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[15] Cell-Based Assays Using Primary Endothelial Cells to Study Multiple Steps in Inflammation

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Abstract

Cell-based assays are powerful tools for drug discovery and provide insight into complex signal transduction pathways in higher eukaryotic cells. Information gleaned from assays that monitor a cellular phenotype can be used to elucidate the details of a single pathway and to establish patterns of cross talk between pathways. By selecting the appropriate cell model, cell-based assays can be used to understand the function of a specific cell type in a complex disease process such as inflammation. We have used human umbilical vein endothelial cells to establish three cell-based, phenotypic assays that query different stages of a major signaling pathway activated in inflammation. One assay analyzes the tumor necrosis factor α (TNFα)-induced translocation of the transcription factor NF-κB from the cytoplasm into the nucleus 20 min after stimulation with TNFα. Two more assays monitor the expression of E-selectin and VCAM-1, 4 and 24 h after stimulation with TNFα. Indirect immunofluorescence and high-throughput automated microscopy were used to analyze cells. Imaging was performed with the IN Cell Analyzer 3000. All assays proved to be highly robust. Z' values between 0.7 and 0.8 make each of the three assays well suited for use in high-throughput screening for drug or probe discovery.

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Cellular Mechanism of the Inflammatory Response

Chronic inflammatory disease is believed to pose a tremendous medical burden in the developed world, both in terms of patient suffering and in the cost of treatment and loss of worker productivity. The more common inflammatory diseases include rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, chronic obstructive pulmonary disease, and psoriasis. Although each disease has unique aspects regarding the affected tissues and the clinical symptoms, they all share some common biological mechanisms for the establishment and maintenance of the disease state.

Inflammatory disease requires that endothelial cells detect and amplify a proinflammatory signal. This results in the adherence, activation, and ultimately transmigration of lymphocytes at the site of damage. Drugs that block this process would significantly alleviate the symptoms of inflammation.

Some of the major signaling molecules that act on the endothelial cell include the cytokines, tumor necrosis factor α (TNFα), interleukin 1 (IL-1), and CD40L (Aggarwal, 2003; Martin and Wesche, 2002). In each case, the signaling molecule binds to a receptor on the surface of the endothelial cell and activates a cascade of internal events. A key event in the early inflammatory process is the translocation of the transcription factor NF-κB from the cytoplasm to the nucleus of the endothelial cell, an early event (20 min) after receptor stimulation (Ding *et al.*, 1998). NF-κB then activates the transcription of an array of proinflammatory proteins (Senftleben and Karin, 2002). These include proteins that regulate the attachment of lymphocytes to the wall of the blood vessel such as proteins of the selectin family (Karmann *et al.*, 1996). One of these, E-selectin, is clearly detectable on the endothelial cell surface 4 h poststimulation. At later times (24 h poststimulation), lymphocyte recruitment is also mediated by VCAM-1, a cell adhesion molecule, on the endothelial cell surface (Gorczynski *et al.*, 1996). VCAM-1 expression is dependent on activation of the MAP kinase in addition to NF-κB. Disruption of the mechanisms that regulate and limit the inflammatory response results in the development of chronic disease.

This chapter describes three assays that monitor three different phases in the inflammatory process.

Cell-Based Assays Used to Monitor the Effects of Proinflammatory Cytokines

Selection of a Suitable Cell System

Selecting an appropriate cell type is an important consideration in setting up a cell-based assay. Obviously the cell type must be a reliable model system for the underlying biology that the assay is designed to

measure, but other considerations are also essential. The cells should be readily available. Their growth characteristics and conditions (media, doubling time, etc.) should be compatible with the desired workflow and enhance assay stability. Cell morphology affects imaging and analysis; large relatively flat cells yield data that are easier to interpret. Also, any special safety risks, such as increased viral risks from human cell types, should be taken into account.

