Technical Bulletin #457

Optimized Chemotaxis Conditions for Primary Blood Monocytes or THP-1 Cells Using Corning® FluoroBlok™ 96-Multiwell Insert Plates

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Introduction

Recruitment of monocytes from peripheral blood into tissues represents a critical step in vascular injury and inflammation. Trafficking of monocytes to their final destination requires activation of locally released integrins and chemokines, and depends on chemotactic gradients and specific expression of adhesion molecules, which are regulated by inflammation. In particular, monocyte chemoattractant protein 1 (MCP-1) has an important influence on monocyte migration into sites of inflammation.[1]

In vitro chemotaxis assays can provide valuable insight into the progression of inflammation by identifying factors that regulate directional migration of leukocytes. In the current study, we used Corning FluoroBlok 96-Multiwell Insert Plates (Cat. No. 351161 or 351162) coated with human fibronectin (hFN) (Cat. No. 354008) to optimize chemotaxis of monocytes in response to MCP-1. For this, we used both freshly isolated monocytes from peripheral blood and a monocytic cell line, THP-1, which mimics many of the properties of monocyte chemotaxis.

Materials and Methods

Cell Culture and Reagents

THP-1 cells (ATCC) were cultured in complete RPMI medium (RPMI 1640 containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 10% FBS).

Corning Fluorescent Dyes

Corning Fluorescent Dyes are available for labeling cells when performing chemotaxis assays, tumor cell invasion, angiogenesis assays, and other cell-based assays. For additional information about Corning Calcein AM (Cat. Nos. 354216 and 354217) and Corning $DiIC_{12}(3)$ (Cat. No. 354218), visit our website at: **www.corning.com/lifesciences**.

Monocyte Isolation from Leukopacks

Primary blood monocytes (PMBC) were obtained from the lymphocyte layer (buffy coat) of peripheral blood from normal donors. Each leukopack (Interstate Blood Bank) was diluted with an equal volume of PBS, and 35 ml of blood preparation was over-laid onto 15 ml of Ficoll-Paque™ PLUS (GE Healthcare). Tubes were centrifuged at 700 x *g* without breaks for 30 minutes at room temperature. The buffy coat interface was removed, added to 40 ml of PBS, and centrifuged at 300 x *g*. Any remaining erythrocytes in the cell pellet were lysed by incubating cells in 10 ml of red blood cell lysis buffer (R&D Systems) for 10 minutes at room temperature. Following lysis 40 ml of PBS was added and the cells were centrifuged for 5 minutes at 200 x *g*. The pellet was washed once with PBS and resuspended in complete RPMI and seeded onto a Falcon® 100 mm tissue culture dish (Cat. No. 353003). After one hour, the media was aspirated and the adherent cells (predominantly monocytes) were harvested using a cell scraper and used in chemotaxis assays.

Chemotaxis Assay

Chemotaxis was assayed in 96-well plates with Corning FluoroBlok Multiwell Inserts with 3.0 µm pores (Cat. No. 351161 or 351162) that were coated with various concentrations of hFN. Briefly, THP-1 cells or freshly isolated monocytes were resuspended in chemotaxis assay buffer (HBSS supplemented with 0.1% BSA) at a density of 2 x 10^6 cells/ml. Cells were labeled with 1.5 µM calcein AM or CellTracker™ Orange (Life Technologies) for 30 minutes at 37°C, 5% CO_2. Following incubation, cells were washed once and resuspended in assay buffer at a density of 0.2-2 x 10^6 cells/ml. Labeled cell suspension was added onto inserts (50 µl/well) and set aside. In a separate Falcon 96-Square well, flat-bottom plate (Cat. No. 353928), 200 µl of chemoattractant was added. The multiwell insert containing cells was gently lowered into the plate containing chemoattractants and immediately placed into a bottom fluorescence plate reader (Victor2™, PerkinElmer). Fluorescence emitted from cells that had migrated to the bottom surface of the insert was measured at various time points. Cells labeled with calcein AM were read at 485/530 nm (Ex/Em) wavelength and CellTracker Orange-labeled cells were read at 530/572 nm.

Results and Discussion

Effect of hFN Coating Concentration on THP-1 Chemotaxis

We tested THP-1 chemotaxis on various concentrations of hFN-coated Corning® FluoroBlok™ 96-Multiwell Insert Plates (Cat. No. 351161 or 351162) (***Figure 1***). The highest concentration of hFN coating tested facilitated an increase in MCP-1-induced cellular migration (2.7-fold over baseline), whereas, its effect on RANTES-induced migration was minimal (1.4-fold). At lower concentrations, hFN did not significantly increase cellular migration in response to either MCP-1 or RANTES.

