor of macrophage function, effecting cytokine, chemokine, NO and MMP production. It also inhibits the expression of major histocompatibility complex (MHC) class II molecules. TGFβ has similar effects as IL-10. However, its most important function is in limiting the production of ROS and nitrogen intermediates by cells either activated by IFNγ or LPS (Ma et al., 2003).

Recent work on the phenotyping of macrophage subsets has indicated that macrophages are capable of adapting to their microenvironment and do not terminally differentiate into one particular phenotype. Instead, unless the signal initiates an apoptotic cascade, the macrophage will eventually revert to its original, functional status after the cytokine signalling ceases (Stout & Suttles, 2004).

1.2.3 The Macrophage Foam Cell

Macrophages are capable of internalising LDL through the surface LDL receptor (LDLR). They are protected from accumulating LDL through the down regulation of the LDL receptors and through the trafficking of cellular cholesterol to apolipoprotein A1 (ApoA1), a constituent of HDL, in the blood via the ATP-binding cassette transporter A1 (ABCA1). However, by taking up modified LDL particles, macrophages turn into fat-loaded macrophages or foam cells. Modified LDL, either oxidised, myloperoxidased or acetylated, has an affinity for the series of aforementioned transmembrane scavenger receptors. These receptors have been found to be induced during the differentiation of monocytes into macrophages (Osterud & Bjorklid, 2003). The expression of the scavenger receptors is regulated by peroxisome proliferator-activated receptor γ (PPARγ), whose ligands include
oxidised fatty acids, and by cytokines such as TNFα and IFNγ (Linton & Fazio, 2003; Linton & Fazio, 2001; Lusis, 2000). Since IFNγ has been found to downregulate ABCA1 and cholesterol efflux from foam cells, this cytokine cascade may promote cholesterol accumulation in the lesion, and may be responsible for the accelerated transformation of macrophages into foam cells (Wang et al., 2002). Foam cells are capable of releasing further amounts of inflammatory mediators such as IL-1β, IL-6, TNFα, IL-8 and M-CSF. This can result in increased monocyte recruitment, further LDL oxidation and production of MMPs consequently leading to plaque instability and possible rupture (Osterud & Bjorklid, 2003; Libby, 2000).

1.2.4 Macrophage Apoptosis

Macrophage death in atherosclerotic lesions is almost certainly multifactorial involving high levels of oxLDL, oxysterols, TNFα, Fas ligand, NO, growth factor withdrawal, hypoxia/ATP depletion, and intracellular accumulation of unesterified, or ‘free’, cholesterol. It is likely that some of the death inducers in early lesions are different from those in the more advanced lesions (Tabas, 2005). In the early lesion, the phagocytic clearance of apoptotic macrophages is very efficient and physiologically beneficial. However, in advanced lesions apoptotic macrophages are more numerous suggesting that the phagocytic clearance is defective (Tabas, 2005). Here, the inefficient clearance leads to secondary necrosis of the macrophage which leads to the generation of the necrotic core, increased inflammation, plaque instability and acute lesional thrombosis (Tabas, 2005; Li & Glass, 2002).
1.3 The Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is a 170-kDa glycoprotein and is the prototype of the type-1 growth factor receptor family with tyrosine kinase activity. The complete amino acid sequence of the EGFR was deduced from its gene sequence in the 1980's (Hunter & Cooper, 1985; Ullrich et al., 1984). These data also revealed that the EGFR is homologous to the v-erb-B oncogene of avian erythroblastosis virus (Downward et al., 1984b).

The EGFR family consists of four members: ErbB1 (also termed EGFR, HER1), ErbB2 (also termed HER2, p185, or neu), ErbB3 (also termed HER3 or p160) and ErbB4 (also termed HER4 or p180). All four family members are expressed by a wide variety of cell types and tissues throughout the body (Casalini et al., 2004; Yano et al., 2003), including the vasculature (Table 1.3).

1.3.1 EGFR Structure

The extracellular region or ectodomain of the EGFR is responsible for the binding of epidermal growth factor (EGF) and the other ligand family members. This region consists of four domains which provide the receptor with its ligand specificity and contact with the plasma membrane (Jorissen et al., 2003; Lax et al., 1991; Lax et al., 1989) (figure 1.1A). Evidence has indicated that in order to create the ligand binding site, all four regions would fold into a configuration that forms a ligand binding pocket (Cadena & Gill, 1992; Ullrich & Schlessinger, 1990) (figure 1.1B).
Table 1.3 Expression and sites of action of the EGF family of growth factors with reference to atherogenesis.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Cells &amp; Tissues of Production</th>
<th>Receptor</th>
<th>Sites of Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal Growth Factor</td>
<td>Platelets (bone marrow)</td>
<td>EGFR</td>
<td>Vascular smooth muscle and endothelial cells</td>
<td>(Styren et al., 1993; Oka &amp; Orth, 1983; Bhargava et al., 1979; Gospodarowicz et al., 1978)</td>
</tr>
<tr>
<td>Receptor (EGF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin-Binding Epidermal</td>
<td>Macrophages, monocytes, T</td>
<td>EGFR, ErbB4</td>
<td>Vascular smooth muscle cells</td>
<td>(Matsumoto et al., 2002; Blotnick et al., 1994; Nakano et al., 1994; Yoshizumi et al., 1992; Higashiyama et al., 1991)</td>
</tr>
<tr>
<td>Growth Factor (HB-EGF)</td>
<td>lymphocytes, smooth muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and endothelial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transforming Growth Factor</td>
<td>Macrophages, vascular smooth</td>
<td>EGFR</td>
<td>Endothelial cells</td>
<td>(Lee et al., 1995; Derynck, 1992; Mueller et al., 1990; Rappolee et al., 1988; Madtes et al., 1988)</td>
</tr>
<tr>
<td>α (TGFα)</td>
<td>muscle cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betacellulin (BTC)</td>
<td>Vascular smooth muscle cells</td>
<td>EGFR, ErbB4,</td>
<td>Vascular smooth muscle cells</td>
<td>(Dunbar &amp; Goddard, 2000; Riese et al., 1996; Shing et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>macrophages</td>
<td>ErbB2:ErbB3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphiregulin (AR)</td>
<td>Activated human monocytes</td>
<td>EGFR; EGFR:ErbB2</td>
<td>Vascular smooth muscle cells</td>
<td>(Shin et al., 2003; Mograbi et al., 1997)</td>
</tr>
<tr>
<td>Epiregulin (EPR)</td>
<td>Peripheral blood macrophages</td>
<td>EGFR, ErbB4</td>
<td>Smooth muscle cells</td>
<td>(Koo &amp; Kim, 2003; Toyoda et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>vascular smooth muscle cells</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 1.1 Schematic representations of the domains and proposed structure-function topology of the EGF Receptor. (A) EGF receptor domains with amino acid positioning. Abbreviations used: L for ligand binding domain and CR for cysteine-rich domain. The 4 domains are also known as I(L1), II(CR1), III(L2) and IV(CR2). TM - transmembrane domain, JM - juxtamembrane domain and CT - carboxy-terminus (Jorissen et al., 2003). (B) Subdomains I and IV represent the cysteine-rich regions of the extracellular domain. Left: side view. Right: top view. S and R represent the proposed interaction sites for substrates and regulatory molecules (Ullrich & Schlessinger, 1990).
The transmembrane domain of the EGFR consists of a single alpha-helix and its main function is to anchor the receptor in the plasma membrane (Ullrich & Schlessinger, 1990; Kashles et al., 1988). The juxtamembrane domain is conserved between members of the EGFR family. It has been suggested that this region is involved in regulatory functions such as receptor downregulation (via threonine 654), ligand-dependent internalization events, basolateral sorting and interaction with proteins such as calmodulin and heterotrimeric G proteins (Jorissen et al., 2003; Wells, 1999; Ullrich & Schlessinger, 1990).

The cytoplasmic domain has been found to carry the structural determinants that define kinase specificity, high affinity binding, mitogenic and transforming potential, and receptor trafficking (Van der Heyden et al., 1997; Riedel et al., 1989). All four EGFR family members have one uninterrupted tyrosine kinase domain in the cytoplasmic region however, ErbB3’s region is inactive as a result of three point mutations which disable the receptor’s ability to facilitate the transfer of phosphates between ATP and the receptors substrates (Wells, 1999; Guy et al., 1994). This domain is the most highly conserved region of all the receptor tyrosine molecules (Fantl et al., 1993; Carpenter & Cohen, 1990). There is a consensus sequence, GlyXGlyXXGlyX(15-20)Lys, that functions as part of the binding site for ATP. Replacement of the consensus Lys721 residue completely abolishes kinase activity (Ullrich & Schlessinger, 1990; Moolenaar et al., 1988; Honegger et al., 1987). Various receptor mutants have been developed to investigate the importance of the tyrosine kinase domain (Prywes et al., 1986). Although kinase activity was not found to be essential for receptor expression and targeting to the cell membrane, it was essential for signal transduction and induction of both early and late cellular
responses, including mitogenesis and transformation (Chen et al., 1987). Protein tyrosine kinase activity was also found to be important for EGFR targeting to lysosomes upon ligand-induced activation. This implied that lysosomal targeting requires the phosphorylation of specific substrates, or that components of the intracellular routing system may only recognise the conformation of an activated receptor (Ullrich & Schlessinger, 1990). The binding of EGF to its receptor in vitro is associated with the generation of hydrogen peroxide (H$_2$O$_2$) through phosphoinositol 3-kinase (PI3-K) in a variety of cell types (Gamou & Shimizu, 1995). H$_2$O$_2$ has been found to reversibly oxidize protein tyrosine phosphatases (PTPases) which can lead to a more sustained receptor phosphorylation (Lee et al., 1998; Bae et al., 1997; Gamou & Shimizu, 1995).

The carboxy-terminal tail sequences of the EGFR family are among the most divergent of the known receptor tyrosine kinases (Ullrich & Schlessinger, 1990). Phosphorylation sites have been mapped to the carboxy-terminal tail of the EGFR at tyrosine residues 992, 1068, 1086, 1148 and 1173 (Fantl et al., 1993; Walton et al., 1990; Margolis et al., 1989; Downward et al., 1984a). Autophosphorylation of the receptor is mediated by the intrinsic kinase activity through an intermolecular process. This involves the catalytic domain of one receptor partner phosphorylating its neighbouring receptor partner (Moriki et al., 2001; Yarden & Schlessinger, 1987b).

### 1.3.2 EGFR Localisation

All receptors are synthesized and directed to specific cellular locations on the cell membrane. Their distribution is a dynamic event, which can be dependent on the
type of cell (Wiley, 2003). Experiments have shown that a region in the extracellular juxtamembrane domain of the receptor targets it to caveolae/raft regions (Yamabhai & Anderson, 2002). However, it is still unclear as to where precisely the unstimulated receptors are directed. Some studies indicate that more than half of the unstimulated EGFRs on the cell surface are located within caveolae; these are cholesterol- and sphingolipid-rich invaginations of the plasma membrane containing the protein caveolin-1 (Jorissen et al., 2003; Lai, 2003; Anderson, 1998). However, other studies have shown that they are located in non-caveolar membrane domains (Roepstorff et al., 2002; Waugh et al., 1999). It has been observed that with short exposure to ligand, receptors become associated with lipid rafts, whereas, with longer stimulation, the receptors are associated with caveolae where they became dephosphorylated. This is consistent with the fact that caveolin is a negative regulator of the receptor (Matveev & Smart, 2002; Couet et al., 1997), and that cholesterol affects the function of the receptor. Cholesterol depletion increases ligand binding, the signalling activity of the receptor and decreases internalization (Roepstorff et al., 2002; Pike & Casey, 2002). It has also been proposed that a variety of proteins that are involved in cell signalling are concentrated in cholesterol-rich microdomains known as membrane rafts. It is thought that membrane rafts function as sites of assembly of proteins involved in cell signalling including the receptors (Lai, 2003; Schlessinger, 2000; Simons & Ikonen, 1997).

1.3.3 Receptor Activation

The EGFR family is activated by a family of peptide growth factors (Table 1.4). Among these are: epidermal growth factor (EGF), transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR),
betacellulin (BTC), epiregulin (EPR), epigen, and the four neuregulins (NRGs). NRG-1 is also known as Neu differentiation factor (NDF), heregulin (HRG), acetylcholine receptor-inducing activity (ARIA) and glial growth factor (GGF) (Harris et al., 2003; Falls, 2003; Massague & Pandiella, 1993).

EGF family members are commonly grouped with respect to their structural homology and biological activity. All members contain one or more repeats of a conserved six cysteine-containing motif in their extracellular domain. These six cysteine residues are contained within a sequence of 35-40 amino acids CX7CX4CX10-13CX8GXRC (C, cysteine; G, glycine; R, arginine; X, any amino acid), and have the potential to form three intra-molecular disulfide bond pairings between C1-C3, C2-C4 and C5-C6 to produce three loops that are essential for high-affinity binding to the receptor (Harris et al., 2003; Lee et al., 1995; Massague & Pandiella, 1993).

The EGF family of growth factors are derived from type-I transmembrane glycoprotein precursors. These precursor molecules comprise an extracellular region containing the growth factor sequence, a hydrophobic trans-membrane domain and a cytoplasmic domain. Within the plasma membrane they undergo proteolytic cleavage resulting in the release of soluble, biologically active growth factors (Massague & Pandiella, 1993). This cleavage is achieved by members of the family of a disintegrin and metalloproteinase (ADAMs) which have a wide tissue distribution and are essential for mammalian development (Primakoff & Myles, 2000; Peschon et al., 1998). Certain factors are capable of enhancing growth factor shedding. Treatment of cells with the phorbol ester 12-O-tetradecanoyl-phorbol-13-
acetate (TPA), an activator of protein kinase C (PKC), induces a strong increase in the level of TGFα synthesis in keratinocytes and other cells as well as increased cleavage of the membrane bound form (Lee et al., 1995).

Some membrane-bound forms of EGF and TGFα have been found to be biologically active by promoting juxtacrine stimulation of EGFR receptors on adjacent cells (Singh & Harris, 2005; Lee et al., 1995; Massague & Pandiella, 1993; Anklesaria et al., 1990). It has also been suggested that proHB-EGF can promote juxtacrine stimulation of the EGFR through its association with CD9 and integrin α3β1, and that this may generate a different EGFR-mediated signal than that generated by the soluble form (Harris et al., 2003; Iwamoto & Mekada, 2000). For example, a transmembrane HB-EGF has been found to protect rat hepatoma cells from TGFβ-induced apoptosis, whereas the soluble mature HB-EGF has no protective effect (Raab & Klagsbrun, 1997).

The biological activities of the different members of the EGF family of growth factors are similar. All the members have been shown to induce cell migration, differentiation and gene expression, and are involved in processes such as angiogenesis, wound healing, bone reabsorption, atherosclerosis, blastocyst implantation and tumour growth (Lee et al., 1995).

Ligands that bind to the EGFR family of receptors can be divided into two classes (Table 1.4). The first group includes EGF, TGFα, HB-EGF, BTC, AR, EPR and epigen; these bind to ErbB1 homodimers. Unlike the other members of this group, BTC, HB-EGF and EPR are also able to bind and activate ErbB4 (Piepkorn et al.,
1998; Elenius et al., 1997; Beerli & Hynes, 1996). Furthermore BTC is also capable of activating all possible combinations of heterodimeric receptors (Riese et al., 1996). The second group of ligands are the NRG family. They are known to bind ErbB3 and ErbB4 homodimers but are unable to bind ErbB1 (Falls, 2003).

Ligand-induced receptor oligomerization of the EGF receptor family has been demonstrated in living cells (Sako et al., 2000; Cochet et al., 1988), intact isolated cell membrane (Sako et al., 2000), in detergent-solubilized (Ge et al., 2002; Hurwitz et al., 1991) and purified receptor preparations (Yarden & Schlessinger, 1987a). The binding affinity of ligands for the EGF receptor may be high or low, and this may be determined in part by the presence of pre-existing bound ligand, or whether the receptor is in a dimerized form (Jorissen et al., 2003; Hurwitz et al., 1991; Prywes et al., 1986). There is still controversy as to how the ligand-receptor complexes form. Many theories have been presented including the conjugation of ligand-bound receptor monomers (Schlessinger, 2000; Lemmon et al., 1997; Sherrill & Kyte, 1996), asymmetric ligand binding mechanisms (Ge et al., 2002) and conformational changes in the extracellular region with ligand binding, with subsequent cytoplasmic domain conformational changes (Tanner & Kyte, 1999). It has also been suggested that inactive EGFR monomers are in equilibrium with active receptor dimers without bound ligand (Moriki et al., 2001; Lemmon et al., 1997).

Once the receptor-ligand dimers are formed, autophosphorylation occurs. The catalytic domain adopts an open configuration that permits access to ATP and substrates, and enables phosphotransfer from MgATP to tyrosines on the receptor itself and on cellular proteins involved in signal transduction (Schlessinger, 2000).
Table 1.4 The discovery of the epidermal growth factor receptor family of major ligands and the ErbB homodimers and heterodimers they bind.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Cells of Discovery</th>
<th>Receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>Submaxillary glands of adult male mice</td>
<td>ErbB1</td>
<td>(Carpenter &amp; Cohen, 1990; Starkey et al., 1975; Cohen, 1962)</td>
</tr>
<tr>
<td>TGFα</td>
<td>Murine 3T3 fibroblasts</td>
<td>ErbB1</td>
<td>(Anzano et al., 1983; De Larco &amp; Todaro, 1978)</td>
</tr>
<tr>
<td>AR</td>
<td>Human breast adenocarcinoma cell line (MCF-7)</td>
<td>ErbB1, ErbB1:ErbB2</td>
<td>(Johnson et al., 1993; Shoyab et al., 1989; Shoyab et al., 1988)</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Human U937 macrophage-like cells</td>
<td>ErbB1, ErbB4</td>
<td>(Raab &amp; Klagsbrun, 1997; Higashiyama et al., 1991)</td>
</tr>
<tr>
<td>BTC</td>
<td>Mouse pancreatic β cell tumours</td>
<td>ErbB1, ErbB4, ErbB2:ErbB3</td>
<td>(Miura et al., 2002; Shing et al., 1993)</td>
</tr>
<tr>
<td>EPR</td>
<td>Mouse fibroblast-derived tumour cell line NIH3T3</td>
<td>ErbB1, ErbB4</td>
<td>(Toyoda et al., 1997; Toyoda et al., 1995)</td>
</tr>
<tr>
<td>Epigen</td>
<td>Mouse keratinocytes</td>
<td>ErbB1</td>
<td>(Strachan et al., 2001)</td>
</tr>
<tr>
<td>NRG-1</td>
<td>Conditioned medium of human MDA-MB-231 breast carcinoma cells</td>
<td>ErbB3, ErbB4</td>
<td>(Holmes et al., 1992)</td>
</tr>
<tr>
<td>NRG-2</td>
<td>Sequence analysis &amp; molecular cloning</td>
<td>ErbB3, ErbB4</td>
<td>(Carraway, III et al., 1997; Busfield et al., 1997)</td>
</tr>
<tr>
<td>NRG-3</td>
<td>Sequence analysis &amp; molecular cloning</td>
<td>ErbB4</td>
<td>(Zhang et al., 1997)</td>
</tr>
<tr>
<td>NRG-4</td>
<td>Sequence analysis &amp; molecular cloning</td>
<td>ErbB4</td>
<td>(Harari et al., 1999)</td>
</tr>
</tbody>
</table>
The EGFR was the first protein tyrosine kinase (PTK) to be shown to dimerize after ligand binding. Since then, many studies have demonstrated the combinatorial hetero-oligomerization of different pairs of members of the EGFR family (Schlessinger, 2000). This ability to heterodimerize firstly increases the diversity of cellular signalling pathways that can be activated by PTKs, and secondly, increases the repertoire of ligands that can be recognised by each receptor on its own (Lemmon & Schlessinger, 1994). Since no ligand has yet been identified for ErbB2, it has been proposed that it functions as a promiscuous heterodimeric partner of the other EGFR family members providing a platform for the recruitment of more diverse intracellular signalling pathways as a result of differences in their C-terminal domains (Qian et al., 1994). It also appears that ErbB2 is the preferred partner of all ErbB proteins. If all the receptors are present then ErbB3 and ErbB4 will preferentially bind ErbB2. However, if ErbB2 is not present, only then will the two receptors interact with ErbB1 (Graus-Porta et al., 1997). ErbB3 lacks kinase activity and so this receptor also serves as a docking protein to recruit a broader spectrum of signalling molecules after phosphorylation by EGFR or ErbB2 (Wells, 1999).

1.3.4 Receptor Signalling

Ligation of the EGFR has pleiotropic biological effects. Cellular responses, that include mitogenesis, apoptosis, chemotaxis and differentiation, depend on the context of the cell and include cell density, extracellular matrix composition, and priming by other cytokines (Wells, 1999) (Figure 1.2). Autophosphorylation allows the receptor to recruit adapter proteins such as Grb2 (Lowenstein et al., 1992) and Shc (Batarz et al., 1994) which bind via their Src homology 2 (SH2) (Koch et al., 1991) and phosphotyrosine-binding (PTB) domains (Schlessinger & Lemmon, 1999).
These adapter proteins further recruit other molecules such as the guanine nucleotide exchange factor Sos which activates the small G-protein Ras (Lowy & Willumsen, 1993). Once in its active GTP-bound state, Ras interacts with other effector proteins leading to the activation of the MAP (mitogen activated protein) kinase pathway. This pathway is highly conserved in evolution and results in the control of metabolic processes, cell cycle, migration, proliferation and differentiation (Nishida & Gotoh, 1993). The binding of c-Cbl to the activated bound Grb2 targets the EGFR for degradation in the lysosome (Duan et al., 2003; Fukazawa et al., 1996).

Phospholipase Cγ (PLCγ) binds directly to the activated EGFR via its two SH2 domains (Chattopadhyay et al., 1999; Meisenhelder et al., 1989). Once active, PLCγ catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Toker, 1998). IP₃ mediates calcium release from intracellular stores which, amongst activating a host of Ca²⁺-dependent enzymes such as NFκB (Sun & Carpenter, 1998), has been found to be responsible for inducing cellular motility (Chen et al., 1994). DAG however, is known to activate PKC (Crotty et al., 2006) which in turn phosphorylates the EGFR inhibiting its activity (King & Cooper, 1986).
Figure 1.2 EGFR signalling pathways. The EGFR, once activated by its family of ligands, is capable of activating numerous signalling pathways leading to essential cellular processes. The recruitment of the adapter proteins Shc, Grb2 and Gab1 leads to the activation of c-Src, known to be involved in mitogenesis; c-Cbl, which targets the receptor for degradation and the MAP kinase pathway leading to proliferation. The EGFR can also directly bind the STAT transcription factors, which are involved in the transcription of numerous target genes, and PLCγ, which via IP₃ liberates calcium resulting in actin reorganisation and migration, and via DAG activates PKC which is involved in the downregulation of EGFR activation. Adapted from (Oda et al., 2005).
Activated EGF receptors are also able to activate the signal transducer and activator of transcription (STAT) transcription factors, notably STAT-1, 3 and 5. After causing rapid tyrosine phosphorylation, they migrate to the nucleus where they are involved in the transcription of many target DNA genes such as caspase-1 and the cell cycle inhibitor p21^{WAF/CIP} (Darnell, Jr., 1997). This could be a means by which PTKs play a role in the negative control of cell proliferation, as seen sometimes in A431 epithelial cells (Jorissen et al., 2003; Hackel et al., 1999).

1.3.5 Receptor Regulation

Receptor tyrosine kinases (RTKs) must be tightly regulated in order to mediate their normal control over cellular tasks and physiological responses. Therefore, there are many mechanisms that exist that attenuate and terminate the activity of the receptor induced by stimulatory ligands including PKC (Lund et al., 1990), protein tyrosine phosphatases (PTPases) (Tiganis, 2002) and receptor internalisation (Sweeney & Carraway, III, 2004). PKC is capable of phosphorylating Thr654 located in the juxtamembrane domain. This results in the abolition of EGF high-affinity binding and receptor kinase activity in the same cell, as well as inhibition of internalisation and downregulation (Lund et al., 1990). Protein tyrosine phosphatases dephosphorylate regulatory phosphotyrosine residues which results in the inhibition of tyrosine kinase activity and the biological responses mediated by downstream effectors that rely on tyrosine activity. In the presence of PTPase inhibitors, virtually all RTKs can be activated, even with the absence of ligand (Schlessinger, 2000).

Growth factor stimulation results in receptor localization to plasma membrane clathrin-coated pits, internalization of coated pits and delivery to endosomes, and
sorting of receptors for trafficking back to the cell surface or to the lysosomes for degradation (Sweeney & Carraway, III, 2004). Following ligand binding and dimerization, activated receptors serve as a docking site for Cbl. This protein contains a phosphotyrosine binding domain and a RING finger E3 ubiquitin ligase domain. Cbl-mediated EGFR ubiquitination is required for efficient sorting of activated EGFR to the lysosome for its degradation (Duan et al., 2003).