We selected a primary cell type, human umbilical vein endothelial cells (HUVEC), for these assays. These cells are well established as a model system for studying inflammation and behave similarly to cells in the context of a tissue in a human body. Although they are a primary cell type, HUVECs are readily available as well-characterized, pooled preparations. Using pooled cells from multiple individuals reduces the variation that would result from differences in individual phenotypes. Furthermore, HUVECs can be grown for multiple passages *in vitro* (Gimbrone *et al.*, 1974), which is an important consideration when planning cell-based assays that use nonimmortalized cell types. A 100,000 well screen, with a seeding density of 10,000 cells per well, consumes 1×10^9 cells. This number can be grown easily from a few vials (1,000,000 cells per vial) of frozen stocks. We routinely use cells up to passage 10 for pilot experiments and cells between passage 6 and 9 for screens. We selected a growth medium formulation that contains low serum (2%) and defined supplements to provide controlled growth conditions, thus increasing assay robustness by minimizing the impact of lot-to-lot variations of the serum. Because HUVECs are human cells, it is essential to use preparations that have been screened for pathogens.

Selection of the Optimal Probe

Selecting an appropriate probe (assay readout) is another important consideration when setting up cell-based assays. Commonly used probes include immunofluorescent detection of endogenous proteins, green fluorescent protein (GFP)-reporter proteins, and other reporter proteins. Each has advantages and disadvantages.

A major advantage of using GFP-reporter proteins is that these probes are compatible with live cell assays and allow measurements of a single well to be made at multiple time points. Alternatively, the samples can be fixed and analyzed at a later time. Because GFP reporter proteins remain fluorescent after fixation, there is no need for complex immunostaining protocols, which simplifies assay workflow and reduces cost in reagents.

A major disadvantage of using GFP-reporter proteins is that the reporter must be introduced into the cells. This is usually achieved either by using

a specially engineered cell line that stably expresses the reporter protein or by transiently introducing an expression vector (either plasmid or viral) encoding the reporter. Another disadvantage is that endogenous proteins are not monitored. GFP-reporter proteins might not faithfully represent the behavior of their endogenous counterparts. This may occur if the GFP moiety affects the proteins function, if the expression levels or stability of the GFP and native proteins are different, or for other reasons. Developing an engineered cell line or transient expression system and validating the reporter protein can be time-consuming, especially if specific expression levels are desired. High-level expression of proteins can deregulate pathways and important cellular functions, further complicating efforts to validate the reporter. Furthermore, immortalized, engineered cell lines are often highly transformed. Immortalized cell lines accumulate mutations over time in culture and may lose the full spectrum of response that is found in the parental cells, again making assay validation essential.

Immunofluorescent detection of endogenous proteins avoids many of the aforementioned disadvantages because endogenous proteins are monitored. Once a suitable antibody has been identified and its availability in sufficient quantities to complete the screen has been ensured, then assay development is usually straightforward. Because this approach minimizes manipulation of the cells, the risk of creating cell systems with new properties is relatively low. Despite these significant advantages, choosing immunofluorescent probes does have some disadvantages. Validating the specificity of primary antibodies for cell imaging is challenging because antibody cross-reactivity cannot be distinguished from the specific signal; indeed, cross-reactivity of antibodies is essentially impossible to detect based on immunomicrograph data alone. Live cell assays are almost impossible to perform using immunofluorescent probes because cells must be permeabilized as a necessary part of the immunostaining procedure. This means that kinetic studies need additional wells for each time point rather than simply monitoring a single well several times during an experiment. The availability of antibodies and batch-to-batch consistency are also factors to consider. These last two can both be more problematic when using polyclonal antibodies compared to monoclonal antibodies.

Sample processing (fixation, permeablization, and staining) for assays that use immunofluorescent probes is also more complicated than for GFP-reporter assays and includes multiple liquid-handling steps. These extra steps add to the cost and labor needed to screen an assay at high throughput.