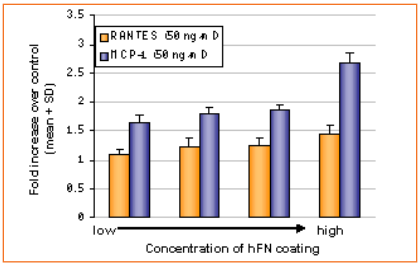

Figure 1. *The effect of various concentrations of hFN coating on the inner surface of Corning FluoroBlok 96-Multiwell Inserts was tested to determine the best conditions for THP-1 chemotaxis in response to RANTES and MCP-1. Fold increase over control depicts a ratio of RANTES- or MCP-1-induced chemotaxis as compared to cells treated with assay buffer alone. Each bar on the graph represents a mean ± SD of four replicate wells.*

Comparison of THP-1 and Primary Monocyte Chemotaxis on Corning BioCoat™ Angiogenesis System: Endothelial Cell Migration 96-Multiwell Insert Plates

Figure 2 illustrates a comparison of THP-1 and monocyte chemotaxis in response to a range of MCP-1 concentrations on Corning BioCoat Angiogenesis System: Endothelial Cell Migration 96-Multiwell Insert Plates (Cat. No. 354147 or 354148). The inner surface of Corning FluoroBlok membrane inserts in these plates is evenly coated with an optimal concentration of hFN. MCP-1 induced a similar concentration-dependent migration response in both THP-1 and primary monocytes. Maximal chemotaxis was observed with 25 nM MCP-1, resulting in a 3.3-fold increase of THP-1 migration, and a 2.3-fold increase in primary monoctye migration. In the same experiment, 5% FBS was also tested for its ability to stimulate migration of THP-1 and monocytes. However, FBS failed to induce a detectable increase in chemotaxis of either cell type (data not shown). This observation suggests that the increase in cellular migration observed in response to MCP-1 is indeed directional chemotaxis rather than a general increase in cellular motility. The background, or baseline counts, of cells treated with assay buffer alone was higher for monocytes compared to THP-1. This may be attributable in part to the difference in cell size (THP-1 cells are larger than primary monocytes).

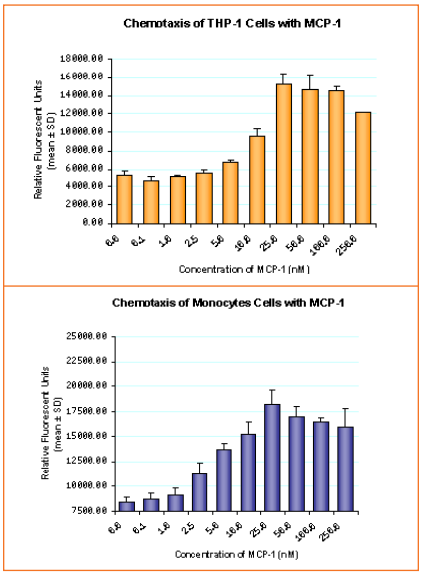

Figure 2. *MCP-1 induced chemotaxis in THP-1 and monocytes. Cells pre-labeled with 1.5 µM calcein AM were incubated with varying concentrations of MCP-1 or assay buffer for 20 minutes. The data presented in the graphs are representative of two independent experiments. Each bar on the graph represents mean ± SD of four replicate wells.*

Time-study for Optimal Measurement of THP-1 and Monocyte Chemotaxis

The effect of 25 nM MCP-1 on migration of pre-labeled THP-1 and primary monocyte chemotaxis was determined at varying time points (***Figure 3***). The highest signal-to-ratio for THP-1 chemotaxis was achieved at approximately 25 minutes following exposure to the chemoattractant in the bottom chamber. Further incubation led to a steady decline in the signal-to-noise ratio. A likely explanation for this observation is that THP-1 cells, which are known to detach easily, fell off the bottom surface of the insert membrane and scattered in the well below following migration. This was confirmed using an inverted fluorescent microscope. Monocytes on the other hand, remained adhered to the bottom of the insert following migration. However, with prolonged incubation, a general decrease in signal-to-noise ratio was observed for both cell types.

Comparison for Cell Seeding Density for Optimal Chemotaxis of THP-1 and Primary Monocytes

To identify an optimal number of cells to use in the monocytic chemotaxis assay, we tested several cell seeding densities (***Figure 4***). Maximal signal-to-noise ratio for THP-1 was observed with 50,000 cells/well, whereas for monocytes, 100,000 cells/well resulted in a more robust chemotaxis response.

Optimal Concentration of Calcein AM

We compared the effect of various concentrations of calcein AM and CellTracker™ Orange (5 nM, 100 nM, 1.0 µM, and 5.0 µM) for pre-labeling THP-1 cells prior to performing chemotaxis studies (***Figure 5***). A positive migration signal with CellTracker Orange could not be detected. The highest signal-to-noise ratio was observed with 100 nM calcein AM, however, this ratio began to decline very rapidly after 20 minutes. With 1.0 µM calcein AM, the signal-to-noise ratio was ~2.8-fold and more stable over time. Using concentrations higher than 1.0 µM calcein AM resulted in increasing background counts but not the overall signal-to-noise ratio.