After internalization, activated receptors are sorted into the lysosomal pathway (Wiley, 2003). The ligand-receptor complexes dissociate in acidic endocytotic vesicles (Sorkin & Carpenter, 1991) with the ligand then being degraded in the lysosomes. The receptor is either degraded or recycled back to the cell surface (Massague & Pandiella, 1993). This difference in sorting pathways is thought to depend upon protein tyrosine kinase activity, as kinase-negative receptor mutants have been shown to recycle back to the cell surface for reutilization, even when coexpressed in the same cell as wild-type receptors (Schlessinger, 2000; Ullrich & Schlessinger, 1990). It has also been shown to depend upon the receptors heterodimerization status. EGFR homodimers are quickly endocytopsed and degraded in the lysosomes, whereas, a heterodimeric complex with EGFR and either ErbB2 or ErbB3 reduces the internalization rate and causes dissociation of the complex in the early endosome resulting in the EGFR being recycled back to the plasma membrane (Lenferink et al., 1998).

There is the possibility that internalization is required for signalling as certain critical substrates may not be located at the cell surface but further within the cytoplasm (Cadena & Gill, 1992). It has been documented that an endosomal fraction is the
primary site for the EGF-stimulated Shc phosphorylation and Grb2 recruitment (Leof, 2000). It has also been suggested that the receptor could signal in multiple locations depending on the particular signalling pathway activated. For example, PLCγ1-stimulated hydrolysis of PIP₂ is restricted to the plasma membrane, whereas Ras activation can occur at both the membrane and within an internal compartment (Leof, 2000).

1.3.6 Biological Effects

Previous studies have shown that all members of the group one family of ligands compete with each other for binding to the EGFR. TGFα is capable of competing with EGF for the EGFR (Massague, 1983). However, it has been suggested that these ligands bind in different manners to the receptor and that this may account for the differences in the biological activities (Winkler et al., 1989). For example, TGFα is a more potent stimulus for keratinocyte migration and angiogenesis than EGF (Lee et al., 1995). Both HB-EGF (Thompson et al., 1994) and AR (Thorne & Plowman, 1994) contain a heparin-binding domain, which allow binding to cell surface heparin-sulphate proteoglycans which may in turn modulate their biological activity. For example, HB-EGF is a more potent smooth muscle cell mitogen than EGF or TGFα (Massague & Pandiella, 1993). Also, although HB-EGF is able to stimulate proliferation and chemotaxis in cells expressing EGFR, only chemotactic responses have been demonstrated in cells expressing ErbB4 alone (Iwamoto & Mekada, 2000; Elenius et al., 1997; Thompson et al., 1994). Hence, HB-EGF can bind and activate two different types of receptors leading to different biological outcomes.
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Many cells that synthesise TGFα also possess EGF receptors, allowing the possibility of autocrine responses (Derynck, 1992). This can also been seen in some cells responsive to AR (Johnson et al., 1993). In addition to interactions between the soluble forms of growth factor with the EGFR, membrane bound forms of TGFα and EGF are known to exist, and these appear to promote juxtacrine stimulation which causes the autophosphorylation of EGF receptors in adjacent cells (Lee et al., 1995; Massague & Pandiella, 1993; Anklesaria et al., 1990). It has also been suggested that proHB-EGF can promote juxtacrine stimulation of the EGFR through its association with CD9 and integrin α5β1 and that this may generate a different EGFR-mediated signal than that generated by the soluble form (Harris et al., 2003; Iwamoto & Mekada, 2000). For example, transmembrane HB-EGF has been found to protect a rat hepatoma cell line from TGFβ-induced apoptosis, whereas soluble mature HB-EGF had no protective effect (Raab & Klagsbrun, 1997).
1.4 The Role of the EGFR Family in Atherogenesis

The EGFR and the EGF family of growth factors are expressed by cells involved in atherogenesis and appear to mediate important biological effects relevant to the atherogenic process (figure 1.3).

EGF receptors have been identified immunocytochemically on intimal smooth muscle cells within human atherosclerotic plaques (Tamura et al., 2001) as well as vascular endothelial cells (Styren et al., 1993). EGF receptors have also been demonstrated on cultured rat aortic smooth muscle cells and can mediate both cell proliferation and DNA synthesis (Nanney et al., 1988; Tomita et al., 1986).

In some cases, the ligands elicit their actions on the same cells to produce the same biological outcome. For example, EGF, TGFα, HB-EGF, BTC and EPR can all mediate the transformation of SMCs from a differentiated non-proliferative phenotype into a dedifferentiated proliferative and migratory phenotype. This change is associated with the development and progression of atherosclerosis (Yamanaka et al., 2001).
Figure 1.3 The expression of the EGFR and its ligands within the atherosclerotic plaque. The EGFR is expressed on endothelial cells, smooth muscle cells (SMCs) and monocytes/macrophages. These cells also express the EGFR ligands, most notably HB-EGF, TGFα, BTC and EPR.
1.4.1 Heparin-Binding Epidermal Growth Factor

Since its discovery in 1991, HB-EGF has been considered to play an important role in atherosclerosis. It was found to be a SMC chemoattractant and mitogen (Higashiyama et al., 1993; Higashiyama et al., 1991) mediating its effects via the MAP Kinase and PI3-K pathways leading to DNA synthesis (Reynolds et al., 2002). HB-EGF is expressed by monocytes (Nakano et al., 1994; Higashiyama et al., 1991), SMCs (Dluz et al., 1993; Temizer et al., 1992; Yoshizumi et al., 1992), T cells (Blotnick et al., 1994) and endothelial cells (Yoshizumi et al., 1992), and their expression of this growth factor is upregulated by several factors associated with atherogenesis (Table 1.5).

HB-EGF mRNA has also been found to be abundantly expressed in human adipose tissue. Plasma HB-EGF levels increase with increasing body fat content and this appears to be associated with risk of coronary artery disease in human subjects (Matsumoto et al., 2002). In 1995, Miyagawa (Miyagawa et al., 1995) reported the expression of HB-EGF protein in SMCs and macrophages associated with human atherosclerotic plaques as well as the expression of the EGF receptor in SMCs. The existence of HB-EGF protein was thereafter discovered in human coronary arteries, and its presence was closely associated with the progression of coronary atherosclerosis. It was suggested that HB-EGF protein produced by SMCs and macrophages could be involved in the atherosclerotic process, especially in the migration and proliferation of SMCs and in the interaction between SMCs and macrophages through the EGFR-mediated signalling pathway (Nakata et al., 1996). This was supported by Reape and colleagues, who reported that HB-EGF mRNA and protein was present in macrophage rich areas of atherosclerotic human carotid
Table 1.5 Factors stimulating the production of HB-EGF expression in cells associated with atherogenesis. Heparin-binding epidermal growth factor is expressed by all the major cell types involved in atherogenesis.

<table>
<thead>
<tr>
<th>Cell Type and Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Smooth Muscle Cells</strong></td>
<td></td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>(Temizer et al., 1992)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>(Nakano et al., 1993)</td>
</tr>
<tr>
<td>Phorbol ester</td>
<td>(Dluz et al., 1993; Temizer et al., 1992)</td>
</tr>
<tr>
<td>Serum</td>
<td>(Dluz et al., 1993)</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>(Dluz et al., 1993)</td>
</tr>
<tr>
<td>bFGF</td>
<td>(Dluz et al., 1993)</td>
</tr>
<tr>
<td>PDGF</td>
<td>(Dluz et al., 1993)</td>
</tr>
<tr>
<td>Vessel injury</td>
<td>(Raab &amp; Klagsbrun, 1997)</td>
</tr>
<tr>
<td><strong>Endothelial Cells</strong></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>(Yoshizumi et al., 1992)</td>
</tr>
<tr>
<td>LysoPC</td>
<td>(Raab &amp; Klagsbrun, 1997)</td>
</tr>
<tr>
<td>High glucose</td>
<td>(Asakawa et al., 1996)</td>
</tr>
<tr>
<td>Hyperosmolarity</td>
<td>(Asakawa et al., 1996)</td>
</tr>
<tr>
<td>Sheer stress</td>
<td>(Raab &amp; Klagsbrun, 1997)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>(Yoshizumi et al., 1992)</td>
</tr>
<tr>
<td>VEGF</td>
<td>(Arkonac et al., 1998)</td>
</tr>
<tr>
<td><strong>Monocytes/macrophages</strong></td>
<td></td>
</tr>
<tr>
<td>OxLDL</td>
<td>(Ouchi et al., 1997)</td>
</tr>
<tr>
<td>LysoPC</td>
<td>(Nakano et al., 1994)</td>
</tr>
<tr>
<td>LPS</td>
<td>(Nakano et al., 1994)</td>
</tr>
<tr>
<td>Platelet activating factor (PAF)</td>
<td>(Pan et al., 1995)</td>
</tr>
<tr>
<td><strong>T Lymphocytes</strong></td>
<td></td>
</tr>
<tr>
<td>LysoPC</td>
<td>(Nishi et al., 1997)</td>
</tr>
</tbody>
</table>
arteries, as well as the expression of HB-EGF mRNA in both normal and atherosclerotic human arteries (Reape et al., 1997). Furthermore, in differentiated macrophages and SMCs, HB-EGF and CD9 mRNA and protein are upregulated in response to oxLDL or lysoPC which could lead to juxtacrine stimulation (Nishida et al., 2000; Ouchi et al., 1997). In SMCs, HB-EGF was found to cause an upregulation of PDGF and bFGF mRNA, as well as M-CSF receptor gene expression which suggests a possible role in the phenotypic transformation of SMCs to 'macrophage-like' smooth muscle cell-derived foam cells in atherosclerotic lesions (Raab & Klagsbrun, 1997; Peifley et al., 1996). HB-EGF has also been shown to mediate the transactivation of EGFR by G-protein coupled receptors (GPCRs) and remnant lipoproteins in cultured vascular SMCs, thus suggesting that the EGFR may be important in the regulation of vascular SMCs (Kawakami et al., 2003; Kalmes et al., 2001).

1.4.2 Betacellulin

Betacellulin (BTC) and its receptors were reported to be present in the human aortic wall (Tamura et al., 2001). The percentage of BTC-positive medial SMCs increased significantly in the aorta in individuals with atherosclerosis. Intimal SMCs also showed strong BTC immunoreactivity indicating that this growth factor may be involved in intimal SMC proliferation as the two known receptors for BTC, EGFR and ErbB4, were also found to be expressed in SMCs with the same distribution patterns. This study suggested that BTC is produced by macrophages, and it has also been detected in the human monocytic THP-1 cell line. It was also speculated that macrophages or foam cells could stimulate or modulate the proliferation of SMCs by
secreting BTC. This evidence strongly suggests that BTC may be one of the factors involved in atherosclerotic plaque formation.

1.4.3 Epidermal Growth Factor

Although platelets are a putative source of EGF during atherogenesis, they lack protein biosynthetic capability, and are involved late in the atherogenic process. It is possible that EGF is synthesized in megakaryocytes and stored in platelets, or that EGF is derived from some other source and is subsequently taken up by platelets. It has also been suggested that EGF originates from the bone marrow and is released into the plasma during the coagulation process (Oka & Orth, 1983).

EGF has been shown to possess mitogenic activity for smooth muscle cells in vitro (Thyberg et al., 1990), the effects of which can be enhanced by TGFβ, angiotensin II and insulin growth factor-1 (IGF-1), and inhibited by HDL (Ko et al., 1993a; Ko et al., 1993b). Furthermore it appears that intimal SMCs are more sensitive to the mitogenic effects of EGF compared to medial smooth muscle cells (Mitsumata et al., 1994). EGF stimulation of the EGFR in vascular SMCs activates the MAPK pathway, and inhibits angiotensin II action (Gui & Zheng, 2003; Ullian et al., 1997).

1.4.4 Transforming Growth Factor α

There is limited evidence for the role of TGFα in atherogenesis. It was discovered that TGFα could stimulate arterial blood flow (Gan et al., 1987), angiogenesis (Schreiber et al., 1986) as well as prostacyclin (PGI₂) release from endothelial cells which plays a role in vasodilation and inhibition of platelet aggregation (Ristimaki,
1989). Since then TGFα has been reported to be produced by activated macrophages and vascular smooth muscle cells and as it can promote endothelial proliferation TGFα could contribute to the pathogenesis of atherogenesis (Mueller et al., 1990; Rappolee et al., 1988; Madtes et al., 1988).

1.4.5 Others

Recent data also suggest that amphiregulin and epiregulin may play a role in atherogenesis. These growth factors have been found to be produced by human monocytes and peripheral blood macrophages respectively, and stimulate smooth muscle cell mitogenesis both in vitro and in vivo (Shin et al., 2003; Kato et al., 2003; Mograbi et al., 1997; Toyoda et al., 1997). Epiregulin, produced by SMCs, can be induced by angiotensin II, endothelin-1 and thrombin (Taylor et al., 1999). It has also been found to be the major mediator in factor Xa-induced rat aortic smooth muscle cell mitogenesis and can be considered as an autocrine growth factor (Koo & Kim, 2003).
Chapter 1 – Introduction

1.5 EGFR, Monocytes, Macrophages and Atherogenesis

Although there is already evidence of the expression of three distinct families of receptor tyrosine kinases (RTKs) (M-CSF-1R, Axl/Tyro3/Mer and murine STK/human RON) in the monocyte/macrophage lineage, there is limited evidence for the expression of the EGF receptor family (Correll \textit{et al.}, 2004). There is evidence that EGF receptors are expressed on brain-derived microglial cells. Furthermore these tissue macrophage-like cells respond chemotactically to EGF (Nolte \textit{et al.}, 1997). Some forms of malignancy are associated with an inflammatory response, and it has been shown that macrophages associated with this response in uveal melanoma express high levels of EGFR (Scholes \textit{et al.}, 2001). Although Mograbi (Mograbi \textit{et al.}, 1997) found that the human monocytic cell line, THP-1 and activated human monocytes express HB-EGF, AR and HRG, they reported that mRNA for EGFR, c-erbB2 and c-erbB4 was undetectable. Nevertheless, the EGFR has been reported to be expressed on another human monocytic cell line, U937 using flow cytometry analysis with anti-EGFR antibodies (Eales-Reynolds \textit{et al.}, 2001). Furthermore, it has been shown that ErbB1 is present on rabbit peripheral blood leukocytes, and co-localises with macrophages within lesions of atherosclerosis in the cholesterol-fed rabbit (Lamb \textit{et al.}, 2004). In these experiments, EGFR mediated both chemotactic responses towards monocytes and mitogenic responses towards monocyte-derived macrophages \textit{in vitro}. Although EGF was found to be a less potent chemotactic factor for peripheral blood monocytes than MCP-1, it was more potent than MCP-1 for monocyte-derived macrophages (Lamb \textit{et al.}, 2004). Therefore, ligands for ErbB1 may only play a limited role in attracting monocytes to the atherosclerotic plaque, but they may be important in targeting macrophages to different areas of the plaque, resulting in macrophage-rich areas, such as the shoulder.
region. Upon reaching their destination, the mitogenic effects of EGF on monocyte-derived macrophages may result in proliferation at these sites (Lamb et al., 2004). Indeed, proliferating macrophages have been identified in atherosclerotic lesions from both humans (Gordon et al., 1990) and cholesterol-fed rabbits (Rosenfeld & Ross, 1990). Together, these data add to the potential importance of the EGFR during atherogenesis.
1.6 Aims of Thesis

1.6.1 Hypothesis

Monocytes and macrophages are an important determinant of plaque growth and stability. The EGFR family of ligands modulate monocyte and macrophage function via activation of the EGF receptor.

1.6.2 Aim

To investigate the expression, cellular location and function of the epidermal growth factor receptor on human monocytes and macrophages using a wide range of molecular and immunochemical techniques.

In this study, EGFR expression will be assessed using THP-1 cells and peripheral blood-derived monocytes and the effect of cellular differentiation will be investigated. Gene expression and inducibility of the EGFR will be assayed following EGF ligand family and cytokine treatment and the functionality of the receptor in response to ligands will also be investigated within these cells. Finally, expression of the EGFR and its possible co-localisation with macrophages within human atherosclerotic plaques will be investigated.
Chapter Two

General Reagents & Methods

2.1 Tumour Cell Lines

The human monocytic cell line THP-1 was purchased from the European Collection of Cell Cultures. This cell line was established from the blood of a 1 year old boy with acute monocytic leukaemia (Tsuchiya et al., 1980).

In addition to THP-1 cells, three human carcinoma cell lines expressing high (HN5), intermediate (EJ) and undetectable (MCF-7) levels of EGFR were used in this study (Modjtahedi & Dean, 1996). Of these, the head and neck carcinoma cell line, HN5 and the human bladder carcinoma cell line, EJ express $1.4 \times 10^7$ and $<2 \times 10^5$ EGFR receptors per cell respectively. Expression in the human breast carcinoma cell line MCF-7 is undetectable.

2.1.1 Cell Culture Growth Media

THP-1 cells were cultured routinely in RPMI-1640 medium with GLUTAMAX (GIBCO Cell Culture Systems, Invitrogen Ltd., Paisley, UK) supplemented with 1% penicillin (50IU/ml) and streptomycin (50μg/ml) (GIBCO, UK).
Chapter 2 – Materials & Methods

All adherent cell lines were cultured routinely in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, UK) supplemented with 1% penicillin (50IU/ml) and streptomycin (50μg/ml) (GIBCO, UK).

Both media were routinely supplemented with 10% heat inactivated foetal calf serum (FCS; GIBCO, UK) unless otherwise stated. All media was pre-warmed to 37°C prior to use unless otherwise stated.

2.1.2 Passaging of Tumour Cell Lines

All cells were routinely cultured in large (175cm²), medium (75cm²), or small (25cm²) tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂.

Monocytic THP-1 cells exist as a suspension culture. They were maintained at a cell density of 2-9x10⁵ cells per ml, as described below. Non-adherent cells were transferred to a centrifuge tube and centrifuged at 1200rpm (400g) for 3 minutes (Fisherbrand 3R, Fisher Scientific UK, Loughborough, UK). After removal of the supernatant, the cell pellet was resuspended in warm (37°C) sterile phosphate buffered saline (PBS; BDH, UK) and centrifuged once again. After removal of the PBS supernatant, the cell pellet was resuspended once again in PBS in preparation for determination of cell viability. A 20μl aliquot of cell suspension was mixed with 20μl of trypan blue solution (0.4% solution; Invitrogen Ltd., UK) and loaded onto a haemocytometer chamber (Neubauer). In each of the four corner squares the number of clear viable cells and blue non viable cells was counted and the total cell number for that square was calculated. The average number of viable cells and total cells
from each of the four squares was then calculated. Percentage cell viability was also calculated using the equation below:

\[
\% \text{ Cell Viability} = \frac{\text{Average viable cell number}}{\text{Average total cell number}}
\]

The number of viable cells per ml was calculated by multiplying the average viable cell number by any dilution factors that had been applied and then multiplying that number by $1 \times 10^4$. This whole process was repeated on the other side of the haemocytometer and the averages for viable cell number per ml and cell viability of both sides was calculated. The required aliquot of viable cells was then removed, centrifuged, and resuspended in the required volume of growth medium to give a final concentration of $2 \times 10^5$ cells per ml once seeded into a new tissue culture flask.

Adherent cell monolayers approaching confluency were washed thrice with PBS (37°C) prior to incubation with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (T4049; Sigma-Aldrich Company Ltd., Dorset, UK) at 37°C until the cells were dissociated. The trypsin was inactivated with an equal volume of growth medium and the cell suspension was centrifuged at 1200rpm (400g) for 3 minutes. The cell pellet was resuspended in pre-warmed growth medium and cells were seeded in new tissue culture flasks at the required density.

2.1.3 Long Term Storage of Cells

All tumour cells were stored in liquid nitrogen for long term storage. Near confluent monocytic THP-1 cells were washed by centrifugation in PBS prior to cell re-
suspension in FCS containing 10% sterile glycerol (Sigma Ltd., UK). One millilitre aliquots were transferred to 1.5ml cryovials (Nunc™, Fisher Scientific, UK) and stored at -80°C for 24 hours in a Cryo 1°C freezing vessel (Nalge (Europe) Limited, Hereford, UK) containing propan-2-ol. Cells were then transferred to liquid nitrogen. When required, cells were removed from liquid nitrogen, thawed rapidly at 37°C and transferred to 10ml RPMI-1640 containing 20% FCS, 15mM N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES; GIBCO, UK) and 1% penicillin (50IU/ml) and streptomycin (50μg/ml) (GIBCO, UK) in a 25cm² tissue culture flask. The flask was stood upright and the cells were incubated at 37°C in this position for 7 days. Cells were then centrifuged and the cell pellet was resuspended in 7ml routine culturing RPMI-1640 growth medium. Cells were once again incubated at 37°C for 7 days or until the culture had become a sufficient cell suspension. Cells were then cultured as described in section 2.1.2.

Near confluent adherent tumour cells were dissociated using trypsin and centrifuged at 1200rpm (400g) for 3 minutes as described in section 2.1.2. Following removal of the supernatant, the cell pellet was resuspended and frozen as outlined above. When required, cells were removed from liquid nitrogen, thawed rapidly at 37°C and transferred to 10ml routine culturing DMEM medium. Following centrifugation, the cell pellet was resuspended in fresh routine culturing DMEM medium and cultured as described under section 2.1.2.

### 2.2 THP-1 Cell Differentiation

Monocytic differentiation was achieved by incubation of 4x10^5 cells per ml in routine culturing RPMI-1640 with 10nM Phorbol 12-myristate 13-acetate (PMA;
Sigma-Aldrich, UK). Cells were incubated as described in section 2.1.2 for 72 hours, and during this time developed into adherent macrophage-like cells (Basheeruddin et al., 1992; Auwerx, 1991; Rehfeldt et al., 1991; Tsuchiya et al., 1982).

2.3 Primary Human Mononuclear Cell Culture

Human blood from a total of nine individuals was used in this study (in accordance to the University of Surrey’s ethics policy on the donation and use of human specimens in teaching and research). Venous blood was collected via syringe from an antecubital vein into sterile 3.8% sodium citrate (Sigma-Aldrich, UK) in the ratio of 9 parts blood to 1 part citrate. The anticoagulated blood was then diluted 1:1 in warm PBS and carefully layered onto an equal volume of Histopaque-1077 (Sigma-Aldrich, UK). The blood was centrifuged at 1200rpm (400g) for 30 minutes at room temperature. The buffy mononuclear cell layer was removed along with the plasma and washed twice in PBS at 200g for 10 minutes. Isolated peripheral blood mononuclear cells (PBMCs) were resuspended in serum-free RPMI-1640 medium. Cells were counted using a haemocytometer, diluted to 1x10^6 cells per ml in serum-free RPMI-1640 medium and incubated at 37°C to allow monocytes to adhere. After 2 hours, the media containing the unattached cells was replaced with routine culturing RPMI-1640 medium. Primary monocytes were incubated as above into macrophage-like cells with a medium replacement at 24 and 48 hours and at every 48 hour interval up to 8 days in culture.
2.4 Serum Starvation of THP-1 Cells

THP-1 cell proliferation and viability was investigated in culture under different percentages of FCS. THP-1 cells were cultured to near confluency as described in section 2.1.2. Cells were centrifuged at 1200rpm (400g) for 3 minutes and the pellet resuspended in PBS (37°C). Cell number and viability was determined using trypan blue solution (0.4%; Invitrogen Ltd., UK) as described in section 2.1.2. Cells were diluted to $1 \times 10^5$ viable cells per ml in RPMI-1640 medium containing either 10%, 5% or 2% FCS. Cells were cultured in a 12-well plate in triplicate wells with 2ml of cells ($2 \times 10^5$) per well. At 24 hour intervals, the cells were mixed to create a full suspension and an aliquot of cells was collected and the cell count and viability was determined as described in section 2.1.2.

2.5 Growth Factors and Antibodies

Several cellular growth factors were used in this study (Table 2.1). Growth factors were reconstituted with 0.1% bovine serum albumin (BSA A9418; Sigma-Aldrich, UK.) in either PBS or 10nM acetic acid. All reconstituted growth factors were stored in working aliquots at -80°C and thawed as required. Once thawed the aliquot was kept at 4°C for up to 1 month.

Several commercial primary and secondary antibodies were used in this study (Table 2.2). The rat monoclonal antibodies ICR62 (IgG2b) and ICR16 (IgG2a) used in this study were developed and characterised by Dr Helmout Modjtahedi (Modjtahedi et al., 1993). ICR62 and ICR16 were raised against the external domain of the EGFR.
on the human breast carcinoma cell line MDA-MB468 and the human head and neck carcinoma cell line HN5 respectively.

### 2.6 EGFR mRNA Analysis

#### 2.6.1 Cell Culture Preparation

THP-1 cells were cultured to near confluency as described in section 2.1.2. THP-1 cells were collected, centrifuged and resuspended in warm PBS. Cells number and viability was determined and THP-1 cells were diluted and seeded at $1.5 \times 10^6$ viable cells per well (2ml) in a 24-well plate in RPMI-1640 media supplemented with 2% or 10% (control) FCS. Cells were incubated for 18 hours to acclimatise.

For differentiated THP-1 experiments, THP-1 cells were collected and counted as described above. Cells were seeded at $8 \times 10^5$ viable cells per well (2ml) in a 24-well plate in routine culturing RPMI-1640 medium in the presence of a final concentration of 10nM PMA (Sigma-Aldrich, UK). Cells were incubated for 72 hours into macrophage-like cells as described in section 2.2.