Based on the relative difficulty of transiently transfecting primary cell types, such as HUVEC, and on our desire to model the behavior of

endogenous human endothelial cells as closely as possible, we decided to use immunofluorescent probes in this set of assays. An alternative possibility that we considered was to use a viral system, such as recombinant retro- or adenoviruses, to introduce reporter proteins. However, this would have necessitated additional investment of time and labor in the development and optimization of viral delivery. It would also have added a level of complexity, as, in addition to obtaining specific and constant levels of expression of the target, a homogeneous level of infection among the target cells would need to be ensured. This is especially important for phenotypic cell-based assays (compared to biochemical assays), as the number of individual cells analyzed is limited; typically fewer than several hundred cells are captured in one image. In order to partially alleviate the drawbacks associated with sample processing for immunofluorescent assays, we have developed a standardized protocol (described later) designed to minimize liquid-handling steps compared to conventional staining protocols.

Materials and Methods Common to All Three Assays

We have developed standardized cell culture and postfixing staining procedures for the three assays described in this chapter. This section describes these standardized procedures.

Cell Culture

HUVECs are obtained as frozen stocks (Cambrex). Cells are grown in EGM-2 medium with supplements (Cambrex). To passage cells, the monolayer is washed first with PBS (HyQ HyClone) and then with a trypsin/EDTA solution (0.05% trypsin/0.02% EDTA in PBS). Cells are detached by incubation with 5 ml of trypsin per T175 flask, resuspended in 20 ml of DMEM containing 10% fetal calf serum (Cambrex), and centrifuged for 5 min at 800*g* (Beckman CS 6KR centrifuge). Cells are resuspended, seeded at dilutions of 1:3, and grown until they are confluent. Experiments are performed with cells kept in culture up to passage 10. Cells are grown at standard conditions (37°, 5% CO_2, and 100% and humidity) in a Nuaire 12 Autoflow Incubator (Nuaire).

Cell Seeding and Stimulation

For phenotypic assays, HUVECs are seeded at 10,000 cells per well in 96-well ''ViewPlates'' (Perkin Elmer) and grown for 24 h under standard conditions. Proinflammatory signaling is initiated by removing the growth medium and replacing it with 100 μl prewarmed medium containing the desired cytokine, either TNFα (R&D Systems) or IL1 (R&D Systems).

Compound Addition

To test the effects of various compounds on proinflammatory signaling, we seeded cells in view plates, grew them for 16 h, and then replaced the growth medium with 100 μl prewarmed medium containing the desired compound. The cells are preincubated in the presence of compound for a known time: 3 h 40 min for NF-κB translocation assay or 1 h for E-selectin and VCAM-1 assays. Then proinflammatory signaling is initiated by the addition of cytokine, directly to the medium (1 μl of 1 μg/ml TNFα). The incubation is continued for a further known time: 20 min for NF-κB , 4 h for E-selectin, and 24 h for VCAM-1. Then the cells are fixed and stained (described later). All tested compounds are first dissolved as stock solutions in DMSO at a sufficiently high concentration to ensure that the final concentration of DMSO in the assay does not exceed 0.1% (v/v). This concentration of DMSO does not itself affect any of the three assays.

siRNA Transfection

For assays where siRNA is used, cells are seeded at a density of 5000 cells per well in 96-well view plates and grown for 16 h before transfection. The transfection mix is assembled as follows: 100 n*M* final concentration of siRNA is added in 15 μl Opti-MEM (Invitrogen) and kept for 5 min at room temperature. In a separate tube, 0.5 μl of Oligofectamine (Invitrogen) is added to 2.5 μl of Opti-MEM. Both reactions are combined, mixed gently, and incubated at room temperature for 20 min to allow RNA/lipid complexes to form. Cells are washed once with PBS and 80 μl of Opti-MEM is added. The formed RNA/Lipid complexes are then added to the cells. After 4 h, medium is supplemented with 150 μl EGM-2 media. Cells are incubated for an additional 48 h under standard conditions before being assayed. After 48 h the medium is removed and cells are stimulated with cytokines as described earlier.