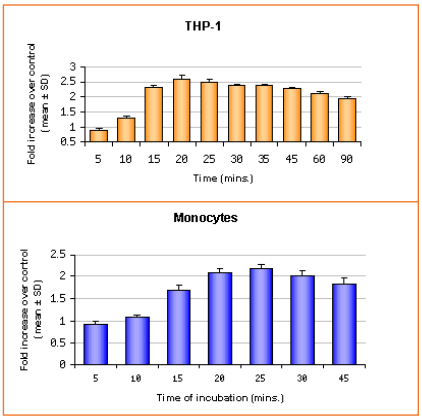

Figure 3. *Time course of MCP-1 induced chemotaxis in THP-1 and monocytes. Pre-labeled cells in the inserts were incubated with 25 nM MCP-1 in the bottom chamber. Bottom fluorescence was measured at varying time points. Data on the graph are representative of a typical experiment. Each bar represents the mean ± SD of four replicate wells.*

Summary

- MCP-1 induces directional migration of THP-1 cells and primary monocytes in a concentration-dependent manner and maximal response is observed with 25 nM MCP-1 on Corning® BioCoat™ Angiogenesis System: Endothelial Cell Migration 96-Multiwell Insert Plates (Cat. No. 354147 or 354148) containing 3.0 µm pores coated with an optimal concentration of hFN (Cat. No. 354008).
- To attain optimal signal-to-noise ratio of MCP-1-induced chemotaxis, 25-30 minutes of incubation is adequate.
- Optimal cell seeding density for monocytes was 100,000 cells/well and 50,000 cells/well for THP-1 cells.
- 0.5 to 1.0 µM calcein AM was a suitable concentration for pre-labeling cells.
- Under these assay conditions assay reproducibility was high and intra-assay variability was low (%CV is <10).

Update

- ***Figure 6*** data generated with individually coated (hFN) 24 well permeable membranes with the new (black) membrane. The fluorescence blocking range of this new membrane is 360-690 nm.

  For details, see Technical Bulletin **CLS-DL-CC-042**: *New PET Membrane for Corning® FluoroBlok™ 3.0 µm and 8.0 µm Pore Size Cell Culture Inserts*.

Reference

1. Charo, I.F. and Taubman, M.B. Chemokines in the pathogenesis of vascular disease. *Circulatory Research*, **95**:858-66 (2004).

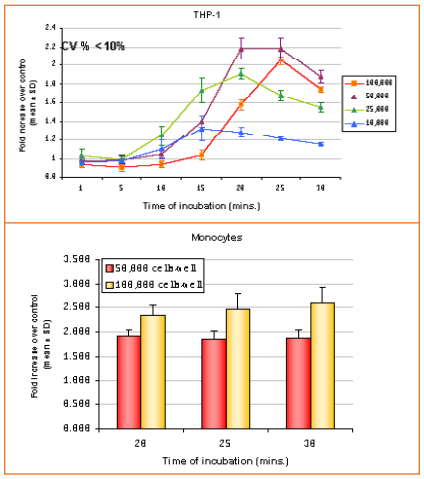

Figure 4. *Effect of cell seeding density on THP-1 and monocytes chemotaxis. Pre-labeled cells were seeded at various densities and their ability to migrate towards 25 nM MCP-1 was measured at different time points.*

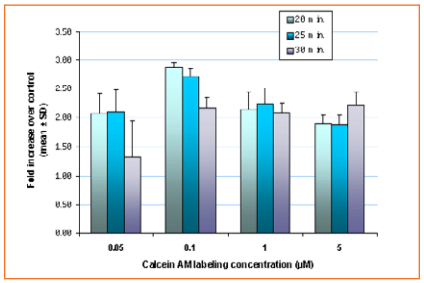

Figure 5. *Effect of calcein AM pre-labeling concentration on signal-to-noise ratio of THP-1 chemotaxis at 20, 25, and 30 minutes post-incubation with 25 nM MCP-1.*

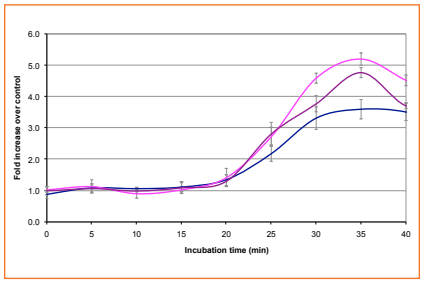

Figure 6. *THP-1 cells were seeded at 100,000 cells/well on fibronectin coated inserts and chemotaxis to 25 nM MCP-1 (R&D Systems®) was measured. Peak response time in three separate assays (n=3) was 30-35 min.*

Corning acquired the BioCoat™, Falcon®,and FluoroBlok™ brands. For information, visit www.corning.com/discoverylabware.

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