At the start of the experiment (0 hours), the previous medium was removed and replaced with 2ml RPMI-1640 medium containing 2% FCS supplemented with the four EGFR ligands (EGF, HB-EGF, TGFα or BTC) at 25nM or 50nM, or the monocyte activators IFNγ (100U/ml), IL-1β (2.5ng/ml) and LPS (100ng/ml) in duplicate or triplicate tissue culture wells. The treatment concentrations for the monocyte activators had been previously optimised for THP-1 cells within the
Table 2.1 Commercial growth factors used in this study. PBS: Phosphate buffered saline; BSA: Bovine serum albumin

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Reconstitution</th>
<th>Stock Dilution</th>
<th>Product Code/ Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betacellulin (BTC) Human Recombinant</td>
<td>0.22µm-filtered PBS containing 0.1% BSA</td>
<td>10µg/ml</td>
<td>B-3670 Sigma-Aldrich Company Ltd., Dorset, UK</td>
</tr>
<tr>
<td>Epidermal Growth Factor (EGF) Human Recombinant</td>
<td>0.22µm-filtered 10nM acetic acid containing 0.1% BSA</td>
<td>200µg/ml</td>
<td>E-9644 Sigma-Aldrich Company Ltd., Dorset, UK</td>
</tr>
<tr>
<td>Heparin-Binding Epidermal Growth Factor-Like Growth Factor (HB-EGF) Human Recombinant</td>
<td>0.22µm-filtered PBS containing 0.1% BSA</td>
<td>50µg/ml</td>
<td>E-4643 Sigma-Aldrich Company Ltd., Dorset, UK</td>
</tr>
<tr>
<td>Interleukin-1β (IL-1β) Human Recombinant</td>
<td>0.22µm-filtered PBS containing 0.1% BSA</td>
<td>1ng/ml</td>
<td>201-LB R&amp;D Systems Europe Ltd., Abingdon, UK</td>
</tr>
<tr>
<td>Interferon γ (IFNγ) Human Recombinant</td>
<td>0.22µm-filtered PBS containing 0.1% BSA</td>
<td>100U/µl</td>
<td>285-IF R&amp;D Systems Europe Ltd., Abingdon, UK</td>
</tr>
<tr>
<td>Monocyte Chemotactic Protein-1 (MCP-1) Human Recombinant</td>
<td>0.22µm-filtered PBS containing 0.1% BSA</td>
<td>10µg/ml</td>
<td>M-6667 Sigma-Aldrich Company Ltd., Dorset, UK</td>
</tr>
<tr>
<td>Macrophage Colony Stimulating Factor (M-CSF) Human Recombinant</td>
<td>0.22µm-filtered PBS containing 0.1% BSA</td>
<td>5µg/ml</td>
<td>216-MC R&amp;D Systems Europe Ltd., Abingdon, UK</td>
</tr>
<tr>
<td>Transforming Growth Factor α (TGFα) Human Recombinant</td>
<td>0.22µm-filtered 10nM acetic acid containing 0.1% BSA</td>
<td>100µg/ml</td>
<td>239-A R&amp;D Systems Europe Ltd., Abingdon, UK</td>
</tr>
</tbody>
</table>
### Table 2.2 Commercial primary and secondary antibodies used in this study.

FC: Flow Cytometry; IHC: Immunohistochemistry; ICC: Immunocytochemistry; WB: Western Blotting; RPE-Cy5: R-phycoerythrin-cyanine5; FITC: Fluorescein isothiocyanate.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Stated Specificity</th>
<th>Experimental Use and Dilution</th>
<th>Product Code/Company</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primaries</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse anti-human CD14: RPE-Cy5 conjugate (IgG2a; clone Tuk4)</td>
<td>Human CD14 cell surface antigen</td>
<td>FC (1/20)</td>
<td>MCA1568C Serotec Ltd., Oxford, UK</td>
</tr>
<tr>
<td>Rabbit anti-human EGFR (polyclonal, affinity purified)</td>
<td>C-terminus of human EGFR</td>
<td>WB (1/200)</td>
<td>SC-03 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>Mouse anti-human smooth muscle cell actin (IgG2a, kappa; clone 1A4)</td>
<td>a-smooth muscle cell isoform of actin</td>
<td>IHC (1/500)</td>
<td>M 0851 DAKO Ltd., Ely, UK</td>
</tr>
<tr>
<td>Mouse monoclonal anti-human CD68, macrophage (IgG1, kappa; clone KP1)</td>
<td>Human CD68 cell surface antigen</td>
<td>IHC (1/100)</td>
<td>M 0814 DAKO Ltd., Ely, UK</td>
</tr>
<tr>
<td>Mouse monoclonal HAM56 to macrophage (IgM, kappa; clone HAM56)</td>
<td>Human macrophages (specific antigen has not been characterised)</td>
<td>IHC (1/30)</td>
<td>ab8186 Abcam Ltd., Cambridge, UK</td>
</tr>
<tr>
<td>Mouse IgG2a negative control: RPE-Cy5 conjugate</td>
<td>Negative control in human tissues</td>
<td>FC (1/20)</td>
<td>MCA929C Serotec Ltd., Oxford, UK</td>
</tr>
<tr>
<td>Mouse IgG1 negative control</td>
<td>Negative control in human tissues</td>
<td>IHC (1/25)</td>
<td>MCA928 Serotec Ltd., Oxford, UK</td>
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<tr>
<td>Mouse IgM negative control (IgM, kappa; clone DAK-G08)</td>
<td>Negative control in human tissues</td>
<td>IHC (1/1000)</td>
<td>X 0942 DAKO Ltd., Ely, UK</td>
</tr>
<tr>
<td>Rat IgG2b negative control (IgG2b, clone LO-DNP-11)</td>
<td>Negative control in human tissues</td>
<td>FC (1/100) ICC (1/5)</td>
<td>MCA1125R Serotec Ltd., Oxford, UK</td>
</tr>
<tr>
<td>Rat IgG2a negative control (IgG2a, clone LO-DNP-16)</td>
<td>Negative control in human tissues</td>
<td>IHC (1/20)</td>
<td>MCA1124R Serotec Ltd., Oxford, UK</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F(ab')2 rabbit anti-rat IgG: FITC conjugate</td>
<td>Rat IgG</td>
<td>FC (1/100) ICC (1/300)</td>
<td>STAR17B Serotec Ltd., Oxford, UK</td>
</tr>
</tbody>
</table>
laboratory. Control cells in routine culturing RPMI-1640 medium had fresh medium re-applied. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

At appropriate time points (6, 12, 24 and 48 hours), 1ml of THP-1 cells were transferred from the well to a 1.5ml microcentrifuge tube and centrifuged at 200g for 3 minutes at room temperature. The supernatant was removed and the remaining 1ml of cells was transferred from the well to the microcentrifuge tube. The cells were once again centrifuged at 200g for 3 minutes at room temperature and the supernatant was removed. The pellet was resuspended in 500µl TRIzol (Invitrogen Ltd., UK) and mixed to ensure complete cell lysis. For differentiated THP-1 cells, the medium was removed at the appropriate times points (0, 6, 12, 24 and 48 hours) and 500µl TRIzol (Invitrogen Ltd., UK) was added. The TRIzol (Invitrogen Ltd., UK) was mixed to ensure complete cell lysis. All samples were stored at -20°C for short term storage or -80°C for long term storage prior to RNA extraction.

Mononuclear cells were isolated and cultured as described in section 2.3. Cells were cultured in 24-well plates at 2x10⁶ cells (2ml) per well. At the required time points the medium was removed and 500µl TRIzol (Invitrogen Ltd., UK) was added. The TRIzol (Invitrogen Ltd., UK) was mixed to ensure complete cell lysis. All samples were stored as above prior to RNA extraction.

2.6.2 RNA Extraction & Quantification

TRIzol cell lysates were defrosted at room temperature and then mixed gently. Total cellular RNA was isolated as per manufactures instructions – 200µl chloroform was added per 1ml TRIzol and shaken vigorously for 15 seconds. Samples were left to
settle for 5 minutes at room temperature and subsequently centrifuged at 12,000g for 15 minutes at 4°C. The top, aqueous layer was carefully removed, transferred to a fresh microcentrifuge tube and mixed by inversion with an equal volume of propan-2-ol. The sample was again centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet was resuspended and vortexed in 500μl ice-cold 70% ethanol made in autoclaved diethylpyrocarbonate (DEPC; Sigma-Aldrich, UK)-treated MilliQ water. The RNA was centrifuged at 10,000g for 5 minutes at 4°C. The supernatant was removed and the RNA pellet was left to air-dry. Pellets were resuspended in 20μl DEPC-treated MilliQ water per 500μl TRIzol and stored at -80°C prior to quantification.

RNA concentration was measured using a Nanodrop spectrophotometer (Agilent Technologies, Stockport, UK). RNA integrity was assessed by gel electrophoresis. 2μg/10μl RNA in 10x loading dye (glycerol buffer containing bromophenol blue, Sigma-Aldrich, UK) was separated on a RNase-free 2% agarose (Invitrogen Ltd., UK) 1x Tris-Borate-EDTA (TBE) gel (10x solution - 108g Tris base, 55g boric acid and 9.3g Na₂EDTA to 1 litre with deionised water, pH 8.3) with 1% ethidium bromide (Promega, Southampton, UK). The gel was run at 120V for 30 minutes with appropriate ssRNA ladder (New England Biolabs (UK) Ltd., Hitchin, UK) to separate the 28 S, 18 S and 5 S ribosomal RNAs and visualised under ultra violet light (figure 2.1).

Samples were diluted to a concentration of 10ng/μl in autoclaved DEPC-treated MilliQ water. 100ng RNA was treated with RNase-Free DNasel (Promega) for 40 minutes at 37°C. The reaction was terminated with the addition of 1μl DNase STOP
Figure 2.1 Gel electrophoresis of isolated RNA. Isolated RNA was subjected to electrophoresis on a 2% RNase-free TBE agarose gel at 120V for 30 minutes to show integrity of the samples. The bands of the 28 S, 18 S and 5 S ribosomal RNAs are indicated. The example samples run are; Lane A – no sample, B – RNA ladder, C – EJ standard curve sample, D – THP-1 standard curve sample, E – human monocyte sample, F – THP-1 sample, G – differentiated THP-1 sample and H – no sample.
buffer and with incubation for 10 minutes at 65°C. Samples were then stored at -80°C.

2.6.3 Quantitative Real-Time PCR

Quantitative real-time PCR was performed using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen Ltd., West Sussex, UK). A QuantiTect Primer Assay (QT00085701, GeneGlobe, Qiagen Ltd., UK) was used to detect human EGFR, based upon the NCBI sequence NM_005228 (figure 2.2). Primers were reconstituted to a 10x concentration in DEPC-treated MilliQ water.

Primers for the internal housekeeping gene MLN51 (Metastatic lymph node-51) (Degot et al., 2002; King & Cooper, 1986) were designed based on the NCBI sequence NM_007359; left-hand sequence CAA GAG TGC TGA GGA GTC GG, right-hand sequence TCA TTA GCT TCT GAT TTC AG by HA Khwaja (PhD Thesis University of Oxford, 2005). The primer pair was synthesised by MWG-Biotech (MWG-Biotech Ltd., Milton Keynes, UK) and reconstituted to a concentration of 100pmol/µl (100µM) in DEPC-treated MilliQ water.

Quantitative PCR (QPCR) reaction mixes were composed on ice and shown in Table 2.3. 9.5µl reaction mix was loaded into appropriate wells of Thermo-Fast opaque white 96 well plates (Abgene, Epsom, UK). 20ng (3µl) DNased RNA was added to appropriate wells in duplicate for EGFR and MLN51 reactions resulting in a final reaction volume per well of 12.5µl. Plates were sealed with Absolute QPCR seals (Abgene, UK), centrifuged at 2000rpm for 1 minute, agitated to mix the components and centrifuged once again. The plates were kept on ice at all times.
Figure 2.2 GeneGlobe EGFR primers. The location is based on NCBI sequence NM_005228. The primers are designed to cross the boundary of exon 1 and 2 based on the Ensembl Transcript ENST00000275493.
Table 2.3 Composition of master mixes for EGFR and MLN51 QPCR reactions.

**EGFR**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x QuantiTect SYBR Green RT-PCR Master Mix (Qiagen Ltd., UK)</td>
<td>6.25μl</td>
<td>1x/reaction</td>
</tr>
<tr>
<td>10x QuantiTect EGFR Primer Assay (Qiagen Ltd., UK)</td>
<td>1.25μl</td>
<td>1x/reaction</td>
</tr>
<tr>
<td>QuantiTect RT mix (Qiagen Ltd., UK)</td>
<td>0.125μl</td>
<td>0.125μl/reaction</td>
</tr>
<tr>
<td>RNase-free H₂O (Qiagen Ltd., UK)</td>
<td>1.875μl</td>
<td>-</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>9.5μl</td>
<td></td>
</tr>
</tbody>
</table>

**MLN51**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x QuantiTect SYBR Green RT-PCR Master Mix (Qiagen Ltd., UK)</td>
<td>6.25μl</td>
<td>1x/reaction</td>
</tr>
<tr>
<td>Primer R (10pmol/μl)</td>
<td>0.75μl</td>
<td>7.5pmol/reaction</td>
</tr>
<tr>
<td>Primer L (10pmol/μl)</td>
<td>0.75μl</td>
<td>7.5pmol/reaction</td>
</tr>
<tr>
<td>QuantiTect RT mix (Qiagen Ltd., UK)</td>
<td>0.125μl</td>
<td>0.125μl/reaction</td>
</tr>
<tr>
<td>RNase-free H₂O (Qiagen Ltd., UK)</td>
<td>1.625μl</td>
<td>-</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>9.5μl</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 Cycling conditions for the QPCR of EGFR and MLN51.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>30 minutes</td>
<td>50°C</td>
</tr>
<tr>
<td>PCR initial activation step (cDNA denaturation)</td>
<td>15 minutes</td>
<td>95°C</td>
</tr>
<tr>
<td>3 step cycling (40 cycles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 seconds</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>30 seconds</td>
<td>55°C</td>
</tr>
<tr>
<td>Data Collection</td>
<td>30 seconds</td>
<td></td>
</tr>
</tbody>
</table>
QPCR analysis was performed using the ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) for the monocyte analysis or the 7500 Fast Real-Time PCR System (Applied Biosystems, USA) for THP-1 cell analysis. The cycling conditions for the QPCR reaction are shown in Table 2.4. QuantiTect Primer Assays are pre-optimised to work with QuantiTect SYBR Green RT-PCR kits at an annealing temperature of 55°C so no further optimisation of the reaction was necessary.

At the end of the QPCR reaction, Ct (Cycle Threshold) values were obtained by setting the baseline of the amplification and adjusting the threshold to the start of the exponential region of the amplification curves. For the 7500 Fast Real-Time PCR System (Applied Biosystems, USA) the baseline was set between cycles 3 and 15 and the threshold was adjusted to 0.5. For the ABI Prism 7000 Real-Time PCR System (Applied Biosystems, USA) the baseline was set between cycles 6 and 15 and the threshold was adjusted to 0.02. These settings were used consistently for each machine to ensure consistency between Ct values for each experiment.

Melt curve analysis of the PCR products was performed over a range of 65°C - 95°C to verify their specificity and identity. Gel electrophoresis of the PCR products was also performed to confirm product specificity. Total PCR product (12.5μl) was mixed with 10x loading buffer (Sigma-Aldrich, UK) and loaded onto a 2% agarose gel (Invitrogen Ltd., UK) with 1% TBE buffer. The gel was run at 40V for 1 hour to ensure sufficient separation between the specific product and primer-dimer bands.
2.6.4 Standard Curve Preparation

A standard curve for each gene of interest was prepared from RNA extracted from the EJ cell line which expresses both MLN51 and EGFR mRNA. EJ RNA was prepared and measured as described in section 2.6.2. 5μg RNA was treated with DNase I (Promega, UK) as described previously. A serial dilution of RNA in RNase-free water was made covering the range from 0.01ng to 100ng for each gene. Aliquots of each dilution were kept at -80°C and defrosted when necessary.

Separate standard curves were constructed for human peripheral blood monocyte analysis and THP-1 cell analysis as separate machines were used. The Ct values for each dilution were plotted against their corresponding log ng amounts of RNA to create standard curves for each gene. Reference points were incorporated into each further plate analysis to create a compiled standard curve for a specific experiment and to ensure consistency between each QPCR plate.

The ng amount of EGFR and MLN51 for each test sample was calculated from the Ct value utilizing the equation of the prepared standard curve as shown below:

\[ \text{ng} = 10^{((\text{Ct} \times A) + B)} \]

where A is the gradient and B is the intercept of the specific standard curve

EGFR mRNA expression was normalised to MLN51 expression (ng EGFR divided by ng MLN51 for each individual sample) and reported as a fold induction over experimental plate control values.
2.7 Immunoprecipitation

2.7.1 Preparation of Whole Cell Lysates

A 175cm² tissue culture flask of THP-1 cells, cultured as described in section 2.1.2, were grown to near confluency (6.6x10^7 total cells) and transferred to a centrifuge tube. EJ cells were grown to near confluency in a 75cm² tissue culture flask as described in section 2.1.2. The monolayer was washed with cold wash buffer [0.02% sodium azide (Sigma-Aldrich, UK) and 1mM phenylmethanesulphonylfluoride (PMSF; Sigma-Aldrich, UK) in PBS] before the cells were detached into 5ml wash buffer using a cell scraper. The cells were then transferred to a centrifuge tube. All collected cells were then washed twice by centrifugation at 1200rpm (400g) for 3 minutes in cold wash buffer and resuspended in cold lysis buffer (as wash buffer above containing 1% Triton-X 100 (Sigma-Aldrich, UK) in PBS) (4ml for near confluent 75cm² flask of EJ cells; 1ml per 13x10^6 THP-1 cells). Cell lysates were incubated on ice for 20 minutes and centrifuged at 30,000rpm for 30 minutes at 4°C (Beckman Ultra Centrifuge, Beckman Dickinson, UK). The supernatant was removed and stored at -80°C.

2.7.2 Preparation of Protein-G Sepharose Bead and Antibody Conjugation

Protein-G sepharose beads (Pharmacia, UK) (50μl) were conjugated to 10μg of specific antibody (ICR62) or isotype control antibody (rat IgG2b) as described below. Protein-G sepharose beads were transferred to a 1.5ml microcentrifuge tube and centrifuged at a maximum of 500rpm for 30 seconds. The ethanol supernatant was removed and the beads resuspended in 1ml wash buffer (as above) and
centrifuged once again as before. This was repeated twice. After the final wash, the protein-G sepharose beads were resuspended in wash buffer.

About 10μg of antibody or equal volume of wash buffer (no antibody control) was added to every 50μl prepared protein-G sepharose beads in a microcentrifuge tube. Tubes were rotated for 1 hour at room temperature or at 4°C overnight. The conjugated beads were washed thrice with wash buffer as before and resuspended in 1ml wash buffer and stored upright at 4°C.

2.7.3 Cell Lysate Incubation

Antibody conjugated beads were centrifuged as before and supernatant removed. Beads were resuspended in original bead volume and 1ml of cell lysate was added to every 50μl of protein G sepharose bead conjugate. Beads were rotated as described above and washed thrice in 1ml wash buffer by centrifugation as before. After last wash, the supernatant was removed and an equal volume of NuPAGE® lithium dodecyl sulphate (LDS) sample buffer (details in section 2.8) to beads was added. The sample-buffer mix was heated at 75°C for 10 minutes, cooled and stored at -20°C.

2.8 SDS-PAGE and Western Blotting

The expression of the EGFR was investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Table 2.5 details the products purchased from Invitogen Ltd. (UK). A temporal list of working dilutions for this system is detailed below.
Lysis Buffer (1x): For 100μl solution - 25μl NuPAGE® LDS sample buffer (4x; Invitrogen Ltd., UK), 10μl NuPAGE® reducing agent (10x; Invitrogen Ltd., UK) and 65μl distilled H₂O. The lysis buffer was supplemented with 1μl protease inhibitor cocktail (Sigma-Aldrich, UK) containing 104nM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 80μM aprotinin, 2nM leupeptin, 4nM bestatin, 1.5mM pepstatin A and 1.4mM E-64.

Running Buffer (1x): Consisted of 50ml NuPAGE® 4-morpholine propanesulfonic acid (MOPS) sodium dodecyl sulphate (SDS) running buffer (20x; Invitrogen Ltd., UK) and 950ml distilled water. This is enough for one XCell II™ Surelock™ Mini-Cell tank.

Transfer Buffer (1x): Consisted of 50ml NuPAGE® transfer buffer (20x; Invitrogen Ltd., UK), 200ml methanol (100ml per gel), 1ml antioxidant and 750ml of distilled water. This is enough for one XCell II™ Surelock™ Mini-Cell tank.

Blocking Solution: Consisted of 2ml blocker/diluent A (Invitrogen Ltd., UK), 3ml of blocker/diluent B (Invitrogen Ltd., UK) and 5ml of distilled water. This is enough for one PVDF membrane.

Primary Antibody Diluent: Consisted of 2ml blocker/diluent A (Invitrogen Ltd., UK), 1ml of blocker/diluent B (Invitrogen Ltd., UK) and 7ml of distilled water. This is enough for one PVDF membrane.
**Antibody Wash (1x):** Consisted of 10ml antibody wash solution (16x; Invitrogen Ltd., UK) and 150ml distilled water. This is enough for one PVDF membrane over two steps.

### 2.8.1 SDS-PAGE

SDS-PAGE was performed using the XCell II™ Surelock™ Mini-Cell system (Invitrogen Ltd., UK). The prepared immunoprecipitated cell lysates were removed from -20°C and defrosted on ice. 15µl cell lysate was loaded per well of a 1mm 10% Bis-Tris gel (Invitrogen Ltd., UK) with 5µl of multi-coloured molecular weight standard (Invitrogen Ltd., UK). Gel electrophoresis was performed at a constant 200V for 55 minutes in 1x NuPAGE® MOPS SDS running buffer (Invitrogen Ltd., UK) with added antioxidant (400µl; Invitrogen, Ltd., UK).

### 2.8.2 Western Blotting

Following SDS-PAGE, the proteins were transferred from the gel to a 0.45µm pore polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). This was performed in at a constant 30V for 2 hours in 1x NuPAGE® transfer buffer, using the XCell™ Mini-Cell Blot Module kit (Invitrogen Ltd., UK). Effective transfer was indicated by the presence of all coloured bands of the multi-coloured standard (Invitrogen Ltd., UK) on the PVDF membrane post-transfer. Following Western transfer, the membrane was washed briefly in deionised H₂O before incubation with 10ml blocking solution (Invitrogen Ltd., UK) for 30 minutes at room temperature or overnight at 4°C on a rotary shaker set at 1 revolution per second.
Table 2.5 Reagents purchased from Invitrogen Ltd., UK for SDS-PAGE and Western Blotting.

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>WesternBreeze® chemiluminescent western blot immunodetection kit</td>
<td>Blocker/diluent A and B; secondary antibody solution; wash solution (16x); chemiluminescent substrate</td>
</tr>
<tr>
<td>Blocker/diluent A</td>
<td>Buffered saline containing detergent</td>
</tr>
<tr>
<td>Blocker/diluent B</td>
<td>Concentrated Hammerstein casein solution</td>
</tr>
<tr>
<td>Secondary antibody solution</td>
<td>Alkaline phosphate-conjugated, affinity purified, anti-rabbit IgG</td>
</tr>
<tr>
<td>Wash solution (16x)</td>
<td>Buffered saline containing detergent</td>
</tr>
<tr>
<td>Chemiluminescent substrate</td>
<td>CDP-Star® substrate for alkaline phosphatase</td>
</tr>
<tr>
<td>MultiMark® multicoloured standard</td>
<td>On NuPAGE® 10% Bis-Tris Gels/MOPS: Myosin 188kDa; Phosphorylase B 97kDa; Glutamic Dehydrogenase 52kDa; Carbonic Anhydrase 33kDa; Myoglobin Red 21kDa; Myoglobin Blue 19kDa; Lysozyme 12kDa; Aprotinin N/A; Insulin N/A</td>
</tr>
<tr>
<td>NuPAGE® antioxidant</td>
<td>Constituents not specified</td>
</tr>
<tr>
<td>NuPAGE® Gels</td>
<td>10% Bis-Tris 1mm, 15 well gels</td>
</tr>
<tr>
<td>NuPAGE® LDS sample buffer (4x)</td>
<td>106mM Tris HCl; 141mM Tris base; 2% LDS; 10% Glycerol; 0.51mM EDTA; 0.22mM SERVA® Blue G250; 0.175mM Phenol Red; pH 8.5</td>
</tr>
<tr>
<td>NuPAGE® MOPS SDS running buffer (2x)</td>
<td>50mM MOPS; 50mM Tris base; 0.1% SDS; 1mM EDTA; pH 7.7</td>
</tr>
<tr>
<td>NuPAGE® transfer buffer (20x)</td>
<td>25mM Bicine; 25mM Bis-Tris (free base); 1mM EDTA; pH 7.2</td>
</tr>
</tbody>
</table>
Membranes were washed three times for 5 minutes with 20ml deionised water prior to incubation with primary antibody made up in 10ml of antibody diluent (Invitrogen Ltd., UK). Following one hour incubation at room temperature, the membrane was washed three times for 5 minutes with antibody wash solution (Invitrogen Ltd., UK), and then incubated with 10ml of secondary antibody solution (Invitrogen Ltd., UK) for 30 minutes at room temperature. Following three more 5 minutes washes in antibody wash solution and two 3 minute washes in deionised water, 2.5ml of chemiluminescent substrate (CDP-Star®; Invitrogen Ltd., UK) was evenly added to the membrane. Following 5 minutes incubation, the excess CDP-Star® was blotted from the membrane using filter paper and the membrane was wrapped in cling film in preparation for luminography.