Fixing and Staining Solutions

Permeabilizing/blocking solution: 10% (v/v) newborn calf serum (Gibco/Invitrogen) and 0.2% (w/v) Triton X-100 (Sigma) in PBS (MP Biochemicals)

Primary antibody solutions: 2 μg/ml mouse IgG against p65 (Zymed) in PBS containing 10% (v/v) newborn calf serum, 2 μg/ml mouse IgG E-selectin (Chemicon MAB2150) in PBS containing 10% (v/v) newborn calf serum, and 2 μg/ml mouse IgG against VCAM-1 (SantaCruz-13506) in PBS containing 10% (v/v) newborn calf serum

Secondary antibody solution: 2 μg/ml antimouse coupled to Alexa Fluor 647 nm (Molecular Probes/Invitrogen) and 1 μg/ml Hoechst 33342 (Calbiochem) in PBS containing 10% (v/v) newborn calf serum

Fixation and Staining Protocol

Fixation

1. Wash with 200 μl PBS; aspirate.
2. Add 50 μl fix solution (4% paraformaldehyde in PBS).
3. Fix at room temperature for 20 min.
4. Aspirate fix solution and wash with 200 μl PBS; aspirate.
5. Store cells in 100 μl PBS at 4° until ready to stain.

Staining

6. Aspirate PBS from cells.
7. Wash cells with 200 μl PBS; aspirate.
8. Add 50 μl ''permeabilizing/blocking solution.''
9. Incubate at room temperature for 20 min.
10. Aspirate.
11. Add 50 μl ''primary antibody solution'' per well.
12. Incubate at room temperature for 20 min.
13. Aspirate, wash once with PBS.
14. Aspirate.
15. Add 50 μl ''secondary antibody solution.''
16. Incubate at room temperature for 20 min.
17. Aspirate and wash three times with 100 μl PBS; aspirate.
18. Store at 4° in fresh 100 μl PBS and seal until ready to scan.

Image Acquisition

The fixed cells are scanned and analyzed using an IN Cell Analyzer 3000 (GE Healthcare, USA) automated confocal microscope. View plates containing the fixed and immuno-stained cells are sealed and loaded into a Kendro Cytomat Hotel (Kendro, Germany) from which they are transferred automatically to the IN Cell analyzer 3000 by a Mitsubishi MELFA RU-2AJ arm (Mitsubishi, Japan).

The plates are imaged in two channels (blue and red); the respective images are acquired sequentially. Wells are first excited with the 647-nm laser lines, and images are recorded with the 695BP55 emission filter (red channel). The Hoechst dye is then excited with the 365-nm laser line, and images are recorded with the 450BP25 emission filter (blue channel).

A single square field, with dimensions of 0.561 mm^2, is recorded for each well. An average of 200 to 300 cells is imaged in one field. The exposure time is 1.7 ms and the binning factor is 1.

After finishing the run, data files, including images and description files (''run files''), are transferred from the IN Cell computer to a HP DL360 server (Hewlett Packard). Image analyses are performed on PC work stations using IN Cell Analyzer software (GE Healthcare). The modules and the parameters used for the assays mentioned in this chapter are described later. The text files with results of the image analyses are imported into Excel 2003 workbooks to generate graphs. Raw image data, descriptive files, and analysis results are assembled in an Open Microscopy Environment (Goldberg *et al.*, 2005).

Image Analysis

NF-κB Translocation Assay

Image analysis for the NF-κB translocation assay is done using the ''nuclear trafficking analysis'' module of the IN Cell Analyzer software. An average of 200 to 300 cells per image is analyzed. Individual cells are identified by their stained nuclei (blue channel, Hoechst 33342 staining). The NF-κB transcription factor is identified by immunostaining of its p65 subunit (red channel, Alexa Fluor 647-labeled antibody staining). Identified nuclei are used to define masks that are overlaid onto the red channel images, to define the regions to measure. Cytoplasmic measurements are made by dilating from the nuclear mask by two to three pixels to give a perimeter around the nucleus; measurements taken from this region correspond to cytoplasmic signal. Nuclear measurements are made by eroding the nuclear mask by two pixels and taking measurements. This ensures that sampling of cytoplasmic and nuclear regions is limited to areas within well-defined boundaries so edge-associated artifacts are minimized.

