Journal of Immunological Methods 400–401 (2013) 45–57

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Analysis of the effects of stromal cells on the migration of lymphocytes into and through inflamed tissue using 3-D culture models ☆

Hannah C. Jeffery [a], Christopher D. Buckley [b], Paul Moss [c], G. Ed. Rainger [a], Gerard B. Nash [a], Helen M. McGettrick [b,*]

[a] School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK
[b] School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK
[c] School of Cancer Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK

ARTICLE INFO

Article history:
Received 28 May 2013
Received in revised form 19 September 2013
Accepted 8 October 2013
Available online 16 October 2013

Keywords:
Endothelial cells
Leukocytes
Adhesion
Migration
Inflammation
Fibroblasts

ABSTRACT

Stromal cells may regulate the recruitment and behaviour of leukocytes during an inflammatory response, potentially through interaction with the endothelial cells (EC) and the leukocytes themselves. Here we describe new in vitro methodologies to characterise the effects of stromal cells on the migration of lymphocytes through endothelium and its underlying matrix. Three-dimensional tissue-like constructs were created in which EC were cultured above a stromal layer incorporating fibroblasts either as a monolayer on a porous filter or dispersed within a matrix of collagen type 1. A major advantage of these constructs is that they enable each step in leukocyte migration to be analysed in sequence (migration through EC and then stroma), as would occur in vivo. Migrated cells can also be retrieved from the constructs to identify which subsets traffic more effectively and how their functional responses evolve during migration. We found that culture of EC with dermal fibroblasts promoted lymphocyte transendothelial migration but not onward transit through matrix. A critical factor influencing the effect of fibroblasts on recruitment proved to be their proximity to the EC, with direct contact tending to disrupt migration. Comparison of the different approaches indicates that choice of an appropriate 3-D model enables the steps in lymphocyte entry into tissue to be studied in sequence, the regulatory mechanism to be dissected, and the effects of changes in stroma to be investigated.

1. Introduction

During inflammation circulating leukocytes are recruited by blood vascular endothelium (EC), and migrate into the tissue where they fulfil their function in the destruction of invading pathogens and remodelling of damaged tissue. Once the trigger has been eliminated, recruited cells must be cleared to allow resolution. Uncontrolled or ineffective recruitment may be pathogenic, and thus mechanisms controlling these processes have been widely studied. Historically, leukocyte recruitment has been studied using intravital microscopy in animal models, or by in vitro modelling using isolated leukocytes and cultured EC. Based on these studies, paradigms for entry across EC, based on specific adhesion molecules, chemokines and lipids (so-called address codes), have been developed for T-cells and neutrophils (e.g. reviewed by Springer, 1995; Ley et al., 2007). In the case of lymphocytes, capture from flow by cytokine-activated EC is mainly mediated via $\alpha_4\beta_1$-integrin binding to endothelial VCAM-1, with $\alpha_L\beta_2$-integrin binding to ICAM-1 supporting transmigration (Luscinskas et al., 1995; McGettrick et al., 2009b). Signals from chemokines (which may vary depending on the inflammatory stimulus) activate the integrins to stabilise

* Corresponding author at: College of Medical and Dental Sciences, The Medical School, The University of Birmingham, Birmingham B15 2TT, UK. Tel.: +44 121 414 4043; fax: +44 121 414 6919.
E-mail address: h.m.mcgettrick@bham.ac.uk (H.M. McGettrick).

0022-1759/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jim.2013.10.004

the initial interactions (e.g. McGettrick et al., 2009b; Piali et al., 1998), while a downstream signal from prostaglandin D_2 promotes efficient transendothelial migration (Ahmed et al., 2011).

Less is known about the mechanisms regulating onward migration of leukocytes into tissue and their subsequent behaviour. Intravital and in vitro studies have indicated that T-cells and neutrophils receive signals during transendothelial migration, causing subsequent migratory behaviour and use of adhesion molecules to be modified (Smith et al., 1988; Dangerfield et al., 2002; Burton et al., 2011; Ahmed et al., 2011). Nevertheless, in vitro, lymphocytes appear reluctant to migrate away from the sub-endothelial space into collagen matrix even after hours (Brezinschek et al., 1995; McGettrick et al., 2009a), and they may require additional signals from the tissue stroma to drive efficient penetration (McGettrick et al., 2010). Indeed, it has become increasingly clear that the local stromal environment regulates leukocyte recruitment by endothelial cells (reviewed by McGettrick et al., 2012). For example, we demonstrated that 'transformed' tissue stromal cells with characteristics linked to chronic inflammation (e.g., secretory smooth muscle cells or fibroblasts from rheumatoid joints) could potentiate leukocyte recruitment, but that normal fibroblasts could down-regulate recruitment (McGettrick et al., 2009b; Rainger and Nash, 2001).