For visualisation of the transferred proteins the membrane blot was exposed to Kodak BioMax XAR scientific imaging film (Sigma-Aldrich, UK). The film was developed in CD18 manual developer and then fixed in CF42 non-hardening fixer (both 1/5 in water; Photosol Ltd., Brentwood, UK).

2.9 Flow Cytometry Analysis

2.9.1 Flow Cytometry Analysis of EGFR Expressing Tumour Cells

Human tumour cells were cultured as described in section 2.1.2 and dissociated using 0.25% trypsin. Following trypsin inactivation as described in section 2.1.2, the cells were washed by centrifugation at 1200rpm (400g) for 3 minutes in PBS and resuspended at 1x10^6 cells per ml of DMEM with 2% FCS. Cells were incubated by rotation with primary antibody or matched isotype control for 1 hour at 4°C. Cells
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were subsequently washed by centrifugation at 1200rpm (400g) for 3 minutes in 1ml DMEM with 2% FCS three times. After last wash cells were resuspended in 1ml DMEM with 2% FCS and incubated by rotation with FITC-conjugated F(abc')2 rabbit anti-rat IgG secondary antibody (Table 2.2) for 1 hour at 4°C. Cells were washed once in 1ml DMEM with 2% FCS and twice more in PBS by centrifugation. The cells were then transferred to flow cytometry tubes (BD Falcon™, Becton Dickinson UK Ltd., Oxford, UK), centrifuged once again and the cell pellet was resuspended in 1ml FACS Flow™ buffer (Becton Dickinson UK Ltd.) prior to analysis. A minimum of 10,000 events were recorded by excitation with an argon laser at 488nm, and analysed using the FL-1 detector (FITC detector; 525nm) using a FACScan flow cytometer (Becton Dickinson UK Ltd.) and CellQuest™ software. Subsequent fluorescent histogram analysis was obtained using Windows Multiple Document Interface (WinMDI) flow cytometry application version 2.9 (facs.scripps.edu).

2.9.2 Flow Cytometry Analysis of THP-1 and Differentiated THP-1 Cells

THP-1 cells were grown to near confluency in a 75cm² tissue culture flask as described in section 2.1.2. THP-1 cells were centrifuged at 1200rpm (400g) for 3 minutes and the pellet was resuspended in cold PBS. Differentiated THP-1 cells were created as described in section 2.2. After 72 hours the medium was removed and the adhered cells were washed with sterile PBS. Adhered cells were dissociated using cold sterile 10mM lidocaine hydrochloride (Sigma-Aldrich, UK)/ 10mM EDTA (pH 8; Sigma-Aldrich, UK) in PBS (Schafer et al., 2004; Nielsen, 1987). Cells were incubated on ice for 5 minutes and periodically agitated to assist dissociation. Dissociated cells were then collected into a centrifuge tube.
Both THP-1 and differentiated THP-1 cells were then centrifuged at 1200rpm (400g) for 3 minutes and resuspended in cold flow cytometry buffer (1% BSA (A9418, Sigma, UK), 0.05% sodium azide (Sigma-Aldrich, UK) in PBS) and counted. Cells were diluted to 1x10^7 cells per ml and aliquots of 100μl cells (1x10^6 cells) were dispensed per flow cytometry tube. Cells were incubated for 30 minutes in darkness at 4°C with primary antibody or appropriate isotype control, along with FITC-conjugated F(ab')2 rabbit anti-rat secondary antibody (Table 2.2). A final concentration of 2% human serum was added to reduce non-specific FcγR binding. Cells were washed with flow cytometry buffer by centrifugation and the resulting pellet was resuspended in 4% paraformaldehyde (Sigma-Aldrich, UK) in PBS and stored at 4°C to await analysis. Cells were analysed as described in section 2.9.1.

2.9.3 Flow Cytometry Analysis of Primary Human Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as described previously in section 2.3. After 2 hours adherence in serum-free media the non-adherent cells were removed. Adhered cells were dissociated using cold sterile 10mM lidocaine hydrochloride (Sigma-Aldrich, UK)/10mM EDTA (pH 8; Sigma-Aldrich, UK) in PBS (Schafer et al., 2004; Nielsen, 1987). Cells were incubated on ice for 5 minutes and periodically agitated to assist dissociation. Dissociated cells were then collected into a tube and centrifuged at 1200rpm (400g) for 3 minutes. The pellet was resuspended in flow cytometry buffer (as in section 2.9.2) and the cells were counted. Cells were diluted to 1x10^7 cells per ml and aliquots of 100μl cells (1x10^6 cells) were dispensed per flow cytometry tube. Cells were incubated for 30 minutes in darkness at 4°C with primary antibody or appropriate isotype control, along with FITC-conjugated F(ab')2 rabbit ant-rat IgG secondary antibody where
appropriate (Table 2.2). A final concentration of 2% human serum was added to reduce non-specific FcYR binding. Cells were washed with flow cytometry buffer by centrifugation and the resulting pellet was resuspended in 4% paraformaldehyde (Sigma-Aldrich, UK) in PBS and stored at 4°C to await analysis. Events (20,000) were recorded by excitation with a 20mW argon laser at 488nm, and emission collected at 525nm for FITC (FL-1) and 682nm (for the PerCP fluorochrome) for RPE-Cy5 (FL-3) using a BD FACSCanto™ flow cytometer (BD Biosciences UK Ltd., Oxford, UK) and BD FACSDiva™ v.4.0 software (BD Biosciences UK Ltd.). Compensation between RPE-Cy5 and FITC was set at 2.18.

2.9.4 Flow Cytometry Analysis of 8 Day Monocyte Derived Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and cultured in 6-well plates (1x10⁷ cells per well) into monocyte-derived macrophage cells as described previously in section 2.3. After 8 days in culture, the growth medium removed and replaced with RPMI-1640 medium with 2% FCS containing 0.1nM, 10nM or 25nM HB-EGF. After 2 hours the medium was removed and the adhered cells were dissociated and prepared for flow cytometry analysis as described in section 2.9.3.

2.10 Immunocytochemistry

2.10.1 Cytospin Preparation

THP-1 cells were grown to near confluency as described in section 2.1.2. THP-1 cells were differentiated as described in section 2.2 and dissociated with 10nM lidocaine hydrochloride/EDTA as described in section 2.9.2. EJ cells were grown to
near confluency and dissociated as described in section 2.1.2. All cells were washed in PBS, counted and diluted to 2x10^5 cells per ml in routine culturing RPMI-1640 or DMEM media. Aliquots of 200μl cells (4x10^4 cells) were loaded into the funnels of a ThermoShandon Cytospin 4 machine (Thermo Electron Corporation, Cheshire, UK). Cells were spun onto pre-washed, uncoated glass slides at 1000rpm for 5 minutes. The cells were left to air dry on the slides for 1 hour at room temperature and were subsequently wrapped in tin foil and stored at -20°C until staining.

2.10.2 Staining Procedure

Slides were removed from -20°C storage and air-dried for 1 hour at room temperature. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, UK) in PBS for 15 minutes then washed three times in cold PBS. Cells were then permeabilised with cold 0.5% Triton-X 100 (Sigma-Aldrich, UK) in PBS for 15 minutes at room temperature. Slides were washed three times in cold PBS containing 0.5% BSA (A9418, Sigma-Aldrich, UK). Primary antibodies and appropriate matched isotype controls were diluted to their working concentrations in cold 0.5% BSA in PBS. Slides were incubated for 1 hour in darkness at 4°C. Slides were washed three times in cold 0.5% BSA in PBS before application of the FITC-conjugated F(ab')2 rabbit anti-rat IgG secondary antibody (Table 2.2) for 30 minutes in darkness at 4°C. Slides were again washed three times in cold PBS. The nuclear Hoechst 33258 stain (B2883, Sigma) was diluted to 2μg/ml in PBS and applied to the cells for 5 minutes in darkness at room temperature. Slides were washed in cold PBS and mounted with a coverslip in Vectashield mounting medium (H1000; Vector Laboratories Ltd., Peterborough, UK). Slides were wrapped in tin-foil and stored at 4°C until analysis. Cells were visualised using a Leica DMLB microscope with Leica Qwin image
analysis software (Leica Microsystems Ltd., Milton Keynes, UK). The microscope was equipped with a colour JVC TK-C1380 video camera with the integration times set at 3 frames for FITC fluorescence and 0 frames for Hoechst fluorescence.

2.11 Chemotaxis Assay

THP-1 cells were grown to near confluency in a 75cm$^2$ tissue culture flask as described in section 2.1.2. THP-1 cells were collected and centrifuged at 1200rpm (400g) for 3 minutes and the pellet was resuspended in cold PBS. Cells were once again centrifuged and the cell pellet was resuspended in RPMI-1640 medium containing 1% IgG-free BSA (A2058, Sigma-Aldrich, UK). Cells were counted using a haemocytometer and subsequently diluted to 1x10$^6$ cells per ml.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and cultured as described previously in section 2.3. Monocytes were cultured in 25cm$^2$ tissue culture flasks at 1x10$^6$ cells per ml. For monocyte experiments, after 2 hours incubation in serum-free RPMI-1640 medium the non-adherent cells were dissociated as described below. For experiments involving 48 hour monocytes and 8 day monocyte-derived macrophages, after 2 hours incubation the serum-free RPMI-1640 medium was replaced with routine culturing RPMI-1640 medium. The medium was replaced again at 24 and 48 hours, and at every 48 hour interval up to 8 days in culture. At 48 hours and 8 days the cells were dissociated as described below.

All adhered cells were dissociated using cold sterile 10mM lidocaine hydrochloride (Sigma-Aldrich, UK) 10mM EDTA (pH 8; Sigma-Aldrich, UK) in PBS. Cells were
incubated on ice for 5 minutes and periodically agitated to assist dissociation. Dissociated cells were then collected into a tube and centrifuged at 1200rpm (400g) for 3 minutes. The cell pellet was resuspended in RPMI-1640 with 1% IgG-free BSA (A2058; Sigma-Aldrich, UK). Cells were counted using a haemocytometer and subsequently diluted to 1x10^6 cells per ml.

A microBoydon chemotaxis chamber (Neuroprobe, Bethesda, MD, USA) was assembled with 28μl control medium (RPMI-1640/1% IgG-free BSA) or the chemokine of interest (prepared in RPMI-1640/1% IgG-free BSA) being placed in triplicate wells in the lower reservoir. A polycarbonate polyvinyl pyrrolidone (PVP)-free membrane with 5μm pores (Poretics Corp, Livermore, CA, USA) was placed on top and the top section of reservoirs was assembled. 50μl of cell suspension (5x10^4 cells) was placed into each well in the upper reservoir. The chamber was incubated for 2-4 hours at 37°C in a humidified 5% CO₂ atmosphere. The chamber was disassembled and the membrane removed, fixed and stained using Diff-Quik solutions; fixative (2 minutes), stain I (2.5 minutes) and stain II (1 minute) (Dade, Gamidor Technical Services, UK). The membrane was washed in deionised water to rinse off excess stain and mounted on a microscope slide so that the cells faced down. The membrane top was wiped with a damp cotton bud to remove unmigrated cells and another microscope slide was placed on top to keep the membrane in place. It was then left to air dry. Total migrated cells were counted in 10 fields of view at x400 magnification within each well using a Leica DMLB microscope (Leica Microsystems Ltd., Milton Keynes, UK). Chemotactic activity was normalised by expressing it as fold migratory increase over control cells.
2.12 Proliferation Assay (MTT)

THP-1 cells were cultured to near confluency as described in section 2.1.2. Cells were collected and washed by centrifugation in PBS at 1200rpm (400g) for 3 minutes. The cell pellet was resuspended in PBS and cells were counted using a haemocytometer. Cell viability was also checked using trypan blue solution (0.4%; Invitrogen Ltd., UK) as described in section 2.1.2. Cells were subsequently diluted to 2x10^5 cells per ml in RPMI-1640 medium with 2% FCS. Cells were distributed in 100μl aliquots (2x10^4 cells) to each well of a 96-well plate (Thermo-Fisher Scientific, Denmark) and incubated for 4 hours at 37°C in a humidified 5% CO₂ atmosphere. For differentiated THP-1 cell experiments, THP-1 cells, after being counted, were diluted to 4x10^5 cells per ml in routine culturing RPMI-1640 medium containing a final concentration of 10nM PMA (Sigma-Aldrich, UK). Cells were distributed in 100μl aliquots (4x10^4 cells) to each well of a 96-well plate and incubated for 72 hours at 37°C in a humidified 5% CO₂ atmosphere. After the incubation period, the medium was replaced with RPMI-1640 medium with 2% FCS in preparation for treatment.

The EGFR ligands (EGF, HB-EGF, BTC and TGFα), the monoclonal antibody ICR62 and the tyrosine kinase inhibitor gefitinib (IRESSA) ((Ciardiello et al., 2000); a kind gift to Dr Helmout Modjtahedi from Astra Zenica, UK) were diluted in RPMI-1640 medium with 2% FCS and 100μl aliquots were added to triplicate wells. Cells were incubated for 72 hours at 37°C in a humidified 5% CO₂ atmosphere. For analysis, 50μl of sterile MTT (5mg/ml, thiazolyl blue tetrazolium bromide; Sigma-Aldrich, UK) was added to each well and incubated for 4 hours. Plates were centrifuged at 400g for 5 minutes and the supernatant removed. The water-insoluble
MTT-formazan blue crystals were solubilized with 200µl dimethyl sulfoxide (DMSO) and the plates were shaken vigorously to ensure proper dissolution. Colour intensity/absorbance was read at 540nm using a Labsystems MultiSkan RC plate reader (Thermo Electron, UK).

2.13 Statistical Analysis

A $P$ value equal to or less than 0.05 was considered statistically significant. Data are expressed as mean ± SEM or SD where indicated for each variable. Statistical analysis was performed using an unpaired $t$ test or a one- or two-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison or Bonferroni post hoc tests where appropriate using GraphPad Prism (version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).
3.1 Introduction

The epidermal growth factor receptor has been found to be expressed on a wide variety of cell types and tissues throughout the human body and is important for embryogenesis and development, mediating cellular events such as differentiation, migration, proliferation and apoptosis (Casalini et al., 2004; Yano et al., 2003). Tissues expressing EGFR include those from epithelial, mesenchymal and neuronal origins (Normanno et al., 2006; Yano et al., 2003), as well as cells within the vasculature, particularly intimal (Tamura et al., 2001) and medial (Nakata et al., 1996) smooth muscle cells and endothelial cells (Styren et al., 1993). There is however, limited evidence of its expression on the human mononuclear cell lineage. Only two examples exist: brain derived microglial cells (Nolte et al., 1997) and macrophages associated with the inflammatory response in uveal melanoma (Scholes et al., 2001).

To date only one human monocytic cell line, U937, has been found to express the EGFR on its cell membrane (Eales-Reynolds et al., 2001). The receptor in this case was detected with flow cytometry using the monoclonal antibody ICR62. Furthermore, the phorbol ester phorbol 12-myristate 13-acetate (PMA) was shown to significantly increase the percentage of EGFR-positive cells.
3.1.1 Aim

This study aimed to investigate whether the EGFR is expressed on other human mononuclear cells, either primary cells or the cell line THP-1. Expression of the EGFR was established on monocytic THP-1 cells using flow cytometry and immunocytochemistry techniques. The effect of differentiation into a macrophage-like cell was also investigated. The detection of protein within THP-1 cells was performed using SDS-PAGE and western blotting techniques. Similarly, human peripheral blood monocytes and human macrophage-like cells were investigated.
3.2 The Cell Surface Expression of EGFR on Human Tumour Cells by Flow Cytometry

Three human carcinoma cell lines that express EGFR were used to validate the use of the anti-EGFR rat monoclonal antibody ICR62 in the flow cytometry system used for THP-1 cells. The three cell lines have different levels of EGFR expression. MCF-7 cells have an undetectable expression, EJ cells display medium expression (<2 x 10^5 receptors per cell) and HN5 cells display extensive expression (1.4 x 10^7 receptors per cell) (Modjtahedi & Dean, 1996).

The cell surface expression levels of EGFR as determined by flow cytometry in the three tumour cell lines is presented in figure 3.1. MCF-7 cells had a mean fluorescence intensity (MFI) of 3.44, which was barely above the MFI of the unstained control cells at 3.14. The EJ cells had a MFI of 179.11 and the high expressing HN5 cell line had a MFI of 4057.5. Controls for both the antibody isotype and secondary antibody showed no non-specific binding (data not shown).

In order to validate the use of ICR62 with the flow cytometry protocol designed for cells of the monocytic lineage, the level of EGFR expression in the EJ cell line was determined using the method described in section 2.9.2. This method had been previously optimised within the laboratory (Lamb et al., 2004). EJ cells were incubated with ICR62 along with the F(ab')2 rabbit anti-rat IgG FITC conjugated secondary antibody for 1 hour at 4°C (figure 3.2). Although the resulting MFI of 21.68 was considerably lower than for the previous method, the THP-1 flow
cytometry method was still considered a suitable method of detecting cell surface EGFR expression.
Figure 3.1 EGFR expression on the human carcinoma cell lines. The three tumour cell lines MCF-7 (A), EJ (B) and HN5 (C) were labelled as described in section 2.9.1 with the rat monoclonal antibody ICR62 (10μg/ml) (iii) for 1 hour at 4°C. Cells were then washed prior to incubation with the F(ab')2 rabbit anti-rat IgG FITC conjugated secondary antibody (10μg/ml) for 1 hour at 4°C. Unstained cells are shown in forward versus side scatter dot plot (i) and FL1-H FITC (ii) histogram form. The mean fluorescence intensity (MFI) of each sample is shown as the figures in the top left hand corner of each histogram. These are representative plots of two experiments.
Figure 3.2 EGFR expression on the EJ cell line using the same flow cytometry method used to stain THP-1 cells. The EJ tumour cell line was incubated with the rat monoclonal antibody ICR62 (10μg/ml) or IgG2b isotype control (10μg/ml) and the F(ab')2 rabbit anti-rat IgG FITC conjugated secondary antibody (10μg/ml) for 1 hour at 4°C as described in section 2.9.2. Unstained cells (A) were gated in a forward versus side scatter dot plot with the resulting FL1-H (FITC) histograms of unstained cells (B), IgG2b isotype control (C) and ICR62 (D). The mean fluorescence intensity (MFI) of each sample are shown as the figures in the top left hand corner of each histogram. This is a representative result of three experiments.
3.3 The Cellular Surface Expression of EGFR on the Monocytic THP-1 Cell by Flow Cytometry

Monocytic THP-1 cells were incubated in the presence of the anti-EGFR monoclonal antibody ICR62 and the appropriate isotype control as described in section 2.9.2. For flow cytometry analysis, THP-1 cells were gated in a forward versus side scatter dot plot and transformed into a FL1-H fluorescence histogram. An example is shown in figure 3.3A. Here, unstained cells had a MFI of 2.67 and the IgG2b isotype control had a MFI of 3.23. THP-1 cells incubated with ICR62 had a MFI 19.32. The table (figure 3.3B) shows the MFI of THP-1 cells incubated with ICR62 from repeat experiments. THP-1 cells had an average MFI of 21.62 ± 2.92 (mean ± SD) and therefore could be considered to express EGFR on their cell surface.
Figure 3.3 The cell surface expression of the EGFR on THP-1 cells. Monocytic THP-1 cells were incubated for 1 hour at 4°C in the presence of the monoclonal antibody ICR62 (10μg/ml) and the F(ab')2 rabbit anti-rat IgG FITC conjugated secondary antibody (10μg/ml) as described in section 2.9.2. Unstained control THP-1 cells were gated in a forward versus side scatter dot plot (Ai) with the resulting FL1-H (FITC) histogram of ICR62 (Aii). Histogram populations in the example shown are for unstained (black), IgG2b isotype control (blue) and ICR62 (red). The mean fluorescence intensity of each population is shown on the histogram. This is a representative result of six experiments as shown in the table (B).
3.4 The Cell Surface Expression of EGFR on the Differentiated THP-1 Cell by Flow Cytometry

THP-1 cells were differentiated into macrophage-like cells with 10nM PMA over 72 hours and examined for their EGFR cell surface expression by flow cytometry analysis as described in section 2.9.2. Differentiated THP-1 cells were incubated in the presence of the anti-EGFR monoclonal antibody ICR62 and the appropriate isotype control. Differentiated THP-1 cells were gated in a forward versus side scatter dot plot (figure 3.4A) and transformed into a fluorescence histogram (figure 3.4B). Unstained control cells had a MFI of 3.61 and the IgG2b isotype control had a MFI of 3.47. THP-1 cells incubated with ICR62 had a MFI 3.96 and therefore did not express EGFR on their cell surface.
**Figure 3.4** The cell surface expression of the EGFR on differentiated THP-1 cells. Monocytic THP-1 cells were differentiated into macrophage-like cells in the presence of 10nM PMA over 72 hours as described in section 2.2. Differentiated THP-1 cells were incubated for 1 hour at 4°C in the presence of the monoclonal antibody ICR62 (10µg/ml) and the F(ab’)2 rabbit anti-rat IgG FITC conjugated secondary antibody (10µg/ml) as described in section 2.9.2. Unstained control differentiated THP-1 cells were gated in a forward versus side scatter dot plot (A) with the resulting FL1-H (FITC) histogram of ICR62 (B). Histogram populations shown are for unstained (black), IgG2b isotype control (blue) and ICR62 (red). The mean fluorescence intensity of each population is shown on the histogram. This is a representative result of four experiments.
Chapter 3 – EGFR Expression

3.5 The Cell Surface Expression of the EGFR by Human Peripheral Blood-Derived Monocytes

Human peripheral blood monocytes were isolated from whole blood from one healthy individual and cultured as described in section 2.3. Isolated human peripheral blood monocytes were incubated with an antibody to the monocyte marker CD14 and ICR62 to establish the presence of EGFR as described in section 2.9.3.

The suspected monocyte population was gated on a forward versus side scatter dot plot and is shown as the red cells within P1 (figure 3.5A). 21.3% of the total cell population was located within this gated population. Confirmation that these gated cells were monocytes was achieved by plotting the total cell population in a forward versus RPE-Cy5 dot plot (figure 3.5B). Cells which stained positive with the anti-CD14 antibody are shown as the blue population (figure 3.5Bi). The percentage of CD14 positive cells within the whole cell population was 16.9%. Cells stained with the mouse IgG2a isotype control are also shown (figure 3.5Bii). The percentage of cells stained in P3 with the isotype control was 1.5% of the total cell population. When the CD14 positive cells from P3 were gated on the original forward versus side scatter dot plot they were located within P1.

The gated CD14 positive monocyte cells within P1 (figure 3.5A) were transformed into a FL1-H (FITC) versus FL3-H (RPE-Cy5) dot plot (figure 3.6). When the cells were double stained for CD14 and EGFR 55.2% of the gated cell population expressed both markers (figure 3.6aQ2). The double stained isotype control cells only showed 0.5% of the gated population in this same region (figure 3.6bQ2).
However, cells were still located within sector Q4 (13.2%) indicating non-specific binding from the matched isotype control for EGFR.

When cells were singly stained for CD14, 58.9% of cells in the gated population were found to be positive (figure 3.6cQ1). This percentage was reduced to 0.1% when the isotype control for CD14 was used (figure 3.6dQ1). Therefore, non-specific binding of the CD14 antibody was minimal. When the cells were singly stained for EGFR using ICR62, 9.8% of P1 were considered positive (figure 3.6eQ4). However, when the cells were singly stained with the rat IgG2b isotype control for ICR62, 11.4% of P1 were located within sector Q4 (figure 3.6fQ4). This would suggest that the positive result seen with ICR62 may be non-specific. Although the double stained cells (figure 3.6aQ2) are positive for CD14 and can be considered monocytes, it is not clear whether these cells are really EGFR positive.
Figure 3.5 Isolation of the CD14 positive monocyte population. Isolated human peripheral blood monocytes from one healthy individual were incubated in the presence of antibodies against the monocyte marker CD14 and ICR62 (EGFR) for 1 hour at 4°C as described in section 2.9.3. (A) The suspected monocyte population (P1 – red population; 21.3% of total cell population) was gated within a forward verses side scatter dot plot containing the total cell population (20,000 events). (B) The CD14 positive population (monocytes) within the whole cell population was identified by plotting cells in a forward versus RPE-Cy5 dot plot. Positively expressing CD14 monocytes (P3 – blue population; 16.9% total cell population) (i) and isotype control (1.5% of total cell population) (ii). The CD14 positive cell population in P3 was located within the whole cell population on the forward versus side scatter dot plot (A). These cells are represented as the blue population, with the majority of them falling within P1.
Figure 3.6 The cell surface expression of the EGFR on human peripheral blood monocytes. Human peripheral blood monocytes from one healthy individual were double stained for the monocyte marker CD14 and EGFR with ICR62 as described in section 2.9.3. Gated cells in P1 (figure 3.5A) were transformed into a FL1-H (FITC) for EGFR versus FL3-H (RPE-Cy5) for CD14 dot plot. The dot plots show double CD14/EGFR positive (a), double isotype control (b), single CD14 positive (c), single CD14 isotype control (IgG2a) (d), single EGFR (ICR62) positive (e), single EGFR isotype control (IgG2b) (f) and unstained cells (g). Figures in each quadrant show the percentage of stained cells from P1.
3.6 The Cell Surface Expression of the EGFR on Monocyte-Derived Macrophages

Human peripheral blood monocytes were isolated from whole blood from one healthy individual and cultured as described in section 2.3. Monocyte-derived macrophages were incubated with an antibody to the monocyte marker CD14 and ICR62 to establish the presence of EGFR as described in section 2.9.3.