E-Selectin and VCAM-1 Expression Assays

Image analysis for the E-selectin and VCAM-1 expresssion assays is done using the ''object intensity module'' of the IN Cell Analyzer 3000 software. This module uses two color channels: one for the object ''marker'' and another for the ''signal.'' First, the algorithm identifies all potential objects in the marker channel (the blue channel showing cell nuclei is used for these assays). Then size or intensity filters are applied and a mask is generated from the qualifying objects. Next, the algorithm establishes a measurement region by dilating the object masks by a specified number of

pixels. The masks are then overlaid onto the signal channel (red in these assays) and measurements are taken.

Statistical Analysis

For data presented here, an average value from four wells is used to calculate each data point. Error bars show standard deviations. Z' factors are calculated as described (Zhang *et al.*, 1999), according to the formula:

$$Z' = 1 - [(3 \times \text{SD of the positive controls} + 3 \times \text{SD of the negative controls})/(\text{mean value from the positive controls-mean mean value from the negative controls})]$$

Assay Data

NF-κB Assay

Assay Principle. Cytokines such as TNFα and interleukin 1 (IL-1) activate a proinflammatory response in endothelial cells. An early event in this response is nuclear translocation of the NF-κB transcription factor. This translocation causes the transcription of proinflammatory genes to be induced (Li and Stark, 2002; Pober, 2002). The nuclear translocation of NF-κB in stimulated primary HUVECs therefore provides a readout that can be used to study the effects of bioactive compounds on the responsiveness of endothelium to proinflammatory stimuli.

NF-κB is a heterodimer, composed of p65 and p50 subunits. Nuclear translocation is monitored by immunolocalization of the p65 subunit of NF-κB in fixed cells and is quantified by measuring the ratio of nuclear to cytoplasmic NF-κB signal (Fig. 1A).

The NF-κB Translocation Assay Produced Data with High Confidence. Figure 1 shows a representative result for HUVECs treated with TNFα (10 ng/ml) for 20 min, then analyzed by immunofluorescence for the translocation of the p65 subunit of NF-κB. Almost 100% of treated cells showed translocation of NF-κB from the cytoplasm to the nucleus under these conditions. Similar results were obtained with IL-1 (data not shown).

Time course experiments showed that NF-κB translocation was maximal 20 min after stimulation by either TNFα or IL-1 (Fig. 2A). Both of these cytokines caused a dose-dependent stimulation of NF-κB translocation in HUVECs. The nuclear-to-cytoplasmic ratio of NF-κB concentration increased up to sixfold after stimulation (Fig. 2B). Using TNFα as an activator, the Z' value of this assay was 0.79.

