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Comparison of the pro-inflammatory potential of monocytes from healthy adults and those with peripheral arterial disease using an in vitro culture model

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Abstract

We adapted a monocyte:endothelial cell co-culture model to investigate the pro-inflammatory potential of monocytes from patients with peripheral arterial disease (PAD). Isolated monocytes were cultured with human umbilical vein endothelial cells (HUVEC) for 24 h, after which the ability of the HUVEC to recruit flowing neutrophils was tested. Development of a usable protocol required comparisons of primary HUVEC with cells that had been passaged and/or frozen and thawed, evaluation of optimal culture media and comparison of monocytes from freshly drawn and stored blood. We found, for instance, that expansion of HUVEC was assisted by inclusion of hydrocortisone, but this agent was withdrawn before the test phase because it reduced responses of HUVEC. Using the optimal practical protocol, we found great variation in the ability of monocytes from different donors to cause neutrophil adhesion. Slightly more (∼20%) monocytes from patients with PAD adhered to HUVEC than monocytes from healthy controls, and the monocytes from PAD patients induced ∼70% greater subsequent adhesion of neutrophils. Thus, we developed a functional model of inflammatory potential usable in clinically-related studies and found that patients with PAD had circulating monocytes with greater than normal ability to activate endothelial cells.

Keywords: Endothelial cells; Monocytes; Co-culture; Leukocyte adhesion; Atherosclerosis; Peripheral arterial disease

1. Introduction

Circulating monocytes are continually recruited through vascular endothelium into tissue, where they differentiate predominantly into either macrophages or antigen-presenting dendritic cells [1,2]. The tissue resident macrophages are important regulators of leukocyte recruitment during acute inflammation, acting as a major source of inflammatory cytokines [3,4]. During atherogenesis, it has been postulated that cells of mononuclear lineage resident within the diseased artery wall play an analogous but inappropriate role [5,6]. Indeed, cells of the monocytic lineage constitute the bulk of the inflammatory infiltrate of the earliest recognised arterial lesion, the fatty streak, and also contribute to the cellular infiltrate of the more complex lesions that are associated with pathology [7]. Studies using in vitro co-culture with endothelial cells (EC) have revealed the potent pro-inflammatory potential of monocytes [6,8–10]. Adhesion receptors become upregulated on EC, in part at least through action of tumour necrosis factor-α (TNF) released by the monocytes [10], and an ability to capture flowing leukocytes of all major classes is imparted [6,9,11]. The concept arises, therefore, that monocytes accumulating within the intima of diseased arteries generate inflammatory agents that establish a self-perpetuating cycle of EC activation and leukocyte recruitment [6].

Based on the foregoing, we wondered whether variation in the properties or behaviour of monocytes in human

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disease could influence the formation or progression of atherosclerotic, or indeed other inflammatory disease, and if so, whether manipulation of such behaviour could offer a route to novel therapy. As a starting point, we wished to investigate whether the pro-inflammatory potential of circulating monocytes was greater in patients with peripheral arterial disease (PAD) arising from arteriosclerosis affecting the lower limbs, compared to age-matched healthy controls. Direct demonstration and investigation of the role of monocytes in human disease is difficult. We decided to adapt the co-culture model used previously [6,9] so that it might be used reliably in a relatively high-throughput clinical study. In the existing model, EC were cultured in glass capillaries until confluent, seeded with monocytes of interest, and 24 h later, ability of the EC to support adhesion and migration of neutrophils was tested. To adapt the method, it was necessary to evaluate effects of passaging, freezing and thawing the EC, and of storage of blood used for monocyte isolation. Having developed a practical method, we went on to compare the ability of monocytes from patients and controls to induce endothelial cells to recruit flowing neutrophils. While the assay cannot be considered as a direct model of atheroma (in which the part played by neutrophils may not be great), it does provide a functional readout of ability to activate endothelium, that is likely to be relevant to this and other inflammatory processes. To our knowledge, no study of comparable complexity has been achieved with human tissue before.