Monocyte-derived macrophages were gated within a forward versus side scatter dot plot based upon their CD14 expression as described previously in section 3.5. This gated population (P1) contained 27% of cells from the total cell population (figure 3.7a). Cells within P1 were transformed into a FL1-H (FITC) versus FL3-H (RPE-Cy5) dot plot (figure 3.7b, c & d).

Human monocytes showed an extremely low expression of EGFR following maturation into monocyte-derived macrophages (figure 3.7d). The percentage of cells in Q2, positive for both CD14 and EGFR, was 1% of the cells gated in P1. This is equivalent to 54 cells out of the 5420 cells in P1, or 0.27% of the total 20,000 cell population. When stained with the double isotype controls, only 0.4% of cells in P1 were located in sector Q2 (figure 3.7c). Also, only 0.3% of cells in P1 were located in sector Q4, which would indicate positive EGFR expression and therefore non-specific binding of that isotype of antibody. Therefore it could be suggested that out of the 1% of double positive cells, 0.7% are actually positive (1% total - 0.3% non-specific = 0.7% specific EGFR). This is equivalent to 37 cells out of the 5420 cells in P1, or 0.19% of the total 20,000 cell population.
Figure 3.7 The cell surface expression of the EGFR on human peripheral blood monocyte-derived macrophages. Peripheral blood monocytes were isolated from whole blood from one healthy individual and cultured for 8 days for maturation into monocyte-derived macrophages as described in section 2.3. Cells were assessed for the expression of CD14 (RPE-Cy5) and the EGFR (FITC) with the rat monoclonal antibody ICR62 as described in section 2.9.3. Monocyte-derived macrophages were gated as P1 (a), and transformed into FL-1H (FITC) versus FL-3H (RPE-Cy5) dot plots for unstained cells (b), double isotype control (c) and double CD14 & EGFR (d). Figures in each quadrant show the percentage of stained cells from P1.
HB-EGF is one of the EGFR ligands (Higashiyama et al., 1991). Treatment of monocyte-derived macrophages from one healthy individual with HB-EGF was carried out to investigate any changes in cell surface EGFR expression. A slight increase in EGFR expression was observed in Q2 when the cells were treated with 10nM HB-EGF (figure 3.8c). The percentage of cells within this quadrant increased from 1% in untreated cells (figure 3.8a) to 1.9% in the 10nM treated cells. This increase in percentage within Q2 was combined with an observed increase in MFI within Q1, indicating a shift in the population. Untreated cells had an MFI of 377, whereas 10nM HB-EGF treated cells had an MFI of 515. 0.1nM and 25nM treated cells had MFI values of 321 and 306 respectively. Indeed, on visual inspection of the dot plots a cell population shift to the right is clearly seen.
Figure 3.8 The effect of HB-EGF on the cell surface expression of the EGFR on human peripheral blood monocyte-derived macrophages. Peripheral blood monocytes were isolated from whole blood from one healthy individual and cultured for 8 days for maturation into monocyte-derived macrophages as described in section 2.3. Cells were treated for 2 hours with 0.1nM, 10nM or 25nM HB-EGF and then assessed for the expression of CD14 (RPE-Cy5) and EGFR (FITC) with the rat monoclonal antibody ICR62 as described in section 2.9.3. Monocyte-derived macrophages were gated as P1 (figure 3.7a), and transformed into FL-1H (FITC) versus FL-3H (RPE-Cy5) dot plots for untreated cells (a), 0.1nM (b), 10nM (c) and 25nM HB-EGF (d). Figures in each quadrant show the percentage of stained cells from P1.
3.7 Localization of EGFR in Monocytic and Differentiated THP-1 Cells

Monocytic and differentiated THP-1 cells were immunocytochemically stained with ICR62 as described in section 2.10 to determine the location of their EGF receptors. Monocytic THP-1 cells showed EGFR reactivity within their cytoplasm with slight cell membrane staining (figure 3.9A). There was minimal reactivity following incubation with the isotype control IgG2b or control buffer (figure 3.9B & C). When the images for ICR62 and nuclei were combined the EGFR positive regions could clearly be seen to surround the nuclei (figure 3.9D).

EJ cells in comparison have a more honey comb-like appearance in their pattern of staining with ICR62 (figure 3.10A). This is consistent with the knowledge that this cell line expresses many EGF receptors on its cell surface (Modjtahedi & Dean, 1996).

The staining pattern for the differentiated THP-1 cells was different. Not only are the cells much larger but the EGFR appeared to be located within the cell nucleus as well as within the cytoplasm (figure 3.11A). When both the images for ICR62 and Hoechst were combined it became clear that the blue nuclear staining did overlap the green FITC staining for EGFR (figure 3.11D). Once again, there was no reactivity following incubation with the isotype control IgG2b or control buffer (figure 3.11B & C).
Figure 3.9 Localisation of the EGFR in monocyctic THP-1 cells. Monocyctic THP-1 cells were spun onto microscope slides using a Cytospin and subjected to immunocytochemical staining as described in section 2.10. Cells were air-dried, fixed in 4% paraformaldehyde and permeabilised in 0.5% Triton X-100. Immunofluorescent staining was conducted with the rat monoclonal antibody ICR62 (20μg/ml) (A) and a rat IgG2b isotype control (20μg/ml) (B) as described in section 2.10.2. Control cells (C). Representative staining for FITC staining (left panels) and Hoechst 33258 nuclear staining (right panels) are shown. All images are at x100 magnification. The location of the nuclei is shown in the combined image (D).
Figure 3.10 Localisation of the EGFR in the EJ tumour cell line. EJ tumour cells were dissociated with trypsin and spun onto microscope slides using a Cytospin and subjected to immunocytochemical staining as described in section 2.10. Cells were air-dried, fixed in 4% paraformaldehyde and permeabilised in 0.5% Triton X-100. Immunofluorescent staining was conducted with the rat monoclonal antibody ICR62 (20μg/ml) as described in section 2.10.2. Representative staining for FITC staining (A) and Hoechst 33258 nuclear staining (B) are shown. All images are at x100 magnification. The location of the nuclei is shown in the combined image (C).
Figure 3.11 Localisation of the EGFR in differentiated THP-1 cells. Monocytic THP-1 cells were incubated in the presence of 10nM PMA for 72 hours to produce differentiated THP-1 cells as described in section 2.2. The cells were dissociated and spun onto microscope slides using a Cytospin and subjected to immunocytochemical staining as described in section 2.10. Cells were air-dried, fixed in 4% paraformaldehyde and permeabilised in 0.5% Triton X-100. Immunofluorescent staining was conducted with the rat monoclonal antibody ICR62 (20μg/ml) (A) and a rat IgG2b isotype control (20μg/ml) (B) as described in section 2.10.2. Control cells (C). Representative staining for FITC staining (left panels) and Hoechst 33258 nuclear staining (right panels) are shown. All images are at x100 magnification. The location of the nuclei is shown in the combined image (D).
3.8 THP-1 Cell EGFR Protein Expression

EJ cells are known to express easily detectable levels of EGFR. Therefore, these cells were used as a positive control for the immunoprecipitation reaction. Cell lysates were incubated with Protein-G sepharose beads conjugated with ICR62. Following SDS-PAGE and western transfer, the resulting membrane was investigated for the presence of EGFR protein with a commercial antibody SC-03 (Table 2.2). A positive EGFR band is seen in the ICR62 lane after a 3 minute exposure (figure 3.12). No bands are seen in the IgG2b or control lanes indicating that the immunoprecipitation reaction is specific and that the EGFR protein is only being isolated when incubated with ICR62.

The reaction was then carried out with THP-1 cell lysates. An EGFR band can be seen in the ICR62 lane (figure 3.13) however, there is high background on this blot as the film had to be exposed for 30 minutes which can cloud the positive result. There are however no bands of the same molecular weight visible in the IgG2b or control columns which strongly suggests that the band seen is due to EGFR.
Figure 3.12 Immunoprecipitation of EGFR in EJ cells. EJ cells were lysed and subjected to immunoprecipitation with the monoclonal antibody ICR62 as described in section 2.7. As controls, equal volumes of the EJ lysate were also immunoprecipitated with the isotype control IgG2b and buffer. Equal volumes (15μl) of the resulting samples were subjected to SDS-PAGE, transferred to PVDF membrane and probed for the presence of EGFR with rabbit anti-human polyclonal antibody SC-03 (1/200) with a chemiluminescent detection system as described in section 2.8. The membrane was exposed to photographic film for 3 minutes. A positive EGFR band is seen in the ICR62 lane.
Figure 3.13 EGFR protein expression in THP-1 cells. THP-1 cells were lysed and subjected to immunoprecipitation with the monoclonal antibody ICR62 as described in section 2.7. As controls, equal volumes of the THP-1 lysate were also immunoprecipitated with the isotype control IgG2b (dilution) and buffer. Equal volumes (15μl) of the resulting samples were subjected to SDS-PAGE, transferred to PVDF membrane and probed for the presence of EGFR with the rabbit anti-human polyclonal antibody SC-03 (1/200) and a chemiluminescent detection system as described in section 2.8. The membrane was exposed to photographic film for 30 minutes. A slight positive EGFR band is seen in the ICR62 lane.
3.9 Discussion

The expression of EGF receptor was investigated in monocytic THP-1 cells, differentiated THP-1 cells, human peripheral blood-derived monocytes and human monocyte-derived macrophages. Receptor expression was investigated using flow cytometry, immunocytochemistry and western blotting techniques.

Validation of the rat monoclonal antibody ICR62 for use with the monocyte flow cytometry protocol was first established with the EGFR expressing human tumour cell lines HN5, EJ and MCF-7 (figure 3.1). EJ cells, characterised as having a mid-range cell surface expression of EGFR had a mean fluorescence intensity of 179.11 when using the human carcinoma flow cytometry protocol described in 2.9.1. However, when analysed using the monocyte flow cytometry protocol as described in 2.9.2, the EJ mean fluorescence intensity fell to 21.68 (figure 3.2). This drop in detection could be attributable to the lack of rotation within the antibody incubation steps which is included in the first protocol. Rotation enables the cells to be constantly free floating in the antibody, whereas without rotation cells naturally form a pellet at the bottom of the incubation tube under the influence of gravity. This could mean that cells within or on the underside of the pellet would have a reduced exposure to the antibodies.

Analysis of THP-1 cells for EGFR expression using the monocyte flow cytometry protocol resulted in an average mean fluorescence intensity of 21.62 ± 2.92 (figure 3.3). This was comparable to the expression on the EJ cells under the same flow cytometry protocol. It could therefore be suggested at this early stage that THP-1 cells express the EGF receptor to the same extent as EJ cells.
The expression of EGFR was subsequently investigated on differentiated THP-1 cells. Monocytic THP-1 cells, when incubated with the phorbol ester PMA for at least 48 hours in culture are considered to become macrophage-like with respect to both morphology and gene expression (Auwerx, 1991; Tsuchiya et al., 1982). Following incubation with 10nM PMA for 72 hours the macrophage-like differentiated THP-1 cells were analysed for EGFR expression. Results from four flow cytometry experiments indicated that there was no EGFR expression on the cellular surface of these differentiated cells (figure 3.4).

Further analysis of the receptor expression on THP-1 cells with immunocytochemistry indicated that the EGFR, present on the cell surface and within the cytoplasm of the monocytic cell (figure 3.9), was located in the nucleus in the differentiated cell (figure 3.11). This provided an explanation for the observed result following the flow cytometry analysis where there was no cell surface reactivity for EGFR with ICR62. What possible role therefore could the EGFR have for being in the cell nucleus of the differentiated THP-1 cell?

All members of the EGFR family have been detected in nuclei (Wang et al., 2004; Offerdinger et al., 2002; Lin et al., 2001; Ni et al., 2001; Marti & Hug, 1995; Xie & Hung, 1994) as well as other PTKs including nerve growth factor receptor (NGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor–2 (VEGFR-2) and type 1 TGFβ receptor. Inflammatory cytokine receptors for IL-1, IL-5 and IFNγ have also been located in the nuclear compartment (Lo & Hung, 2006). Since its first discovery in human adrenocortical carcinoma (Kamio et al., 1990), the nuclear localisation of EGFR has been detected in various tissues and
carcinoma cell types including hepatocytes (Marti et al., 1991), placenta (Cao et al., 1995), thyroid (Marti et al., 2001), breast (Lo et al., 2006), bladder (Lipponen & Eskelinen, 1994), and oral cavity (Psyrri et al., 2005).

The nuclear EGFR pathway can be initiated by ligand binding, and nuclear localisation signals (NLSs) have been identified in all four members of the EGFR family. EGFR’s NLS is located within the juxtamembrane region (Hsu & Hung, 2007; Lo & Hung, 2006). As EGF receptors lack a putative DNA binding domain, it is suspected that they first associate with DNA-binding transcription factors to then enhance target gene transcription via their intrinsic transactivational activity (Lo & Hung, 2006). It is therefore thought that EGFR interacts with STAT3 and E2F1 to upregulate expression of inducible nitric oxide synthase (iNOS) and B-Myb respectively (Hanada et al., 2006; Lo et al., 2005). Nuclear EGFR has also been found to upregulate cyclin D1 expression leading to G1/S cell cycle progression (Lin et al., 2001). All of these factors are associated with increased proliferation in cancerous cells.

The nuclear import of EGFR can also occur in ligand-independent situations, such as exposure to irradiation, heat shock, H$_2$O$_2$ and cisplatin (Lo & Hung, 2006). In these situations, EGFR interacts with DNA-dependent protein kinase (DNA-PK) leading to DNA damage repair and ultimately the radioresistance of tumours (Chen & Nirodi, 2007; Dittmann et al., 2005).

The above evidence strongly suggests that the role for the nuclear localisation of EGFR is to drive cellular proliferation. However, one of the characteristics of a
PMA differentiated THP-1 cell is its inability to proliferate (Schwende et al., 1996; Auwerx, 1991). What, therefore is its role in an essentially non-proliferating cell? It must not be forgotten that THP-1 cells are of leukaemic origin (Tsuchiya et al., 1980) and that the observed result may be due to their oncogenic status rather than their leukaemic origin. Peripheral blood-derived monocytes and macrophage-like cells should be investigated as to whether they also have nuclear localisation of the EGF receptor.

Following the flow cytometry result with the THP-1 cells, isolated peripheral blood monocytes were investigated for their EGFR expression. Since isolated monocytes were investigated from only one individual, care must be taken in the interpretation of these results. The monocytes initially appeared to express the EGF receptor (figure 3.6a) as cells were present in the quadrant representing double staining for both CD14 (monocytes) and ICR62 (EGFR). However, a similar result was obtained with the matched isotype control antibody which would indicate that the result with ICR62 could be due to non-specific binding of the antibody (figure 3.6e & f). In order to confirm the result with ICR62 further experiments with more individuals are needed to investigate any gender or genetic differences in expression and perhaps the use of an alternative isotype control antibody could be investigated.

It is interesting that THP-1 cells express EGFR on their cell surface whereas peripheral blood-derived monocytes from one individual do not. It could be possible that during the oncogenic process, the THP-1 cell developed the expression of the EGFR and now relies in part on it to maintain its growth. Other differences have been observed in the gene expression profiles between monocytic cell lines and
human primary monocytes. For example, the top three genes most highly expressed in THP-1 cells, cathepsin G, neutrophil elastase 2 and proteinase 3 are not generally associated with monocytes. Similarly, ficolin 1, MHC class II DRα1 and interferon γ-inducible protein 30 which are highly expressed in monocytes are not expressed in THP-1 cells. The results of the above study suggested that although THP-1 cells are morphologically similar to monocytes, the two are very different from a transcriptomic point of view (Kohro et al., 2004).

It was subsequently investigated to see whether blood-derived monocyte maturation into a macrophage-like cell would result in EGFR expression. Isolated human peripheral blood-derived monocytes from the same one individual were cultured for 8 days into macrophage-like cells. A very low expression of the EGFR was observed in monocyte-derived macrophages (figure 3.7). However, when these cells were pre-incubated with the EGFR ligand HB-EGF (10nM) there was a slight increase in receptor expression (figure 3.8). It could be suggested that any increase in receptor expression on the cellular surface is immediately counteracted by ligand binding and subsequent internalisation of the receptor. Therefore no extra receptor would be seen.

It could be considered that peripheral blood-derived monocyte maturation into the macrophage-like cell in culture is a very rudimentary process. The main stimulus for monocyte maturation within the tissue culture environment is the presence of a plastic ‘surface’. Within the plaque environment the invading monocyte is exposed to many other different types of ‘surface’ such as other cells and extra cellular matrix components (Ross, 1993). Added to this is the myriad of growth factors and
cytokines which can initiate maturation and activation down specific pathways for example IFNγ and IL-1β (Mosser, 2003; Ma et al., 2003). Perhaps it should be considered that the artificial maturation of cell culture monocytes should occur in the presence of a variety of stimuli such as collagen or fibronectin coated flasks so as to get a representation of the plaque environment. A variety of studies have shown that adherence of monocytes to different surfaces results in the upregulation of different genes such as IL-6, PDGF and IL-8 (Standiford et al., 1991; Shaw et al., 1990; Navarro et al., 1989). Perhaps with a specific activatory stimulus, for example IFNγ the maturing macrophage-like cell may be 'persuaded' to express the EGF receptor.

After discovering that THP-1 cells express the EGFR through flow cytometry and immunocytochemical analysis the expression was also investigated using immunoprecipitation and western blotting techniques. Following a negative result solely with SDS-PAGE and western blotting, the THP-1 cells were subjected to immunoprecipitation with ICR62 to specifically isolate the EGFR protein prior to electrophoresis and western blotting with a commercial anti-EGFR antibody. The technique was optimised with EJ cells in order to demonstrate that indeed ICR62 was capable of precipitating out EGFR (figure 3.12). However, when analysing THP-1 cells, a very low specific EGFR expression was observed following a 30 minute chemiluminescent exposure to photographic film (figure 3.13). Further to this, a total of 6x10⁷ cells were required to obtain this result. This made the further study of the protein expression difficult. The low expression of the EGFR in THP-1 cells maybe outside the detectable range of this system and therefore a more sensitive system has to be employed.
3.10 Conclusions

The main aim of this chapter was to investigate the expression of the EGFR on the THP-1 cell line and peripheral blood-derived monocytes. Flow cytometry analysis of the THP-1 cell line with the monoclonal antibody ICR62 revealed expression of the EGFR which was comparable to the bladder carcinoma cell line EJ which is an intermediate EGFR expresser. When incubated with PMA to induce cellular differentiation, the macrophage-like THP-1 cells displayed no EGFR expression. The EGFR was subsequently shown to be localised within the nucleus of these cells. The purpose of this nuclear localisation remains unclear and blood-derived monocytes and macrophage-like cells should be investigated for their receptor localisation. SDS-PAGE and subsequent western blotting techniques did show specific reactivity to the EGFR following immunoprecipitation with the monoclonal antibody ICR62. However, vast numbers of cells were harvested for this analysis which made future experiments involving primary cells inconceivable.

Peripheral blood-derived monocytes were also investigated for their expression of EGFR by flow cytometry. Minimal receptor expression was observed which was inconsistent with the results seen with the THP-1 cell line. However, although the two types of cell are very similar morphologically, they do exhibit large differences in terms of transcription. Care must be taken in the interpretation of observed results with the THP-1 cell line when referring to a possible role of a blood monocyte. Monocyte-derived macrophages also displayed minimal receptor expression by flow cytometry and this expression increased only slightly following pre-incubation with the EGFR ligand HB-EGF. However, this result was obtained through analysis of
only one individual. More individuals should be investigated to account for any age, gender or genetic differences.
Chapter Four

Analysis of EGFR Gene Expression

4.1 Introduction

The EGFR gene spans 110kb on chromosome 7p12-14 and contains 26 exons with several long introns (Haley et al., 1987; Merlino et al., 1985). The EGFR promoter lacks a characteristic TATA and CAAT box, but is enriched in G + C residues (Haley et al., 1987; Ishii et al., 1985). G + C rich promoters mainly demonstrate transcription from multiple start sites, whereas some, like the TATA-box promoters initiate from a single start site (Hudson et al., 1990).

The four GC boxes bind the ubiquitous, positive transcription factor Sp1 which is essential for EGFR gene transcription (Safe & Abdelrahim, 2005; Kageyama et al., 1988). Three GC rich regions bind the factor EGFR-specific transcription factor-1 (ETF1), which specifically stimulates transcription from promoters without a TATA box (Kageyama et al., 1989). The positive transcription factor ETF2 binds to a single promoter site (Johnson et al., 1988a). The promoter also contains four S1 nuclease-sensitive regions with the motif of TCCTCCTCC. These regions bind Sp1, EGF responsive DNA binding protein (ERDP-1) and the factor TCF (Chen et al., 1993; Johnson et al., 1988b; Ishii et al., 1985). TCF is essential for optimal EGFR gene transcription.
Two repressor proteins have been identified that bind to the EGFR promoter. GCF (GC factor) is a 91kDa protein that binds with high affinity to three GC-rich regions which overlap a ETF1 binding site (Kageyama & Pastan, 1989) and ETR (EGFR transcriptional repressor) is a 128kDa protein which binds to one site on the promoter (Hou et al., 1994).

Regulation of the expression of the EGFR has been extensively studied, but is still not completely understood (Merlino, 1990). A number of agents have been suggested to upregulate the EGFR at a transcriptional level. These include phorbol esters (Bjorge & Kudlow, 1987), IFNγ (Chang et al., 1987), retinoic acid (Oberg & Carpenter, 1991), TGFβ (Fernandez-Pol et al., 1987), and cAMP (Hudson et al., 1989). The ligands EGF and TGFα are also known to stimulate the synthesis of the EGFR (Burgess, 1989; Kudlow et al., 1986).

In this chapter, EGFR gene expression was analysed in monocytic and differentiated THP-1 cells following treatment with four of the EGFR ligands EGF, HB-EGF, TGFα and BTC, as well as three monocyte activators IFNγ, IL-1β and LPS. Human peripheral blood-derived monocytes were also analysed for their gene expression changes during maturation into macrophage cells. Changes in gene expression were determined by analysing mRNA expression using quantitative real-time PCR.
4.2 Effect of Serum Concentration on THP-1 Cells

In order to execute assays in which the effect of the EGFR ligands was being determined, the serum concentration in the experimental medium had to be reduced, in effect, cells were serum starved. This was to ensure that any change in gene expression seen within the experiment was due to the addition of that particular ligand.

THP-1 cells were cultured over a period of 96 hours in RPMI-1640 media containing 10%, 5% and 2% FCS and their growth was investigated. Cells were seeded at $1 \times 10^5$ per ml and a steady increase in cell number was seen with all three serum concentrations (figure 4.1). A drop in cellular proliferation was observed at 96 hours with the cells incubated in 2% FCS ($P<0.05$). Therefore if cells were to be grown in 2% serum for experimental procedures, the maximum duration time of the experiment should be set at 72 hours.

Cell viability was also investigated using the trypan blue exclusion method. At no point during the experiment did viability drop below 95% under any treatment (data not shown).
Figure 4.1 THP-1 cell proliferation under different medium serum concentrations. THP-1 cells were grown in 12-well plates in RPMI-1640 media supplemented with 10%, 5% or 2% foetal calf serum. Total cell counts per ml were taken at 24 hour intervals using a haemocytometer as described in section 2.1.2. Data shown are the mean ± SEM (n = 3). Data were analysed using a two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. *P<0.05 (10% and 5% serum versus 2% serum at 96 hours)
4.3 Preparation of EGFR and MLN51 Standard Curves

Standard curves for both EGFR and MLN51 genes were created with RNA from THP-1 and EJ cells in order to establish the expression range for each cell type. RNA from both EJ and THP-1 cells was diluted to a range of 0.01ng to 100ng. The cycle threshold (Ct) value for each RNA amount was established from the resulting amplification curves (*figure 4.2*) by setting the cycle threshold to 0.5 and the baseline between cycles 3 and 15. The cycle threshold values for each cell line and gene were then plotted against the log ng of RNA (*figure 4.3*).

Detection of EGFR gene expression in EJ cells could be obtained at the lowest amount of 0.01ng RNA. However, this quantity of RNA for the THP-1 cells did not yield a detectable product, as demonstrated by the amplification curve (*figure 4.2Bi*). Both cell lines produced consistent amplification over the range of the diluted amounts as demonstrated by the $r^2$ values on the standard curves (*figure 4.3*). However, the THP-1 cells had lower expression of EGFR as shown by the higher Ct values (*figure 4.3Bi*). MLN51 detection in both cell lines was comparable (*figure 4.3Aii & Bii*).