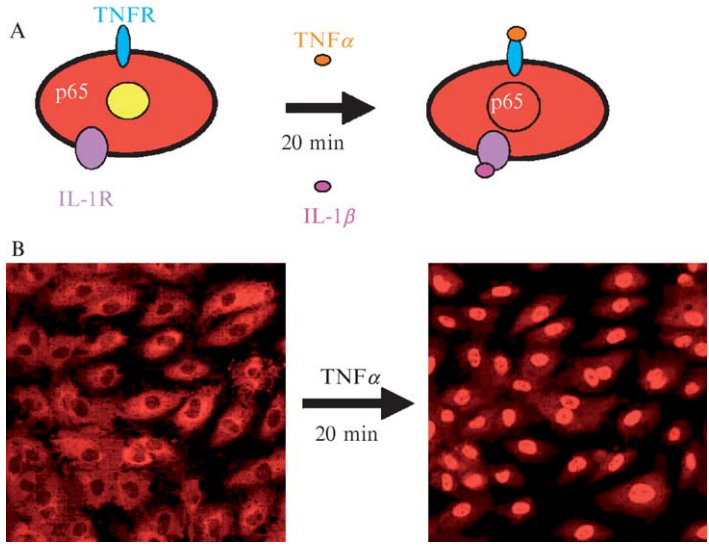

FIG. 1. Nuclear translocation of NF-κB. (A) Translocation of the dimeric transcription factor NF-κB was monitored by immunostaining for the p65 subunit. (B) HUVECs were stimulated with TNFα as described in the text. To detect distribution of the transcription factor, cells were fixed and permeabilized and then incubated with a monoclonal antibody against the p65 subunit of the NF-κB transcription factor and secondary antimouse IgG antibodies coupled to Alexa Fluor 647. Images of nonstimulated (left) and TNFα-stimulated (right) cells.

IL-1 and TNFα Use Different Receptors to Induce NF-κB Translocation

The cytokines IL-1 and TNFα activate different receptors and different upstream arms of the proinflammatory pathway to induce NF-κB translocation (Fig. 3). To prove the specificity and thereby the biological relevance of our assay, we used siRNA technology to demonstrate that the two different cytokines use their natural, physiologically relevant receptors to activate NF-κB. The sequence selection of small double-stranded siRNAs used in these experiments was as described previously (Jagla *et al.*, 2005). Specific knockdown of the receptor for TFNα (TNFR1), by transfecting cells with specific siRNAs 48 h before stimulation, significantly reduced NF-κB translocation in response to TFNα. In contrast, knockdown of the TNFα receptor did not affect IL-1-stimulated NF-κB translocation.

NF-κB Translocation Depends on IκB Kinase (IKK) and Proteasome Activity

We tested the effects of three drugs: curcumin, sulindac sulfide, and RO106–9920. Both curcumin and sulindac target the IKK complex, which

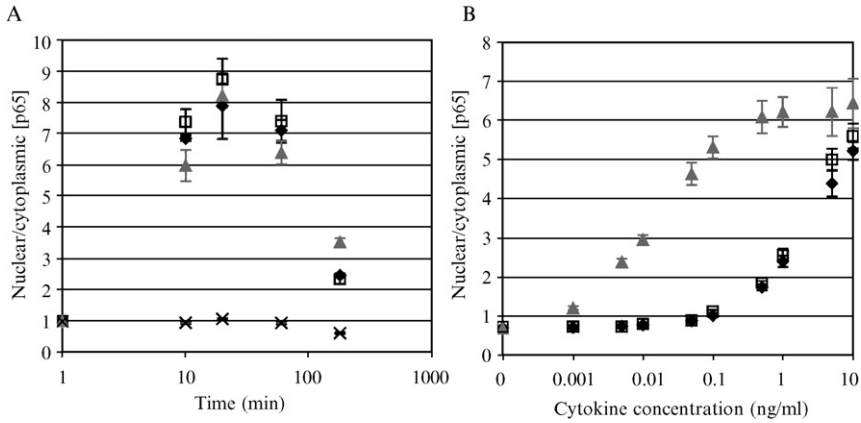

FIG. 2. TNFα and IL-1 induce p65 nuclear translocation. (A) Time course. HUVECs were stimulated with 10 ng/ml TNFα (gray triangles), 20 ng/ml IL-1α (black diamonds), 20 ng/ml IL-1β (open squares), or 1 μg/ml bovine serum albumin (crosses) for the indicated times. Cells were treated as described for Fig. 1B. p65 distribution was calculated as the ratio of nuclear to cytoplasmic staining. (B) Dose response. HUVECs were stimulated (20 min) with the indicated concentrations of TNFα (gray triangles), IL-1α (black diamonds), or IL-1β (open squares). Cells were treated as described for Fig. 1B. p65 distribution was calculated as the ratio of nuclear to cytoplasmic staining.

is a major regulator of the NF-κB pathway (Yamamoto *et al.*, 1999). RO106-9920 blocks an E3-ubiquitin ligase, which has been implicated in translocation of NF-κB (Swinney *et al.*, 2002). All three of these drugs caused a clear dose-dependent inhibition of cytokine-induced NF-κB translocation (Fig. 4). While NF-κB translocation is dependent on IKK kinase activity, as shown by curcumin and sulindac sulfide inhibition data, the process is not dependent on p38 MAP kinase activity. We have tested several p38 inhibitors, but none showed an inhibitory effect on translocation (data not shown).