2. Methods

2.1. Blood withdrawal

Venous blood was collected into citrate-phosphate-dextrose-adenine (CPDA; concentrate diluted (1:10) in blood to give final concentrations of 20 mM D-glucose, 11 mM citric acid, H_2O, and 0.2 mM adenine; pH 7.4), into EDTA-tubes (final EDTA concentration 1.6 mg/ml; Sarstedt, Leicester) or into a plastic universal tube without anticoagulant. The last was allowed to coagulate for 60 min and serum was retrieved after centrifugation at $800 \times g$ for 10 min.

2.2. Isolation of monocytes or neutrophils

Monocytes were isolated as described [6]. One millilitre dextran (MW 500 kD, 6%, w/v in phosphate-buffered saline, PBS; Sigma Aldrich, Poole, UK) was added to 10 ml EDTA blood, the tube was placed at an angle of 45° from the vertical, and red cells were allowed to sediment for 1 h. The leukocyte-rich supernatant was retrieved and placed on top of 3 ml Nycoprep 1068 medium (Amersham Biosciences, Chalfont St. Giles, UK), and centrifuged at $400 \times g$ for 15 min. The plasma-rich top was discarded and the middle layer which was rich in monocytes was decanted, leaving behind a residual pellet containing neutrophils, lymphocytes and red blood cells. The monocytes were washed twice in Ca^{2+}/Mg^{2+}-free PBS/bovine serum albumin (0.15% culture tested BSA; Sigma), resuspended in Medium199 (M199; Invitrogen, Paisley, UK) containing 20% autologous serum and counted using a Coulter Multisizer II (Coulter Electronics, Luton, UK). This device yields a cell volume frequency distribution which allows analysis of the percentage contamination of the monocyte preparation with other smaller leukocytes.

Neutrophils were isolated from EDTA blood within 1 h of withdrawal, using two-step density gradients of Histopaque 1119 and 1077 (Sigma), as described [12]. Cells were washed twice in PBS containing 1 mM Ca^{2+}, 0.5 mM Mg^{2+}, 0.15% BSA and 5 mM glucose (PBS/BSA), and adjusted to 10^6 ml^{-1} in the same medium.

2.3. Culture of endothelial cells in microslides

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins as previously described [13]. Primary HUVEC were cultured in M199 containing 20% fetal calf serum (FCS), 28 μg/ml gentomycin, 1 μg/ml hydrocortisone and 10 ng/ml epidermal growth factor (all from Sigma) until confluent (approximately 4–6 days). The HUVEC from individual confluent 25 cm^2 flasks were detached using trypsin/EDTA (Sigma), re-seeded in two flasks (first passage), and cultured until confluent. This process was repeated to obtain expanded second and third passage cultures. When desired, confluent HUVEC at chosen passage were detached, centrifuged and suspended in a mixture of FCS (90%) and DMSO (10%). Aliquots equivalent to one 25 cm^2 flask were slow frozen at −80 °C overnight and then transferred to liquid nitrogen until use.

For assays, HUVEC were cultured in glass capillary tubes 5 cm long, with a rectangular cross-section of 3 mm × 0.3 mm (microslides; Camlab Ltd., Cambridge, UK). HUVEC were detached from flasks or a frozen aliquot was rapidly defrosted in a water bath at 37 °C, and cells were seeded and cultured in the microslides as previously described [13,14]. Seeding was at a density yielding confluent monolayers within 24 h.

2.4. Stimulation of HUVEC by co-culture with monocytes or by TNF

Monocytes were co-cultured with the HUVEC as described [9]. Microslides containing confluent HUVEC were injected with 50 μl of isolated monocytes adjusted to 3×10^6 ml^{-1}, yielding a ratio of monocytes to endothelial cells of 1:1 (based on a typical endothelial cell surface density of 1000 mm^{-2}). After incubation for 1 h at 37 °C to allow monocyte adhesion, non-adherent monocytes were washed out with culture medium. The microslides were placed on a phase-contrast, video-microscope and recordings made of a series of eight fields along the centreline for evaluation of the number of adherent monocytes (see below). Microslides were