Based on these results, the EJ cell line was chosen to produce the standard curves for both EGFR and MLN51 genes. This would enable very low quantities of EGFR gene expression to be confidently detected in the test/treatment samples from THP-1 cells and human primary monocytes.
Figure 4.2 Amplification plots for EGFR and MLN51 expression in EJ and THP-1 cells. RNA extracted from EJ cells (A) and THP-1 cells (B) was isolated as described in section 2.6.3, diluted to the indicated amounts per reaction and amplified using a one-step SYBR Green real-time PCR reaction as described in section 2.6.3. Cycle threshold (Ct) values for each amount were obtained for EGFR (i) and MLN51 (ii) expression by manually setting the cycle threshold to 0.5 and the baseline between cycles 3 and 15.
Figure 4.3 Standard curves for EGFR and MLN51 with EJ and THP-1 RNA. Standard curves were created with RNA covering the range of 0.01ng to 100ng from EJ cells (A) and THP-1 cells (B) for EGFR (i) and MLN51 (ii). Cycle threshold (Ct) values derived from the amplification curves were plotted against the log ng amounts of RNA. The correlation between the points was analysed by linear regression and $r^2$ values were obtained to demonstrate ‘best fit’. Points represent the mean ($n = 2$) Ct value $\pm$ SD.
Standard curves were constructed for both human peripheral blood-derived monocytes and THP-1 cells from each analysis QPCR amplification plate with EJ RNA (figure 4.4). Linear regression analysis on each of the curves produced $r^2$ values of greater than 0.9.

Following the cycling reaction, melt-curve analysis was performed to establish the melting point of the specific PCR product (figure 4.5). Melt curves are plotted as temperature (°C) against the change in the rate of dissociation (derivative, $-dF/dT$) of the primers and the template cDNA (Pryor & Wittwer, 2006; Ririe et al., 1997). In EJ cells both EGFR and MLN51 have melting point temperature (Tm) values of 78.7°C. The non template control (NTC) for EGFR runs at the bottom of the graph as there is no template for the primers to anneal to. The NTC for the MLN51 reaction contains a peak at 71.3°C which either demonstrates a contaminant in the reaction or a primer dimer due to the primer sequence.
Figure 4.4 Compiled standard curves for human peripheral blood-derived monocytes and THP-1 cells for EGFR and MLN51. Standard curve reference points with EJ RNA were run on each analysis QPCR plate to compile standard curves for monocytes (A) and THP-1 cells (B) for both EGFR (i) and MLN51 (ii). Ct values obtained from the amplification curve were plotted against the log ng amounts of RNA. The correlation between the points was analysed by linear regression and $r^2$ values were obtained to demonstrate 'best fit'. Points represent the mean (n = 4-24) Ct value ± SEM.
Figure 4.5 Dissociation melt curves in EJ cells for EGFR and MLN51 gene expression. Following the PCR reaction, PCR products were subjected to melt curve analysis. Each standard curve ng value are plotted as temperature (°C) against the change in the rate of dissociation (-dF/dT). Tm values for EJ cells for both EGFR (A) and MLN51 (B) was 78.7°C. NTC – non template control.
4.4 EGFR mRNA Expression in Monocytic and Differentiated THP-1 Cells

Monocytic THP-1 cells were found to have a very low level of EGFR mRNA expression when normalised to the housekeeping gene MLN51 (0.003 ± 0.0004) (figure 4.6). When differentiated with 10nM PMA for 72 hours into macrophage-like cells, EGFR mRNA expression increased three-fold (0.01 ± 0.001). This increase was found to be highly statistically significant ($P<0.0001$).
Figure 4.6 Expression of EGFR mRNA in monocytic and differentiated THP-1 cells. The expression of EGFR mRNA in monocytic and differentiated THP-1 cells was established by real-time QPCR analysis and expression was normalised to the housekeeping gene MLN51. Data shown are the mean ± SEM (THP-1 n = 10; dTHP-1 n = 7). Data were analysed using an unpaired t test. ***P<0.0001 (dTHP-1 versus THP-1).
Due to the vast number of samples generated in a preliminary experiment with monocytic and differentiated THP-1 cells treated with four of the EGFR ligands and three monocyte activators at four time points in duplicate tissue culture wells, the tissue culture samples for each condition were pooled prior to DNase treatment. This, therefore, gave an n of 1. The preliminary experiment of pooled samples gave an indication of possible activity which led on to a more detailed investigation into TGFα, HB-EGF, IFNγ and IL-1β at 12 and 24 hours. In the follow up experiment, each treatment at the two time points was investigated in triplicate tissue culture wells. This ultimately gave an n of 1 for all EGF, BTC and LPS data bars and an n of 4 for HB-EGF, TGFα, IFNγ and IL-1β at 12 and 24 hours and an n of 1 at 6 hours.

Data presented on the graphs are the combination of both the preliminary investigation and the more detailed follow-up investigation for 6, 12 and 24 hours. As no significant data were recorded at 48 hours, these data have been omitted from the graphs.
4.4.1 HB-EGF Induces the Expression of EGFR mRNA in Monocytic THP-1 Cells

Monocytic THP-1 cells were treated with physiologically relevant levels of EGF, HB-EGF, TGFα and BTC for 6, 12 and 24 hours. EGFR mRNA expression in response to EGF treatment remained low (figure 4.7A). The greatest induction (1.6 ± 0.41 fold) in expression occurred with 50nM EGF at 24 hours treatment. Peak EGFR expression following HB-EGF treatment occurred at 12 hours with 50nM with a 2.7 ± 0.66 fold induction over control cells (figure 4.7B). This induction in expression was statistically significant (P<0.01). EGFR expression following treatment with TGFα (figure 4.7C) and BTC (figure 4.7D) showed no significant increases. Peak fold induction occurred with 50nM TGFα following 6 hours treatment (1.7 ± 0.39 fold) and with 50nM BTC following 24 hours treatment (1.4 ± 0.58 fold).

The most notable result with monocytic THP-1 cells was that they responded most following treatment with 50nM of each ligand albeit at different time points.
Figure 4.7 EGFR mRNA expression in monocytic THP-1 cells following treatment with EGFR ligands. THP-1 cells were incubated in the presence of EGF (A), HB-EGF (B), TGFα (C) and BTC (D) at 25nM and 50nM. Cells were harvested after 6, 12 and 24 hours treatment. Bars represent the mean ± SEM of the fold induction over untreated control cells of PCR plate duplicates of tissue culture wells (n = 1, 6 hours; n = 4, 12 and 24 hours). Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (50nM HB-EGF at 12 hours versus control).
4.4.2 IFNγ and IL-1β Induce the Expression of EGFR mRNA in Monocytic THP-1 Cells

Monocytic THP-1 cells were treated with physiologically relevant levels of IFNγ, IL-1β and LPS for 6, 12 and 24 hours. EGFR mRNA expression following IFNγ treatment increased 3.7 ± 0.63 fold over control cells at 12 hours (figure 4.8A). This induction was found to be statistically significant (P<0.01). Expression remained at baseline at 6 hours and 24 hours treatment. A similar induction of expression profile was observed following IL-1β treatment (figure 4.8B). EGFR mRNA expression increased 3.6 ± 1.13 fold at 12 hours and this induction was found to be statistically significant (P<0.01). Once again, expression remained at baseline at 6 hours and 24 hours treatment.

No statistical analysis could be performed on the LPS treatments (figure 4.8C) as they were representative results from one preliminary experiment.
Figure 4.8 EGFR mRNA expression in monocytic THP-1 cells following treatment with IFNγ, IL-1β and LPS. THP-1 cells were incubated in the presence of IFNγ (100U/ml) (A), IL-1β (2.5ng/ml) (B) and LPS (100ng/ml) (C). Cells were harvested after 6, 12 and 24 hours treatment. Bars represent the mean ± SEM of the fold induction over untreated control cells of PCR plate duplicates of tissue culture wells (IFNγ & IL-1β: n = 1, 6 hours; n = 4, 12 and 24 hours; LPS: n = 1). Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (IFNγ and IL-1β at 12 hours versus control).
4.4.3 TGFα Induces the Expression of EGFR mRNA in Differentiated THP-1 Cells

Differentiated THP-1 cells were treated with physiologically relevant levels of EGF, HB-EGF, TGFα, and BTC for 6, 12, and 24 hours. EGFR mRNA expression in response to EGF treatment remained low (figure 4.9A). Peak induction in expression (1.4 ± 0.03 fold) occurred following 6 hours treatment with 25nM EGF. HB-EGF treatment at 50nM resulted in a 1.5 ± 0.30 fold induction over control cells at 12 hours (figure 4.9B). Treatment with TGFα resulted in the largest induction in EGFR mRNA expression out of all the EGFR ligands tested. EGFR mRNA expression was induced 2 ± 0.39 fold when treated with 50nM for 12 hours (figure 4.9C). This induction was found to be very statistically significant (P<0.01). EGFR mRNA expression following treatment with BTC remained low with a peak induction of 1.3 ± 0.34 fold seen with 50nM at 6 hours (figure 4.9D).
Figure 4.9 EGFR mRNA expression in differentiated THP-1 cells following treatment with EGFR ligands. After incubation with 10nM PMA for 72 hours, the differentiated THP-1 cells were incubated in the presence of EGF (A), HB-EGF (B), TGFα (C) and BTC (D) at 25nM and 50nM. Cells were harvested after 6, 12 and 24 hours treatment. Bars represent the mean ± SEM of the fold induction over untreated control cells of PCR plate duplicates of tissue culture wells (n = 1, 6 hours; n = 4, 12 and 24 hours). Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (50nM TGFα at 12 hours versus control).
4.4.4 IFNγ and IL-1β Induce the Expression of EGFR mRNA in Differentiated THP-1 Cells

Differentiated THP-1 cells were treated with physiologically relevant levels of IFNγ, IL-1β and LPS for 6, 12 and 24 hours. EGFR mRNA expression following IFNγ treatment increased 1.8 ± 0.19 fold over control cells after 12 hours (P<0.01) (figure 4.10A). Expression remained low at 6 hours and 24 hours treatment. As with the monocytic THP-1 cells, the same expression profile was seen following treatment with IL-1β (figure 4.10B). EGFR mRNA expression increased 1.7 ± 0.22 fold at 12 hours. This induction was found to be statistically significant (P<0.05). Once again, expression remained low at 6 hours and 24 hours treatment. LPS treatment had no effect on the expression of EGFR mRNA (figure 4.10C). However, it should be considered that these data are from the preliminary experiment and therefore are only representative of one experiment.
Figure 4.10 EGFR mRNA expression in differentiated THP-1 cells following treatment with IFNγ, IL-1β and LPS. After incubation with 10nM PMA for 72 hours, the differentiated THP-1 cells were incubated in the presence of IFNγ (100U/ml) (A) IL-1β (2.5ng/ml) (B) and LPS (100ng/ml) (C). Cells were harvested after 6, 12 and 24 hours treatment. Bars represent the mean ± SEM of the fold induction over untreated control cells of PCR plate duplicates of tissue culture wells (IFNγ & IL-1β: n = 1, 6 hours; n = 4, 12 and 24 hours; LPS: n = 1). Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (IFNγ at 12 hours versus control); *P<0.05 (IL-1β at 12 hours versus control).
4.5 EGFR mRNA Expression in Human Blood-Derived Monocytes

Isolated human monocytes from five healthy individuals were incubated and harvested over a period of 8 days and analysed for changes in EGFR mRNA expression (figure 3.11). Gene expression in the monocytes remained very low for the first 24 hours. Between 48 and 96 hours, EGFR mRNA expression varied greatly between individuals with one individual having a 10-fold increased expression. From 7 days, EGFR expression had returned to the original low levels in all individuals.
Figure 4.11 EGFR expression in peripheral blood monocytes and blood derived macrophages. Isolated human blood monocytes from five healthy individuals were incubated over a period of 8 days into macrophages-like cells as described in section 2.3. Cells were harvested at the times shown and analysed for their EGFR mRNA expression using real-time QPCR analysis as described in section 2.6. EGFR expression was normalised to the house-keeping gene MLN51. Each dot represents the mean of the QPCR well duplicate for each tissue culture well duplicate for each individual. Bars represent the mean of all results for that time point.
With a very low level of mRNA expression the validity of the results was investigated by analysing the product dissociation. The results indicated that the majority of the QPCR results were non-valid (figure 4.12). For example sample Xa, dissociation melt curve analysis revealed dominant Tm peaks under 70°C. Dissociation peaks at this temperature indicate primer dimer formation and therefore no specific primer binding to the target sequence. This can occur when there is a very low amount or no target sequence available. This result was confirmed by gel electrophoresis of the PCR products (figure 4.12B). The double band on the gel for sample Xa corresponds to the double peaks seen on the dissociation curve (figure 4.12A). The top band corresponds to the small dissociation peak at 78°C, and this lies in line with the EJ cell EGFR standards. This indicates that there is some specific binding of the primers to the target sequence, however the peak and band for non-specific binding is larger, resulting in the Ct value for that sample being considered void.

Corresponding analysis of MLN51 expression in sample Xa was investigated. Dissociation melt curve analysis produced a specific Tm peak at 79°C (figure 4.13A). This result was confirmed by gel electrophoresis. A strong band can be observed in line with the EJ cell MLN51 standards (figure 4.13B). This result confirms that, although there is a very low copy number of the EGFR gene, there is sufficient mRNA to be analysed.
Figure 4.12 Melt curve dissociation and gel electrophoresis analysis of monocyte EGFR PCR products. (A) The PCR product of sample Xa was subjected to melt curve analysis. The melt curve is plotted as temperature (°C) against the change in rate of dissociation (-dF/dT). Specific product formation is represented by a prominent peak and Tm value of 78.6°C to 79°C. (B) The PCR product of sample Xa was also subjected to gel electrophoresis to confirm product specificity.
Figure 4.13 Melt curve dissociation and gel electrophoresis analysis of monocyte MLN51 PCR products. (A) The PCR product of sample Xa was subjected to melt curve analysis. The melt curve is plotted as temperature (°C) against the change in rate of dissociation (-dF/dT). Specific product formation is represented by a prominent peak and Tm value of 78.6°C to 79°C. (B) The PCR product of sample Xa was also subjected to gel electrophoresis to confirm product specificity.
With the valid PCR products for each individual established, the results were re-plotted (figure 4.14). At 2 hours, EGFR mRNA expression was low, as seen originally. There were no valid data points at the 12 hour or 24 hour time points. Remaining data for the 48 hour time point again indicated an increase in EGFR mRNA expression in two different individuals. At 72 hours, EGFR mRNA expression decreased to low levels and remained at this level for up until 9 days in culture. With the low number of valid results it is not possible to make a valid conclusion on the EGFR mRNA expression in these samples.
Figure 4.14 Viable results for EGFR expression in peripheral blood monocytes and blood derived macrophages. PCR products were checked for their validity and unvalid results were removed. Each dot represents the mean of the QPCR well duplicate for each tissue culture well duplicate for each individual. Bars represent the mean of all results for that time point.
4.6 Discussion

One of the aims of this chapter was to investigate the gene expression of the EGF receptor following treatment with four of the EGFR ligands EGF, HB-EGF, TGFα and BTC, as well as three monocyte activators IFNγ, IL-1β and LPS.

Baseline mRNA expression of the EGFR on monocytic THP-1 cells, when normalised to the housekeeping gene MLN51, was very low at 0.003 (figure 4.6). Equivalent gene expression to MLN51 would have resulted in a ratio of 1. When the THP-1 cells were differentiated into macrophage-like cells, EGFR gene expression increased 3-fold. This could either have been as a result of the differential status of the cell or due to the PMA treatment. Indeed, PMA has been shown to stimulate EGFR synthesis (Bjorge & Kudlow, 1987). Further studies showed that the effects of phorbol esters are mediated via the transcription factors Activator Protein 1 and 2 (AP1 and AP2) (Angel et al., 1987; Chiu et al., 1987) and that the EGFR promoter contains sequences similar to AP1 and AP2 binding sites. Indeed, EGFR gene transcription has been found to be activated by PMA and that this process was in part, mediated via AP2 in prepared nuclei and nuclear extracts from KB cells (Johnson, 1996).

EGFR mRNA expression was increased significantly \((2.7 \pm 0.66; P<0.01)\) when the THP-1 cells were incubated with 50nM HB-EGF for 12 hours (figure 4.7). No other significant effects resulting from the treatment with the other ligands was seen. HB-EGF has a higher affinity for binding to the EGFR and this is aided by its heparin binding capabilities (Higashiyama et al., 1993). This could, therefore, explain why only HB-EGF evoked a response in the THP-1 cells. On the other hand, EGF and
TGFα are capable of stimulating the synthesis of the EGF receptor in cell lines from human breast cancer and rat hepatic epithelial cells (Burgess, 1989; Bjorge & Kudlow, 1987; Kudlow et al., 1986; Earp et al., 1986). To date, HB-EGF has not been associated with EGFR mRNA up-regulation. However, this observed effect of HB-EGF may only occur in cells of the monocytic lineage and so has remained uninvestigated.

When the THP-1 cells were differentiated into macrophage-like cells, 50nM TGFα significantly \( (P<0.01) \) induced EGFR mRNA expression following 12 hours treatment (figure 4.9). This is interesting as previous experiments within this thesis have shown that the EGFR is not expressed on the cell membrane of differentiated THP-1 cells. How, therefore, is TGFα inducing this response? Both PMA and EGF have been shown to stimulate EGFR mRNA expression in human KB cells. However, EGF was actually shown to stabilize the EGFR mRNA, whereas PMA did not affect mRNA stability (Jinno et al., 1988). Further to this, EGF and TGFα have been shown to be equipotent in stabilizing EGFR mRNA and increasing expression of EGFR protein in KB cells (McCulloch et al., 1998) and prostate cancer cells lines (Seth et al., 1999). It could be suggested that in monocytic THP-1 cells EGFR mRNA levels were so low that any stabilization effects could not be seen. However, in the differentiated THP-1 cells, PMA had increased overall receptor gene expression and therefore the stabilizing effects of TGFα could be observed as an increase in gene expression. Why the possible stabilizing effect is not seen at 6 hours is unclear. However, it should be noted that the result at 6 hours is representative of only one result. Due to the fact that EGF produced no notable results throughout these sets of experiments it could be considered that this source of
growth factor was biologically inactive. Perhaps it would have been wise to include a positive control, for example a cell line which is responsive to all four EGFR ligands used.

Monocytic THP-1 cells were also investigated for their EGFR gene expression following treatment with the inducers of monocyte activation IFNγ, IL-1β and LPS. Both IFNγ and IL-1β significantly \((P<0.01)\) induced EGFR mRNA expression at 12 hours (figure 4.8). LPS also induced mRNA induction of EGFR but at 6 hours and 24 hours. However, as this result was from the preliminary experiment, no statistical tests could be undertaken. When THP-1 cells were differentiated into macrophage-like cells, EGFR mRNA expression was significantly induced by IFNγ \((P<0.01)\) and IL-1β \((P<0.05)\), once again, at 12 hours treatment (figure 4.10). However, fold induction for both cytokines was half that seen when the THP-1 cells were monocytic suggesting that the cytokines have more influence on monocytes. As the response to LPS was completely abolished in the differentiated THP-1 cell, focus was set on IFNγ and IL-1β as both these cytokines are influential players in the atherosclerotic plaque environment (Ross, 1993).

IFNγ appears to be an important activating stimulus for macrophages and has been known to upregulate EGFR gene expression for some time (Ma et al., 2003; Chang et al., 1987). In addition to the rapid JAK/STAT1α-mediated priming response, in macrophage activation IFNγ also induces the expression of a set of transcription factors known as interferon regulatory factors (IRF-1 to IRF-9). IFNγ is now known to regulate EGFR gene expression via the induction in the expression of IRF-1. IRF-
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1 is a regulatory factor that binds to the interferon stimulated response element (ISRE) sequences (Schroder et al., 2004; Rubinstein et al., 1998).

IL-1β also plays a role in modulating macrophage activation. The binding of IL-1β to IL-1R1 activates both mitogen-activated protein kinase (MAPK)-AP1 (activator protein kinase-1) and I-κB kinase (IKK)-NFκB pathways via MYD88 (myeloid differentiation factor 88), IRAK (interleukin-1 receptor associated kinase) and TRAF6 (tumour necrosis factor receptor-associated factor 6) (Ma et al., 2003). It is unclear how IL-1β signalling could lead to the upregulation of EGFR mRNA as up until this point IL-1β has only been associated the EGFR protein (Wan et al., 2001; Murthy et al., 2000; Kracht et al., 1994; Bird & Saklatvala, 1990). One possibility could be that TRAF6 associates with various effector molecules, ultimately resulting in activation of transcription factors such as NF-κB and AP-1 (Boch et al., 2001). It has also been proposed that IRF-1 expression can be induced by IL-1β (Fujita et al., 1989).

LPS is a typical pathogen-associated molecular patterns (PAMPs) which macrophages use to recognise a range of bacteria, virus, parasite and fungi. In order to signal, LPS must complex with LPS-binding protein prior to being recognised by a cell membrane-bound high-affinity LPS PRR (pattern-recognition receptor) known as CD14. CD14 then cooperates with the PRR coreceptor TLR4 (toll-like receptor 4) which shares the signal transduction pathway with IL-1 receptor (Ma et al., 2003). It could be via this signal transduction pathway that LPS induces EGFR expression.
EGFR expression in human peripheral monocytes and macrophage-like cells from five individuals was very low (figure 4.14). This was consistent with the low expression seen following flow cytometry analysis. There was however a peak in expression after 48 hours in culture. A possible explanation for this could be that the EGFR has an increased expression as the monocyte matures into a macrophage in order to prepare it for possible exposure to EGFR ligands as that type of cell. If no activation of the EGFR protein occurs then perhaps gene expression is returned to baseline levels. If activation of the EGFR protein does occur then maybe increased EGFR gene expression is sustained. A further extension to this investigation would be to incubate the monocytes after 48 hours with EGFR ligands and monocyte activators as carried out in the THP-1 cell investigation.
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4.7 Conclusions

The main aim of this chapter was to investigate the gene expression of the EGF receptor using quantitative real-time PCR techniques. Initial investigations with the THP-1 cell line showed that the levels of EGFR gene expression were very low when normalised to the housekeeping gene MLN51. As a comparison, the EJ tumour cell line has a gene expression ratio of 1 when normalised to MLN51 (data not shown). Following differentiation into macrophage-like cells, the differentiated THP-1 cells had increased EGFR gene expression. This could either be as a result of the maturation status of the cell or due to the differentiation procedure as PMA is a known inducer of EGFR gene transcription.

Following incubation with the EGFR ligands, HB-EGF was found to significantly increase EGFR gene expression in the monocytic THP-1 cells. Interestingly, to date out of all the ligands only EGF and TGFα have been shown to increase EGFR gene expression. Perhaps, the gene induction seen here with HB-EGF is solely associated with THP-1 cells. When the THP-1 cells were differentiated into macrophage-like cells gene expression was significantly increased following treatment with TGFα. Instead of an actual increase in expression, it was suggested that this result was due to the stabilizing effects TGFα can have on EGFR mRNA.

When incubated in the presence of IFNγ and IL-1β, EGFR gene expression in both monocytic and differentiated THP-1 cells was significantly induced. IFNγ primes and activates monocytes to act as effector cells in pro-inflammatory Th1 cellular response. Perhaps if exposed to IFNγ, the monocyte upregulates the EGFR to aid in a specific process, i.e. the migration of the cell to sites of inflammation.
Interestingly, IL-1β has not previously been shown to upregulate the EGFR as it is usually associated with its downregulation as a protein.

EGFR gene expression in human peripheral blood-derived monocytes from five individuals was very low. As they matured into macrophage-like cells in culture their EGFR expression spiked at 48 hours. Increased EGFR expression could be initiated during the maturation process in anticipation of ligand exposure to the cell. If no ligand activates the EGFR protein then gene expression is reduced back down to baseline. Perhaps incubation after 48 hours with the EGFR ligands or IFNγ in culture could sustain the increased EGFR gene expression.
Chapter Five

The Functional Properties of the EGFR Ligands on THP-1 Cells and Human Monocytes

5.1 Introduction

Monocytes and macrophages play a crucial role in the development and stability of the atherosclerotic plaque. Monocytes are initially attracted to the developing plaque environment under the influence of MCP-1 (Ikeda et al., 2002). Once inside the plaque, mature macrophages migrate within the lesion under the influence of MIP-1α, MIP-1β and RANTES (Osterud & Bjorklid, 2003; Reape & Groot, 1999; Cross et al., 1997; Uguccioni et al., 1995). LysoPC, oxLDL, TGFβ and the CSFs are also potent chemoattractants (Resat et al., 2003; Ross, 1993; Quinn et al., 1988; Quinn et al., 1987). Macrophages may be transformed into sub-populations of cells with different properties depending on the different stimuli they encounter in the plaque. Although IFNγ and the interleukins play a major role in the activation of macrophages, many other factors including lipids, growth factors and extracellular matrix proteins can influence the process. These sub-populations influence the plaque environment further by either being highly microbicidal and inflammatory or immunosuppressive (Ma et al., 2003). The apoptosis of macrophages within the plaque is highly influential to its stability and is almost certainly multifactorial. Inefficient clearance of the apoptotic macrophage leads to the generation of the necrotic core, increased inflammation and plaque instability (Tabas, 2005).
With respect to atherosclerosis, HB-EGF is a potent VSMC mitogen and chemoattractant *in vitro* (Higashiyama *et al.* 1993; Higashiyama *et al.* 1991). It is capable of upregulating PDGF, bFBF and M-CSF receptor mRNA in SMCs and can mediate the transactivation of the EGFR by G-protein coupled receptors for angiotensin II and thrombin (Kalmes *et al.*, 2001; Raab & Klagsbrun, 1997; Peifley *et al.*, 1996). Both BTC and EGF have been shown to possess mitogenic activity for rat SMCs and cultured human aortic SMCs (Tamura *et al.*, 2001; Shing *et al.*, 1993; Thyberg *et al.*, 1990).