The above studies have mainly shown the effects of stroma on the initial stages of capture and have revealed less about what happens after, or the mechanisms which control migration through the tissue. This is due, in part, to the lack of amenable 3-dimensional experimental models incorporating EC, stromal cells and interstitial matrix. Since signals received at each stage in the migration process appear to condition leukocytes for the next step, we believe that it is necessary to develop integrated models where leukocytes pass through vascular EC into interstitium containing stromal cells, rather than to study each phase separately, as has been done in much previous work on interaction of leukocytes with stroma (reviewed by McGettrick et al., 2012). Here we describe development of such models. We compared different constructs incorporating human endothelial cell monolayers, gels of collagen type I (the predominant protein of interstitium) and dermal fibroblasts, for their utility in studying lymphocyte behaviour. As expected, fibroblasts modified adhesion to the endothelial monolayer and migration through it, but they could also determine the subsequent efficiency with which lymphocytes penetrated the matrix and influence the rate of onward migration.

2. Methods

2.1. Isolation of human lymphocytes, fibroblasts and endothelial cells

Venous blood from healthy individuals was collected in EDTA tubes (Sarstedt, Leicester, UK) following informed consent and with approval from the University of Birmingham Local Ethical Review Committee. Peripheral blood lymphocytes (PBL) were isolated by centrifugation on histopaque 1077 followed by panning on culture plastic to remove contaminating monocytes as described (Rainger et al., 2001). Isolated cells were washed, counted using a Cellometer Auto T4 (Peqlab, Southampton, UK), and adjusted to the desired concentration in Medium 199 (M199; Gibco Invitrogen Compounds, Paisley, Scotland) supplemented with 0.15% bovine serum albumin and 35 μg/ml gentamycin (M199BSA; Sigma-Aldrich, Poole, UK).

Tissue samples from the skin were obtained from patients with rheumatoid arthritis (RA) and fibroblasts were isolated as previously described (Salmon et al., 1997). Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat inactivated foetal calf serum (FCS), 1× MEM-non-essential amino acids (100× stock), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (fibroblast medium; all from Sigma) and were used between passages 5 and 9 (McGettrick et al., 2009b).

HUVEC were isolated from umbilical cords using collagenase as previously described (Cooke et al., 1993) and cultured in M199 supplemented with 20% FCS, 1 ng/ml epidermal growth factor, 35 μg/ml gentamycin, 1 μg/ml hydrocortisone (all from Sigma) and 2.5 μg/ml amphotericin B (Gibco) (McGettrick et al., 2009b). All human tissues were obtained with informed consent and with approval from the Human Biomaterial Resource Centre (Birmingham) or NHS Staffordshire Research Ethics Committee.

2.2. Co-cultures of endothelial cells and fibroblasts on separate filters

Fibroblasts and EC were dissociated using trypsin/EDTA (Sigma) and were cultured in fibroblast medium on the inner surfaces of 24-well and 12-well 3 μm pore Transwell filter inserts (BD Pharmingen, Cowley, UK) respectively, as described (McGettrick et al., 2010) (Fig. 1A). Fibroblasts were seeded at 1.5×10^5 cells/filter and HUVEC were seeded at 1.0×10^5 cells/filter to yield confluent monolayers within 24 h. After 24 h, culture media were removed and the 24-well inserts were fitted into the 12-well inserts, with 200 μl fibroblast medium added to the surface of each filter and 1.5 ml to the lower chamber. Cells were co-cultured together for 48 h, with 100 U/ml TNF alpha (R&D Systems, Abingdon, UK) in combination with 10 ng/ml IFN gamma (Peprotech Inc., London, UK) added for the second 24 h when desired. For comparison, parallel cultures of HUVEC or fibroblasts were maintained alone on their original filters.

2.3. Co-cultures of endothelial cells and fibroblasts incorporating collagen gels

To form collagen gels, ice-cold rat-tail type 1 collagen dissolved in acetic acid (2.15 mg/ml; First Link Ltd, West Midlands, UK) was mixed with ice cold 10× concentrated M199 in the ratio 830:170 and the pH was neutralised by addition of ice cold 1 N NaOH. For each 1 ml of gel, 160 μl FCS was added, yielding a final collagen concentration of ~1.5 mg/ml. Gels were dispensed into 12-well or 6-well plates (400 μl or 1 ml respectively), allowed to set for 15 min at 37 °C and then equilibrated with fibroblast culture medium for at least 24 h.

When desired, fibroblasts were incorporated into the gel (Fig. 1B–D). Fibroblasts were dissociated as above, counted and adjusted to the desired concentration in the ice cold FCS (5×10^4 cells/64 μl for 12-well or 2×10^5 cells/160 μl for 6-well). FCS/fibroblasts were mixed with neutralised gel solution, 64 μl FCS + 400 μl gel or 160 μl FCS + 1 ml gel, before it was dispensed into 12-well or 6-well plates respectively and allowed to gel as above. For some assays, a layer of empty gel was formed on top of a gel containing fibroblasts (Fig. 1D). In this