With respect to monocyte and macrophages, EGF has been found to be chemotactic for microglial cells and rabbit peripheral blood-derived monocytes and macrophage-like cells (Lamb *et al.*, 2004; Nolte *et al.*, 1997). EGF was also been found to be mitogenic for rabbit monocyte-derived macrophages (Lamb *et al.*, 2004).

### 5.1.1 Aim

The aim of this chapter therefore was to investigate the function of the EGFR and its ligands EGF, HB-EGF, TGFα and BTC in monocytic and differentiated THP-1 cells. The possible function was also investigated in human peripheral blood-derived monocytes and monocyte-derived macrophages. Functional analysis was determined using proliferation and chemotaxis assays.
5.2 The Effect of EGFR Ligands on THP-1 Cell Growth

THP-1 cells were assessed for their cellular proliferation with the MTT assay following treatment with EGF, HB-EGF, TGFα and BTC for 72 hours at the concentrations shown (figure 5.1A). Untreated control cells had an average absorbance of 1.162 ± 0.03. Treatment of the cells with EGF, HB-EGF and BTC resulted in no change in their growth as their absorbances remained around the control cell absorbance. Interestingly, TGFα caused a significant (P<0.05) drop in absorbance following 0.4nM treatment (0.818 ± 0.03) when compared to control untreated cells. This could indicate either an inhibition in cellular proliferation or activation of cellular apoptosis.

Differentiated THP-1 cells were also investigated using the MTT assay. Monocytic THP-1 cells were differentiated with 10nM PMA for 72 hours followed by treatment with EGF, HB-EGF, TGFα and BTC for a further 72 hours at the concentrations shown (figure 5.1B). Once again, untreated control cells were analysed and they had an average absorbance of 1.050 ± 0.05. Results for each ligand were extremely variable with no clearly defined trend seen.
Figure 5.1 The effect of EGFR ligands on the growth of monocytic and differentiated THP-1 cells. Monocytic (A) and differentiated THP-1 (B) cells were grown in RPMI-1640 with 2% FCS containing EGF, HB-EGF, TGFα, BTC at the indicated concentrations or control medium for a period of 72 hours. Cell density was analysed by MTT assay as described within section 2.12. Each point represents the mean ± SEM (control n = 6; EGFR ligands n = 3). Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. *P<0.05 (0.4nM TGFα versus untreated control)
5.2.1 The Monoclonal Antibody ICR62 Inhibits the Growth of THP-1 Cells but not Differentiated THP-1 Cells

Monocytic THP-1 cells were grown in the presence of varying concentrations of the monoclonal antibody ICR62 (figure 5.2A). Untreated control cells had an average absorbance of 1.346 ± 0.08. When treated with 1μg/ml ICR62, the absorbance was 0.833 ± 0.04, 38% lower than untreated control cells. This inhibition in growth was found to be statistically significant (P<0.01) and continued with further more concentrated treatments.

Differentiated THP-1 cells were also investigated, but as seen with the ligand treatments the results were extremely scattered with no clearly defined trend seen.
Figure 5.2 The effect of the monoclonal antibody ICR62 on the growth of monocytic and differentiated THP-1 cells. Monocytic and differentiated THP-1 cells were grown in RPMI-1640 with 2% FCS containing ICR62 or control medium for a period of 72 hours. Cell density was analysed by MTT assay as described within section 2.12. Each point represents the mean ± SEM (control n = 6; ICR62 n = 3). Data was analysed using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (1, 2 and 16μg/ml ICR62 versus untreated control).
5.3 Chemotactic Effects of the EGFR Ligands

5.3.1 THP-1 Cells Migrate in Response to MCP-1

THP-1 cells were investigated for their chemotactic activity towards the well characterised monocyte chemokine MCP-1. THP-1 cells were exposed to MCP-1 over a range of 0.01nM to 30nM in a microBoydon chemotaxis chamber for 2 hours as previously investigated by Lamb et al., 2004. Migrated cells were counted in 10 fields of view for each test well and the fold migratory increase over unstimulated control cells was calculated.

Peak chemotactic activity was observed at 1nM where an average of 255 ± 47.52 cells migrated (figure 5.3). This was equivalent to a 2.3 ± 0.19 fold increase in migration over unstimulated control cells and was found to be statistically significant ($P<0.05$).
Figure 5.3 THP-1 cells migrate in response to MCP-1. THP-1 cells were assessed for their chemotactic activity towards MCP-1 over 2 hours in a microBoyden chemotaxis chamber. Total cells were counted in 10 fields of view per treatment well. Data shown are the mean ± SEM (n = 9) of the fold increase over unstimulated control cells. Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. *P<0.05 (1nM MCP-1 versus unstimulated control).
5.3.2 THP-1 Cells Migrate in Response to the EGFR Ligands

Monocytic THP-1 cells were assessed for the chemotactic activity towards the EGFR ligands EGF, HB-EGF, TGFα and BTC. The cells were exposed to physiological concentrations of EGF over a range of 0.1nM to 300nM, and HB-EGF, TGFα and BTC over a range of 0.01nM to 30nM in a microBoydon chemotaxis chamber for 2 hours as previously investigated by Lamb et al., 2004. Migratory cells were counted in 10 fields of view for each test well and the fold migratory increase over unstimulated control cells was calculated. For each ligand, the migratory response to MCP-1 is shown for comparison.

THP-1 chemotactic activity towards EGF was minimal with the peak observed at 1nM with an average of 79 ± 17.67 migrated cells (figure 5.4A). This was equivalent to a 1.04 ± 0.23 fold induction over the unstimulated control cells. Chemotactic activity towards HB-EGF was much greater. Peak activity was observed at 0.1nM with an average of 344 ± 41.50 migrated cells, equivalent to a 3 ± 0.36 fold increase over control cells (figure 5.4B). This migratory effect was found to be statistically significant (P<0.01). The response at 0.3nM (2.6 ± 0.24 fold) was also found to be statistically significant (P<0.01), as were 0.01nM (2.2 ± 0.06 fold, P<0.05) and 0.03nM (2.1 ± 0.35 fold, P<0.05). TGFα also resulted in some chemotactic activity with the peak being observed at 0.3nM with a 2 ± 0.06 fold induction over unstimulated control cells (figure 5.5A). This was found to be statistically significant (P<0.05). As seen with EGF, the chemotactic response to BTC was also minimal with a 1.4 ± 0.13 fold induction in chemotactic activity at 0.3nM (figure 5.5B).
Figure 5.4 The chemotactic response of THP-1 cells to EGF & HB-EGF. THP-1 cells were assessed for their chemotactic ability in response to EGF (A) and HB-EGF (B), over 2 hours in a microBoydren chemotaxis chamber. Total cells were counted in 10 fields of view per treatment well. THP-1 response to MCP-1 is shown as a comparison with each ligand. Data shown are the mean ± SEM (n = 3) of the fold increase over each ligand’s unstimulated control cells. Raw cell count data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (0.1nM & 0.3nM HB-EGF versus unstimulated control), *P<0.05 (0.01nM & 0.03nM HB-EGF versus unstimulated control; 1nM MCP-1 versus unstimulated control).
Figure 5.5 The chemotactic response of THP-1 cells to TGFα & BTC. THP-1 cells were assessed for their chemotactic ability in response to TGFα (A) and BTC (B), over 2 hours in a microBoyden chemotaxis chamber. Total cells were counted in 10 fields of view per treatment well. THP-1 response to MCP-1 is shown as a comparison with each ligand. Data shown are the mean ± SEM (n = 3) of the fold increase over each ligand’s unstimulated control cells. Raw cell count data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. *P<0.05 (0.3nM TGFα versus unstimulated control; 1nM MCP-1 versus unstimulated control).
5.3.3 Monocyte Chemotaxis in Response to MCP-1 Following Incubation with Different Blood Anticoagulants

Peripheral blood was collected from the same healthy individual and anticoagulated with either EDTA (BD Vacutainer® EDTA blood tube, BD Diagnostics, UK) or 3.8% sodium citrate as described in section 2.3. Isolated monocytes were then assessed for their chemotactic activity towards MCP-1 over a range of 0.01nM to 30nM (figure 5.6). Between 0.1nM and 30nM MCP-1, monocytes exposed to citrate anticoagulation had a consistently greater chemotactic activity compared to monocytes which had been exposed to EDTA anticoagulation. Peak response to MCP-1 following EDTA anticoagulation occurred at 0.1nM with an average of 292 ± 50.86 migrated cells. This equated to a statistically significant (P<0.01) 3.1 ± 0.52 fold increase in migration over the unstimulated control cells. Peak response to MCP-1 following citrate anticoagulation occurred at 0.3nM with an average of 669 ± 100.2 migrated cells. This equated to a statistically significant (P<0.01) 5.3 ± 0.81 fold increase in migration over the unstimulated control cells. In fact, all responses between 0.1nM and 10nM MCP-1 with citrate anticoagulated cells had statistically significant (P<0.01) increases in their activity when compared to their unstimulated control cells.

Therefore, all blood collected for further monocyte chemotaxis experiments was anticoagulated with citrate as it was felt that this anticoagulant provided the cells with the best opportunity for maximal chemotactic activity to other chemotactic agents.
Figure 5.6 The chemotactic response of human peripheral blood monocytes to MCP-1 with different anticoagulants. Human peripheral blood was collected from the same healthy individual via antecubital vein and anticoagulated in the presence of either EDTA or 3.8% sodium citrate. Isolated monocytes were assessed for their chemotactic ability in response to MCP-1 over 2 hours in a micropore Boyden chemotaxis chamber. Total cells were counted in 10 fields of view per treatment well. Data shown are the mean ± SEM (Citrate, n = 9; EDTA, n = 3) of the fold induction over unstimulated control cells. Raw cell count data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. **P<0.01 (Citrate 0.1nM, 0.3nM, 1nM, 3nM & 10nM versus citrate unstimulated control; EDTA 0.1nM versus EDTA unstimulated control).
5.3.4 Population Control & MCP-1 Responses

From each monocyte chemotaxis experiment with four different healthy individuals the unstimulated control and MCP-1 (0.1nM) treatment data were compiled to produce total population values (figure 5.7). The mean cell count for control cells was 146 ± 11.44. Mean MCP-1 cell count was 345 ± 41.33. This corresponds to a 2.4 ± 0.28 fold increase in migration compared to control cells when exposed to MCP-1. This fold increase was found to be extremely statistically significant (P<0.001) using an unpaired t test.

The total population control was subsequently used to normalise all the experimental data between individuals in calculating the folds over the control. The total population MCP-1 response was subsequently used as a reference point against which experimental data could be compared.
Figure 5.7 Total population control and MCP-1 responses. All experimental data for the control and MCP-1 (0.1nM) responses was consolidated from individual monocyte chemotaxis experiments from four healthy individuals. Total cells were counted in 10 fields of view per treatment well. Data shown are the mean ± SEM fold chemotactic response for MCP-1 over the population control. Mean cell count was 146 ± 11.44 for control cells and 345 ± 41.33 for MCP-1 exposed cells. The population was 2.4 ± 0.28 fold. Raw cell count data (control, n = 37; MCP-1 n = 30) were analysed using an unpaired t test. ***P<0.0001 (MCP-1 versus unstimulated control).
5.3.5 Human Monocytes Migrate Significantly in Response to HB-EGF but not EGF

As a result of HB-EGF evoking the greatest migratory response in THP-1 cells, this ligand was focused upon in order to investigate the migratory responses of human peripheral blood monocytes. EGF was also investigated due to its role as the major EGFR ligand.

Using the blood from three healthy individuals, EGF was investigated over a range of 0.1 to 100nM and HB-EGF was investigated over a range of 0.01 to 10nM. Monocytes showed their peak chemotactic response to EGF at 0.1nM (figure 5.8A) with an average of 230 ± 33.75 cells migrating. This response was 1.6 ± 0.23 fold over the control cells. Peak monocyte chemotactic response to HB-EGF was seen at 10nM (figure 5.8B). The average cell count of 355 ± 41.16 corresponded to a 2.4 ± 0.28 fold increase in chemotactic response over control cells. The chemotactic response of monocytes to HB-EGF at 10nM was found to be very statistically significant (P<0.01). Further to this, the response seen with HB-EGF at 10nM was comparable to that seen with the population response for MCP-1 at 0.1nM.
Figure 5.8 The chemotactic response of human peripheral blood monocytes to EGF and HB-EGF. Human peripheral blood monocytes isolated from three healthy individuals were assessed for their chemotactic response to EGF and HB-EGF in a microBoydon chemotaxis chamber for 2 hours. Total cells were counted in 10 fields of view per treatment well. Data shown are the mean ± SEM (control n = 37; MCP-1 n = 30; EGF/HB-EGF n = 6). Raw cell count data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (10nM HB-EGF versus unstimulated control).
5.3.6 Blood Monocyte Response to MCP-1 Decreases with Cellular Maturation

Peripheral blood monocytes from four healthy individuals were assessed for their ability to respond to MCP-1 during differentiation/maturation into monocyte-derived macrophages. Control unstimulated cells did not alter their chemokinetic activity over the period of maturation (figure 5.9A). Mean control cell count for monocytes was 146 ± 11.44. This reduced to 94 ± 9.52 cells after 48 hours and increased to 110 ± 29.00 cells at 8 days. As there was no statistical difference between the control cells over the maturation period it could be assumed that the cells were healthy and capable of chemotaxis if induced to migrate.

In order to assess if the cellular migratory response to MCP-1 (0.1nM) changed during maturation, data from each individual experiment was compiled at each time point (figure 5.9B). Monocytes had the largest response to MCP-1 with an average of 345 ± 41 cells migrating which equates to a 2.4 ± 0.28 fold increase in migration over control unstimulated cells. After 48 hours the number migrating cells dropped slightly to 304 ± 28. However, as the number of control cells migrating also decreased, the fold increase in migration increased to 3.2 ± 0.29. At 8 days the number of cells migrating in response to MCP-1 dropped dramatically to 106 ± 16, equal to the unstimulated control cells at this time point. When compared to 48 hour monocytes this decrease in migration was found to be statistically significant ($P<0.05$). This indicated that 8 day monocyte-derived macrophages no longer responded chemotactically to MCP-1 at 0.1nM.
Figure 5.9 The effect of monocyte maturation on the chemotactic response to MCP-1. Peripheral blood monocytes from four healthy individuals were cultured and their chemotactic ability to MCP-1 (0.1nM) was assessed as 2 hour monocytes, 48 hour monocytes and 8 day monocyte-derived macrophages. Total cells were counted in 10 fields of view per treatment well. (A) Unstimulated control cells were compared at the three time points. Data shown are the mean ± SEM (monocytes n = 37; 48 hours n = 11; 8 days n = 6). (B) Migratory response of cells at each time point to MCP-1 (0.1nM). Data shown are the mean ± SEM (monocytes n = 30; 48 hours n = 12; 8 days n = 6) of the fold increase in migration over each time point control. Data were analysed using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison post hoc test. *P<0.05 (8 day macrophages versus 48 hour monocytes).
5.3.7 Chemotactic Response to HB-EGF Increases with Cellular Maturation

As seen in section 5.3.5, peak monocyte chemotactic response to HB-EGF was seen at 10nM (figure 5.10A). The average cell count of 355 ± 41.16 corresponded to a 2.4 ± 0.28 fold increase in chemotactic response over unstimulated control cells. The chemotactic response of monocytes to HB-EGF at 10nM was found to be very statistically significant (P<0.01). Further to this, the response seen with HB-EGF at 10nM was comparable to that seen with the population response for MCP-1 at 0.1nM.

After 48 hours adherence in tissue culture, the monocytes showed their peak chemotactic activity at 1nM HB-EGF (figure 5.10B). The average cell count of 190 ± 25.01 corresponded to a 2 ± 0.27 fold induction in chemotaxis over unstimulated control cells. This response was two-thirds of that seen for MCP-1 at this time point (3.2 ± 0.29 fold over control cells). At 8 days, the monocyte-derived macrophages showed their peak response at 0.1nM HB-EGF with an average cell count of 228 ± 44.14 (2 ± 0.40 fold induction over unstimulated control cells) (figure 5.10C). This response was 2 fold higher than the response seen with MCP-1 with these cells, however, this difference was not found to be statistically significant. Therefore, HB-EGF resulted in being the major chemotactic factor for monocyte-derived macrophages.
Figure 5.10 The effect of monocyte maturation on chemotactic response to HB-EGF. The chemotactic response to HB-EGF of monocytes (A), 48 hour old monocytes (B) and 8 day monocyte-derived macrophages (C) from four healthy individuals was investigated in a microBoydon chemotaxis chamber for 2 hours. Total cells were counted in 10 fields of view per treatment well. Data shown are the mean ± SEM (HB-EGF monocytes n = 6; 48 hours n = 3; 8 days n = 3) of the fold increase over unstimulated control cells. See figure 5.9 for control and MCP-1 (n) numbers. Raw cell count data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test. **P<0.01 (monocyte 10nM HB-EGF versus monocyte unstimulated control).
5.3.8 The Monoclonal Antibody ICR62 Inhibits Monocyte Chemotaxis

Having already established that human peripheral blood monocytes migrate in response to HB-EGF it was investigated whether this could be inhibited by the monoclonal antibody ICR62 (figure 5.11). Peak monocyte migratory response to HB-EGF (0.1nM) from one healthy individual was $1.6 \pm 0.21$ fold higher over control cells (average cell count of $232 \pm 30.21$). This was consistent for the population HB-EGF response at this concentration.

Monocytes incubated with ICR62 (50nM) showed a reduced migratory response with an average cell count of $164 \pm 47.64$, only $1.1 \pm 0.33$ fold over control cells. When monocytes were incubated with ICR62 (100nM) the drop in migratory response compared to HB-EGF was found to be significant ($P<0.05$). Mean cell count at this treatment was $96 \pm 15.59$ which corresponded to a fold of $0.66 \pm 0.11$; less migration than control cells. Similar reductions in chemotaxis were also observed with the IgG2b isotype control at 50nM and 100nM. However, they were not found to be statistically significant.
Figure 5.11 The inhibition of monocyte chemotaxis with ICR62. Isolated peripheral blood monocytes from one healthy individual were incubated with ICR62 or IgG2b isotype control at 50nM and 100nM and their chemotactic response to HB-EGF (0.1nM) was assessed after 2 hours. Total cells were counted in 10 fields of view per treatment well. Data shown are the mean ± SEM (control n = 37; MCP-1 n = 6; HB-EGF treatments n = 6) of the fold increase in migration over unstimulated control cells. Raw cell count data were analysed using one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. *P<0.05 (100nM ICR62 versus HB-EGF).
5.4 Discussion

Within this chapter the functional role of the EGFR and its ligands was investigated on monocytic THP-1 cells, differentiated THP-1 cells, human peripheral blood-derived monocytes and human macrophage-like cells. Functionality was assessed using the MTT assay for cell growth and the chemotaxis assay for migration.

The MTT assay may be used in the measurement of cell proliferation studies that traditionally use the incorporation of radioisotopes as a measurement of cell division (Mosmann, 1983). The yellow MTT solution is converted to a water-soluble MTT-formazan of a dark blue colour by the mitochondrial dehydrogenases of living cells. The blue crystals are solubilized with the addition of acidified isopropanol and the intensity is measured colourimetrically at a wavelength of 570nm. The assay has been modified since its original development and has been found to have enhanced sensitivity compared to methods based on $[^3]$Hthymidine incorporation (Gieni et al., 1995; Twentyman & Luscombe, 1987; Denizot & Lang, 1986; Davis & Czech, 1984). It has also been validated for use with cells of the monocytic lineage (Gebre-Hiwot et al., 1992; van de Loosdrecht et al., 1991; Ferrari et al., 1990).

The treatment of monocytic THP-1 cells with EGF, HB-EGF, TGFα or BTC produced no discernable change in THP-1 cell growth, be it proliferative or apoptotic (figure 5.1A). However, the THP-1 cells were growing within the system as the initial absorbance at the time of treatment was an average of 0.320 ± 0.02 (data not shown). When the untreated control cells were assayed 72 hours later, their absorbance had increased 3.6 fold to an average of 1.162 ± 0.03. This increase was found to be statistically significant (P<0.001). A difference in cellular growth was
perhaps not observed with the ligand treatments due to the fact that the cells were not
maintained in serum-free media. There were perhaps sufficient quantities of
endogenous growth factors remaining to drive THP-1 cell proliferation.

When the differentiated THP-1 cells were investigated, results for each ligand were
scattered and no clear trend for either a proliferative or apoptotic response was
observed (figure 5.1B). The differentiated THP-1 cells were assayed for their initial
absorbance before treatment. This average absorbance was 0.810 ± 0.02 (data not
shown). When the untreated control cells were assayed 72 hours later, their
absorbance had significantly ($P<0.001$) increased by 1.3 fold to an average of 1.050
± 0.05. This again suggested that the cells had been proliferating within those 72
hours but not to the same extent as the monocytic THP-1 cells. This reduction or
lack of proliferative ability in the differentiated THP-1 cell is well documented
(Schwende et al., 1996; Auwerx, 1991).

Inhibition of the growth of the monocytic THP-1 cells was also investigated. The
monoclonal antibody ICR62 is known to inhibit ligand binding to the EGF receptor.
During characterisation, the antibody was found to bind to epitope ‘C’ located within
the ligand binding domain of the EGFR (Modjtahedi et al., 1993). Incubation of
THP-1 cells with 1μg/ml ICR62 resulted in a statistically significant ($P<0.01$)
decrease in cell growth compared to untreated control cells (figure 5.2A). This
inhibition was sustained up through the more concentrated treatments. The head and
neck tumour cell line HN5 has complete inhibition of proliferation following 5nM
treatment with ICR62 (Modjtahedi et al., 1993). The complete loss of proliferation
in the HN5 cell line is probably due to the fact that the cells heavily rely on the
EGFR for continued proliferation. Only partial loss of proliferation is seen with the THP-1 cell line probably because the EGFR only plays a part in the cells proliferation. It could be investigated in the future to determine whether ICR62 is actually inhibiting growth or whether it is inducing apoptosis in THP-1 cells at that concentration. It should be noted that without specific antibody isotype control data this result should be interpreted with caution as the decrease in cellular growth could be due to non-specific binding of the antibody. Further experiments into the inhibition of growth of cells of the monocytic lineage with ICR62 should include a matched isotype control.

Other studies have shown that although treatment of an ovarian cancer cell line with varying concentrations of TGFα did not show significant growth promotion, monoclonal antibodies against TGFα and EGFR significantly inhibited cell growth (Morishige et al., 1991).

As seen with the previous ligand treatments, no effect in growth was seen when differentiated THP-1 cells were incubated with ICR62 (figure 5.2B). This is probably due to the fact that there are no receptors on the cell surface as demonstrated by flow cytometry and immunocytochemical analysis.

HB-EGF and the EGFR have been shown to be responsible for the chemotaxis of smooth muscle cells within the developing plaque resulting in the migration of medial smooth muscle cells in to the intimal space therefore adding to the further developing the plaque (Higashiyama et al., 1993). When THP-1 cells were assessed for their chemotactic activity towards the EGFR ligands tested, HB-EGF proved to
be the most affective chemokine (figure 5.4B). This response was found to be greater than that seen with the well characterised monocyte chemokine MCP-1. Interestingly, peak chemotactic activity towards HB-EGF was observed at a ten-fold lower concentration than that seen with MCP-1 and equivalent peak activity towards TGFα was at a third lower concentration than MCP-1. EGFR ligands may have a higher affinity for their receptor compared to MCP-1 and its receptor CCR2, therefore this may explain why the EGFR ligands exhibit their chemotactic activity at lower concentrations. The differential effects of the ligands could be due to their different affinities for the EGFR. HB-EGF has been shown to have a much greater affinity for the EGFR than EGF on smooth muscle cells (Higashiyama et al., 1991). This could be due to the requirement of cell surface heparin sulphate proteoglycan (HSPG) to aid in the binding of HB-EGF to the EGFR (Aviezer & Yayon, 1994; Higashiyama et al., 1993). This may explain why HB-EGF is a more efficient chemokine than the other ligands tested. When investigating whether differentiated THP-1 cells were chemotactic towards the EGFR ligands no responses were seen (data not shown). Further to this the cells were also non responsive to MCP-1. Studies have shown that THP-1 cell maturation mediated by high concentrations of PMA (50nM to 1μM) resulted in the down-regulation of the MCP-1 receptor CCR2 (Phillips et al., 2005; Denholm & Stankus, 1995). This is consistent with previous observations in this thesis as no receptor expression has been observed. 

On the basis of the results observed with the THP-1 cells, HB-EGF was used to assess the chemotactic activity of peripheral blood-derived monocytes and monocyte-derived macrophages. Peripheral blood-derived monocytes had a statistically significant (P<0.01) 2.4 ± 0.28 fold increase in their chemotactic
Chapter 5 – EGFR Ligand Function

response compared to unstimulated control cells when exposed to 10nM HB-EGF (figure 5.8B). This was equivalent to the response seen for 0.1nM MCP-1 with these same cells. Interestingly, as the monocyte matured into an 8 day old monocyte-derived macrophage the chemotactic response to 0.1nM MCP-1 decreased, whereas peak chemotactic activity to HB-EGF occurred at lower concentrations (figure 5.10). The decrease in monocyte responsiveness to MCP-1 with maturation has been previously observed with a downregulation of CCR2 (Phillips et al., 2005), however, why monocyte-derived macrophages should become responsive to HB-EGF at lower concentrations is unclear. One explanation could be that in the circulation the monocytes are designed to be responsive to MCP-1 and not HB-EGF which explains why the monocytes were only chemotactically responsive to HB-EGF at a high concentration (10nM). However, when they enter the tissues and mature into macrophages they may change their responsiveness to HB-EGF as the requirement to respond to MCP-1 decreases. This would enable the fine tuning of macrophage migration within the tissue at very low levels of HB-EGF. Staining for cell markers in serial sections showed macrophages to be the main cell type expressing HB-EGF mRNA (Reape et al., 1997). Studies have shown that there is increased production of HB-EGF protein by macrophages and SMCs in aortic atherosclerotic plaques (Miyagawa et al. 1995). This could mean that macrophages contained in certain areas within an early lesion could produce HB-EGF in order to guide new macrophages to the areas where they are needed, for example areas of high lipid. It has been suggested that lysoPC, a component of oxidatively modified low-density lipoprotein, could up-regulate HB-EGF production in macrophages in human atherosclerotic lesions (Reape et al., 1997). This could mean that the regulation or modulation of HB-EGF protein synthesis in macrophages might be related to the
metabolism of oxidized LDL in the intima (Nakata et al., 1996). Infiltration of macrophages into the intima appeared to be closely associated with the lesion of pathological thickening suggesting that macrophage-derived HB-EGF may play a crucial role in the process of coronary atherogenesis at a time when macrophages would potentially become a more dominant cell type than SMCs (Nakata et al., 1996).

The monoclonal antibody ICR62 is known to decrease the binding of ligand to the EGFR and subsequently inhibit tumour growth (Modjtahedi et al., 1993). When peripheral blood-derived monocytes were incubated with ICR62 in the chemotaxis chamber their activity towards HB-EGF significantly (P<0.05) reduced in a dose dependent manner (figure 5.11). This meant that ICR62 was possibly blocking the binding of HB-EGF to the EGFR thereby inhibiting chemotaxis. This showed that it was indeed the EGFR that was responsible for the chemotaxis of the blood monocytes. The IgG2b isotype control was also investigated alongside ICR62 and was also found to inhibit chemotaxis, but not significantly. Information gained subsequently concerning this particular antibody revealed that it had become contaminated with ICR62 antibody. Therefore another source of isotype control should be used in future experiments. Other studies by Chan et al. have shown that blockade of the EGFR with a monoclonal antibody (151-IgG) reduced intimal hyperplasia induced by balloon injury in the rat carotid artery. They hypothesised that this effect was due to the inhibition of medial SMC proliferation (Chan et al., 2003).
5.5 Conclusions

The main aim of this chapter was to discover a potential functional role of EGF, HB-EGF, TGFα and BTC on THP-1 cells and human peripheral blood-derived monocytes and macrophage-like cells. Investigation of THP-1 cells with the MTT assay showed that the ligands played no role in the survival status of these particular cells. However, when incubated in the presence of the monoclonal antibody ICR62 THP-1 cell proliferation was significantly inhibited. This would indicate that the EGF receptor does indeed play a role in the maintenance of THP-1 cell growth, and warrants further investigation in serum-free media.

Chemotaxis experiments showed that monocyctic THP-1 cells were chemotactic towards the EGFR ligands, most notably HB-EGF and TGFα. The result seen with HB-EGF was perhaps due to the heparin-binding domain located within this ligand. Interestingly, the chemotactic response to HB-EGF was greater than that observed for the major monocyte chemokine MCP-1. However, when these same experiments were conducted with differentiated THP-1 cells no such chemotactic activity was exhibited. This result ties in with the discovery that the EGFR is not expressed on the cell membrane of differentiated THP-1 cells as shown through flow cytometry and immunocytochemical analysis in chapter 3. Peripheral blood-derived monocytes were also significantly chemotactic towards HB-EGF but not EGF. This may again be due to the heparin-binding domain located within the growth factor which aides it’s binding to the receptor. This chemotactic response to HB-EGF was found to be comparable to the response observed with MCP-1. Interestingly, as the monocyte matured into a macrophage-like cell the chemotactic activity towards MCP-1 decreased and the activity towards HB-EGF increased. This could lead to the
hypothesis that monocytes are initially attracted to the developing plaque under the influence of MCP-1. However, as they mature into tissue macrophages and lose their ability to respond to MCP-1, HB-EGF may take over as the major chemokine responsible for internal plaque macrophage movement and rearrangement. When incubated with the monoclonal antibody ICR62, monocyte chemotactic activity towards HB-EGF was significantly inhibited in a dose dependent manner indicating that the EGFR was indeed responsible for the chemotactic ability of monocytes.
Chapter Six

Expression of EGFR Within Macrophages of Human Arterial Sections

6.1 Introduction

The EGFR has been shown to be expressed within the atherosclerotic plaques of humans and experimental animals (Tamura et al., 2001; Nanney et al., 1988; Tomita et al., 1986). This expression is usually associated with smooth muscle cells (Miyagawa et al., 1995) and endothelial cells (Styren et al., 1993). Interestingly the expression of the EGFR on SMCs seems to be solely related to the plaque environment as the EGFR is not expressed in the normal artery wall (Miyagawa et al., 1995). Furthermore, in the coronary and abdominal arteries of adults, strong EGFR immunostaining has been localized to the SMCs on the border between the intima and the media (Nakata et al., 1996; Miyagawa et al., 1995).

Recently the expression of the EGFR has been found to co-localise with macrophages in the atherosclerotic lesions of cholesterol-fed rabbits (Lamb et al., 2004).

6.1.1 Aim

This study aimed to investigate clinical sections of lesions from patients undergoing abdominal aortic aneurism (AAA) repairs of femoral bypass surgery using
immunohistochemical techniques. Within these samples, the expression of EGFR was investigated in relation to monocytes and macrophages within the lesions.
6.2 Materials & Methods

6.2.1 Sample Collection & Fixation

Samples were collected from patients undergoing abdominal aortic aneurism repairs or femoral arterial bypasses at the Royal Surrey County Hospital, Guildford, Surrey, UK (ethical approval obtained from South West Surrey Local Research Ethics Committee and the University of Surrey Ethics Committee). Tissue samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, UK) for 24 hours. Once fixed, tissues were washed and placed in 70% ethanol for storage prior to embedding.

6.2.2 Tissue Processing & Sectioning

Samples were processed in a histokinette prior to paraffin embedding. Blocks were soaked in 5% formic acid prior to sectioning. Five-micron sections were cut, floated onto Superfrost Plus slides (BDH, UK) and air dried over night.

6.2.3 Immunohistochemical Staining & Light Microscopy

Sections were heated at 50°C for 1 hour and then rehydrated in xylene (10 minutes), 100% ethanol (10 minutes), 95% ethanol (5 minutes), 70% ethanol (3 minutes), 50% ethanol (2 minutes) and water. Antigen retrieval was carried out by microwaving sections in 10mM tri-sodium citrate in H2O (pH6) for 3 minutes on high and 7 minutes on medium/low. Sections were washed twice in PBS containing 0.05% Tween-20 (Sigma-Aldrich, UK) (PBST) for 5 minutes before endogenous peroxidase activity was quenched by incubating sections with 0.3% H2O2 in methanol at room temperature for 30 minutes. Sections were again washed in PBST and subsequently
immunostained using the Vectorstain ABC Elite Reagent kit for mouse or rat primary antibodies (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer’s instructions. All primary antibodies (ICR16, α-SMC actin, HAM56 and matched isotype controls) were diluted in PBT (0.1% BSA, 0.01% Tween-20 in PBS) and incubated on the sections overnight at 4°C in a humidified chamber. Sections were washed in PBST between each staining step. The colour peroxidase reaction was visualised either using Vector VIP (diluted in PBS) or DAB (diaminobenzidine) (diluted in MilliQ H₂O) (both Vector Laboratories, USA). Sections were then washed in water and dehydrated through an alcohol series; 50% ethanol (3 minutes), 70% ethanol (3 minutes), 95% ethanol (5 minutes) and 100% (10 minutes). Sections were then cleared in xylene and mounted in DPX (BDH, UK).

Sections were visualised using a Leica DMLB microscope equipped with a colour JVC TK-C1380 video camera with Qwin image analysis software (Leica, Luton).
6.3 The Expression of the EGFR within Human Atherosclerotic Lesions

The lesion of a male undergoing an AAA repair was investigated for its expression of the EGFR. Sections were stained with the monoclonal antibody ICR16 and a matched isotype control (figure 6.1A&B). Positive EGFR staining was localised to areas of cells with the morphology similar to that of macrophages. Smooth muscle cells were localised to the surrounding area around the clump of macrophages (figure 6.1C).
Figure 6.1 EGFR staining within human atherosclerotic lesion of a male abdominal aortic aneurism. Tissue was collected from a male undergoing an abdominal aortic aneurism repair. The sample was fixed, processed and sectioned as described in sections 6.2.1 and 6.2.2. Sections were stained as described in section 6.2.3 with the monoclonal antibody ICR16 (5μg/ml) (A), IgG2a isotype control (5μg/ml) (B) or α-SMC actin (1/500 dilution) (C) and visualised with a DAB peroxidase system. Sections shown are under x400 (i) and x1000 (ii) magnification for EGFR staining or x100 (i) and x400 (ii) for SMC staining.
6.4 The EGFR Co-localises with Macrophages in Human Atherosclerotic Lesions

Sections from a female undergoing femoral bypass surgery were double stained for EGFR expression with ICR16 and for macrophages with HAM56. Using a single stain a macrophage-rich area was identified within the lesion (figure 6.2A). On an associate section the presence of EGFR within this macrophage-rich region was investigated. Within the macrophages (pink) the EGFR is clearly expressed (brown) as demonstrated by the cell identified by the arrow (figure 6.2B).
Figure 6.2 EGFR and macrophage staining within human atherosclerotic lesion of a female femoral artery. Tissue was collected from a female undergoing a femoral bypass. The sample was fixed, processed and sectioned as described in sections 6.2.1 and 6.2.2. Sections were stained as described in section 6.2.3 with the HAM56 (macrophage) (1/30 dilution) and the monoclonal antibody ICR16 (EGFR) (5µg/ml) and visualised with the Vector VIP (pink) or a DAB (brown) peroxidase system. Sections shown are stained with HAM56 and Vector VIP (A), and double staining with HAM56 (Vector VIP) and ICR16 (DAB) (B). Samples are shown under x1000 magnification. A HAM56 positive cell expressing EGFR (ICR16) is identified by the arrow.
6.5 Discussion

The aim of this chapter was to establish whether the EGFR co-localises with macrophages in human atherosclerotic lesions. Samples of atherosclerotic lesions were collected from patients undergoing AAA repairs (average age, 75 years) and femoral arterial bypasses (average age, 77 years) at the Royal Surrey County Hospital, Guildford, UK. Patients undergoing either type of procedure generally have advanced calcified lesions which make the histological process difficult (Stary et al., 1995). As a result of this, only a selection of the samples from the 12 patients could be sufficiently investigated.

The EGFR was found to be expressed within the lesions of a male AAA with the monoclonal antibody ICR16. The positively stained cells had macrophage morphology. Smooth muscle cells were also stained for within this lesion (data not shown). They were also positive for the EGFR.

Within the sections from a femoral arterial lesion, macrophages were identified using the macrophage-specific antibody HAM56. This antibody has been previously used for the detection of EGFR in human atherosclerotic plaques (Gown et al., 1986). Again, the detection of EGFR expression was carried out using ICR16. Double staining techniques revealed that the EGFR was expressed within those cells also positive for macrophages. Co-localisation of the EGFR with macrophages has previously been observed in uveal melanomas (Scholes et al., 2001).

The main difficulty experienced with this study was the quality of the lesional sample. Many samples were highly calcified which made sectioning extremely
difficult even following decalcification treatment with formic acid. Perhaps in the future in order to confirm the results obtained from this study, tissues could be collected from recently deceased individuals. Samples obtained in this fashion may provide better examples of earlier more easily analysed plaques. Samples could also be obtained from a broad age range from infants to the elderly, as well as from different vessels. It is possible that there could be different expression patterns and possible roles within different plaques i.e. carotid versus aortic.

In summary, this study aimed to provide evidence that macrophages within atherosclerotic plaques express the EGFR. Although the evidence is limited, staining for the EGFR was found to co-localise with cells stained positively for macrophages. Further investigations would need to be executed, perhaps using more sensitive confocal microscopy techniques to confirm the results obtained from this study.
Chapter Seven

General Discussion and Concluding Remarks

In this thesis the expression and potential function of the epidermal growth factor receptor (EGFR) was investigated in the monocytic THP-1 cell line and human peripheral blood-derived monocytes. The EGFR is known to play a role in the atherogenic process by mediating the proliferation and migration of smooth muscle cells (Higashiyama et al., 1993; Higashiyama et al., 1991). The EGF family of ligands, most notably HB-EGF, BTC, TGFα and EPR are also elaborated by smooth muscle cells (Tamura et al., 2001; Taylor et al., 1999; Higashiyama et al., 1994; Mueller et al., 1990), monocytes/macrophages (Tamura et al., 2001; Mograbi et al., 1997; Toyoda et al., 1997; Nakano et al., 1994; Higashiyama et al., 1991; Madtes et al., 1988), endothelial cells (Yoshizumi et al., 1992), T lymphocytes (Blotnick et al., 1994) and platelets (Oka & Orth, 1983). Therefore, it can be concluded that the EGFR family plays an important role in the development of atherosclerosis.

Previous studies had reported the expression of the EGFR on human cells of the monocytic lineage most notably brain-derived microglial cells (Nolte et al., 1997), uveal melanoma macrophages (Scholes et al., 2001) and the monocytic cell line U937 (Eales-Reynolds et al., 2001). Based on this evidence the expression of the EGFR on rabbit peripheral blood leukocytes was investigated (Lamb et al., 2004). These cells were found to express the EGFR and activation resulted in both chemotactic and mitogenic responses. The EGFR was also found to co-localise with macrophages within the atherosclerotic plaques of cholesterol-fed rabbits. However,
to date, no study had investigated the expression of the EGFR on human monocytes and macrophages within the context of atherosclerosis.

The EGFR was found to be minimally expressed on peripheral blood monocyte-derived macrophages and to a greater extent on the monocytic cell line THP-1 with flow cytometry techniques *(figures 3.7 and 3.3 respectively)*. The THP-1 cell line was established from the blood of a 1 year old boy with acute monocytic leukaemia *(Tsuchiya et al., 1980)*. Although characterisation studies concluded that it resembled the human monocyte with respect to numerous criteria such as morphology, secretory products, oncogene expression, expression of membrane antigens and genes involved in lipid metabolism *(Auwerx, 1991)*, recent studies into the gene expression of the two cell types have concluded that they are very different from a transcriptomic point of view *(Kohro et al., 2004)*. This could also be a reason as to why with quantitative real-time PCR analysis, THP-1 cells demonstrated higher levels of EGFR mRNA than the peripheral blood-derived monocytes and macrophages *(figures 4.6 and 4.14 respectively)*. The difference in EGFR expression could be due to the transformed nature of the THP-1 cell in that the EGFR participates in the normal proliferation status of the cell. The EGFR is often over-expressed in human carcinomas and amplification of gene expression has been demonstrated to occur in carcinoma patients *(Normanno et al., 2006)*. Care should therefore be taken when interpreting the results of experiments carried out with THP-1 cells in which it is otherwise assumed that they are representative of the macrophage *(Kohro et al., 2004)*. It should also be noted that a very small number if individuals were used to provide monocytes for this study, especially for the flow cytometry experiments. These individuals differed in gender, age and genetic
Chapter 7 – Final Conclusions

background, therefore for future experiments, an increased number of individuals should be used to provide a more complete picture of the status of EGFR expression on peripheral blood monocytes.

Immunocytochemical studies into the localization of the EGFR within THP-1 cells revealed that monocytic THP-1 cells expressed the receptor on their cellular surface and within the cytoplasm (figure 3.9). Cells treated with PMA demonstrated nuclear localisation of the EGFR (figure 3.11). This result corroborated the discovery that differentiated THP-1 cells did not express the EGFR on their cellular surface with flow cytometry and that no functionality could be established following proliferation and chemotaxis assays. The purpose of the EGFR nuclear localisation within differentiated THP-1 cells is unclear, although in other cells it is associated with increased proliferation and DNA damage repair leading to the radioresistance of tumours (Chen & Nirodi, 2007; Lin et al., 2001). The nuclear localisation could either be a characteristic of the macrophage-like cell or be a specific result of PMA treatment within the THP-1 cell. The interaction between the phorbol ester PMA and the EGFR is well documented. PMA has been shown to decrease the high-affinity binding of EGF to the EGFR and is responsible for its internalisation but not degradation in KB cells (Beguinot et al., 1985). PMA is an activator of PKC which is known to phosphorylate the EGFR at threonine 654 leading to receptor deactivation (King & Cooper, 1986). The loss of EGFR cell surface expression of differentiated THP-1 cells could therefore be due to PMA treatment. It would be interesting to investigate the nuclear localisation of the EGFR following treatment with other initiators of THP-1 cell differentiation for example 1,25-dihydroxyvitamin D3, which do not activate PKC (Schwende et al., 1996). PMA has also been found
to activate EGFR gene transcription via the transcription factor AP-2 (Johnson, 1996). This confirms the finding that differentiated THP-1 cells had a greater EGFR gene expression when compared to monocytic THP-1 cells (figure 4.6).

Investigations into the functionality of the EGFR on THP-1 cells and peripheral blood-derived monocytes revealed that the receptor mediated baseline cellular proliferation and cellular motility. A decrease in the proliferation of THP-1 cells was only observed following treatment with the monoclonal antibody ICR62 (figure 5.2). This antibody binds with high affinity to a distinct epitope (termed 'C') on the extracellular domain of the human EGFR and is known to inhibit ligand binding and completely inhibit cellular proliferation at 5nM (Modjtahedi et al., 1993). Although incubation of THP-1 cells with the EGFR ligands failed to increase cellular proliferation, ICR62 significantly reduced cell growth, indicating that the EGFR is indeed required in part for THP-1 cell growth. This effect was also seen with ICR16, another antibody of a different isotype which binds epitope C and is capable of inhibiting tumour growth (data not shown). To fully confirm that this inhibitory result is due to the functional F(ab')2 region of the antibody, both experiments should be repeated with the appropriate antibody isotype controls. Interestingly, when THP-1 cells were incubated with the tyrosine kinase inhibitor gefitinib no growth inhibition was observed (data not shown). Gefitinib is a member of the first generation of EGFR tyrosine kinase inhibitors that bind to their target, the catalytic site in the intracellular kinase domain, through classic competitive binding with ATP (Sequist, 2007; Wakeling et al., 2002). Perhaps no growth inhibition was observed because THP-1 cells are not solely dependent on the EGFR for proliferation. It might be interesting to repeat the experiment with a second generation tyrosine
kinase inhibitor. Many of the second-generation compounds form covalent, and therefore permanent, bonds with their target, which should theoretically increase their effectiveness by prolonging the inhibition of EGFR signalling to the entire lifespan of the drug-bound receptor molecule (Sequist, 2007). Examples of these inhibitors include EKB-569 (Wissner et al., 2003), HKI-272 (Rabindran et al., 2004) and CI-1033 (Allen et al., 2003).

HB-EGF was found to be the most functionally active of the EGFR ligands investigated. It induced EGFR gene expression in THP-1 cells (figure 4.7) and was the predominant chemokine for both THP-1 cells (figure 5.4) and peripheral blood-derived monocytes and macrophages (figures 5.8 and 5.10 respectively). This functional activity could be due in part to its ability to bind cell surface heparin sulphate which leads to a higher binding affinity to the EGFR (Higashiyama et al., 1993). One possible explanation for increased EGFR gene expression following HB-EGF treatment could be that if HB-EGF is being released in the vicinity, monocytes will be chemotactic towards it. However, in order to increase this response, HB-EGF could also stimulate the increased expression of EGFR in order to allow the cells to respond more effectively to the chemotactic stimulus. Both EGF and TGFα are capable of stimulating the synthesis of the EGFR (Burgess, 1989; Bjorge & Kudlow, 1987) but until now this has not been documented with HB-EGF. However, it is important that this phenomenon was observed within the THP-1 cell line and may not be representative of the blood monocyte. EGFR gene expression within blood-derived monocytes was extremely low with only an increase seen at 48 hours incubation (figure 4.14) which could indicate that the EGFR is required for maturation or that with no activation of the receptor via ligand binding, the gene
expression is switched off. If the cells were maintained in HB-EGF or another stimulus such as a macrophage activator like IFNγ perhaps the EGFR would continue to have increased expression. It was demonstrated that IFNγ increased EGFR gene expression in both monocytic and differentiated THP-1 cells (figures 4.8 and 4.10 respectively). Perhaps within the plaque environment where monocytes are in contact with such activating stimuli they may express the EGFR. This hypothesis could be supported by the fact that within this study the EGFR has been found to co-localise with macrophages in human atherosclerotic plaques (figure 6.2).

Interestingly, as peripheral blood-derived monocytes matured into monocyte-derived macrophages and MCP-1 chemotactic activity was lost, the cells developed an increased sensitivity or affinity to HB-EGF (figure 5.10). It is known that MCP-1 is responsible for the recruitment and infiltration of monocytes to the developing lesion. However, as the monocyte matures into a tissue macrophage and the MCP-1 receptor is downregulated (Phillips et al., 2005) it could be suggested that HB-EGF and the EGFR are involved in the directed migration of macrophages within the plaque perhaps to areas of high lipid content where activated macrophages already exist, which elaborate HB-EGF. Importantly, the monoclonal antibody ICR62 seemed to inhibit the migration of peripheral blood-derived monocytes in response to HB-EGF (figure 5.11) although a more appropriate isotype control antibody should be used to confirm this result. Perhaps this could provide a novel therapeutic avenue in the prevention or treatment of atherosclerosis.
7.1 Future Work

As the expression of the EGFR has only clearly been demonstrated on the THP-1 cell by flow cytometry it would be valuable to investigate routes in which the EGFR could be upregulated on the surface of peripheral blood-derived monocytes and macrophages. This thesis has clearly demonstrated that receptors are present on these cells through functional studies but it is unfortunate that they cannot be ‘seen’ to be expressed. Incubation of peripheral blood-derived monocytes and macrophages with IFNγ, IL-1β, LPS and HB-EGF or combinations of them, could result in the upregulation of EGFR gene and protein expression from which signalling pathways could be elucidated. Further studies into the functionality of the EGFR could involve investigating the phosphorylation status of the receptor following exposure to the different ligands.

Immunocytochemical analysis should also be carried out to determine the localisation of the receptors both in blood monocytes and monocyte-derived macrophages. This would provide a clear understanding as to the location of the receptors in mature macrophages and whether they are also localised within the nucleus as seen in differentiated THP-1 cells. Confocal microscopy could be utilised for this as it enables visualisation deep within cells to create sharply defined optical sections from which three-dimensional images can be created. Radioligand binding assays could also be attempted to investigate receptor number and ligand affinity on all monocyte cell types. Perhaps the expression of the other receptor family members should be investigated. After all, smooth muscle cells will proliferate and migrate in response to HB-EGF binding to the EGFR, but only migrate in response to HB-EGF binding to ErbB4 (Elenius et al., 1997). Peripheral blood-derived
monocytes and monocyte-derived macrophages should also be investigated for their proliferation or apoptosis following treatment with the ligands and the inhibitory antibody ICR62. Inhibition of the receptor at the mRNA level could be achieved with siRNA techniques in order to ascertain the basal functionality of the receptor within THP-1 cells and peripheral blood-derived monocytes and macrophages.

EGF receptor expression could also be investigated in lipid laden foam cells. This may provide an insight into the functioning of the macrophage in an advanced plaque scenario. Activation of the EGFR by different ligands may result in the gene expression of other important players in the advanced atherosclerotic environment such as MMPs. It could be interesting to investigate whether the EGFR is involved in the apoptosis of macrophage foam cells which would of course lead to the development of the necrotic core of the lesion.

Other receptors involved in the progression of atherosclerosis are known to transactivate the EGFR such as the PDGF receptor (Saito et al., 2001) and the G-protein coupled receptors (GPCRs) for thrombin (Kanda et al., 2001), endothelin (Iwasaki et al., 1998) and angiotensin II (Ushio-Fukai et al., 2001).

Since discovering that the EGFR co-localises with macrophages within human atherosclerotic lesions of patients undergoing vascular surgery for advanced atherosclerotic lesions the next stage should be to investigate the expression within plaques at different stages of progression. Clinical samples of this type are usually obtained during autopsy. These would give an insight into the role the EGFR has on
the monocyte or macrophage throughout the progression of the disease and also the location of expressing macrophages within the plaque environment.

An extension to the study performed by Lamb et al., in 2004 could be carried out. Injection of the inhibitory antibody ICR62 into the cholesterol-fed rabbit could provide \textit{in vivo} evidence that the progression of athererosclerosis does depend in part on the EGFR and its family of ligands. Inhibition of the EGFR on monocytes and macrophages could result in a decrease in the migration or proliferation of these cells and thereby potentially halt or decelerate the development of the atherosclerotic plaque.
References


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Appendix

Publications


- *Young Investigator Award Finalist*


MATERIAL REDACTED AT REQUEST OF UNIVERSITY