E-Selectin Assay

Assay Principle. A long-term response to TNFα and other proinflammatory cytokines is the transcription of proinflammatory genes (Li and Stark, 2002; Pober, 2002), such as E-selectin. Cell surface expression of E-selectin following stimulation with cytokines in HUVEC cells provides readout to study the effects of bioactive compounds on the transcriptional activity regulated by proinflammatory stimuli (Fig. 5A).

Figure 5B shows a representative result for HUVECs treated with TNFα (10 ng/ml) for 4 h and then analyzed for expression of E-selectin.

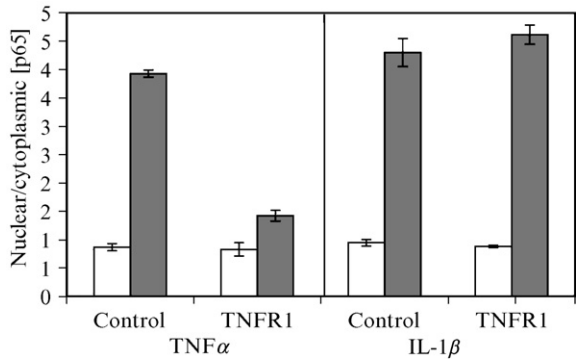

FIG. 3. IL-1 and TNFα use different receptors to induce translocation. HUVECs were seeded and stimulated with 10 or 20 ng/ml IL-1β for 20 min, as described in the text. Where indicated, cells were also treated with siRNA against the TNF receptor 1 (TNFR1) to silence TNFR1 expression in the cells. The distribution of p65 is expressed as a nuclear to cytoplasmic ratio of the fluorescence signal. Silencing of the TNFR1 by siRNA abolishes p65 translocation induced by TNFα but not by IL-1β. Gray bars represent TNFα (left)- or IL-1β (right)-treated cells; white bars represent nontreated control cells.

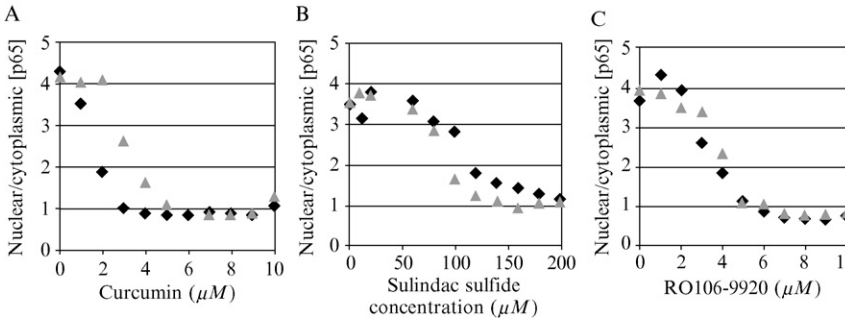

FIG. 4. Effect of compounds on NF-κB translocation. HUVECs were incubated for 1 h (black diamonds) or 4 h (gray triangles) with increasing concentrations of curcumin (A), sulindac sulfide (B), or RO106-9920 (C). Cells were then stimulated for 20 min with TNFα, and distribution was NF-κB measured as described for Fig. 1B.

A dose–response curve (Fig. 6A) indicates that E-selectin can be detected on the surface of approximately 10% of the cells in response to stimulation with as little as 1 pg/ml of TNFα. The response increases with increasing dose up to 50% positive cells at the highest dose tested (100 ng/ml). Time course experiments showed that E-selectin expression was maximal 4 h after stimulation with cytokines (data not shown).

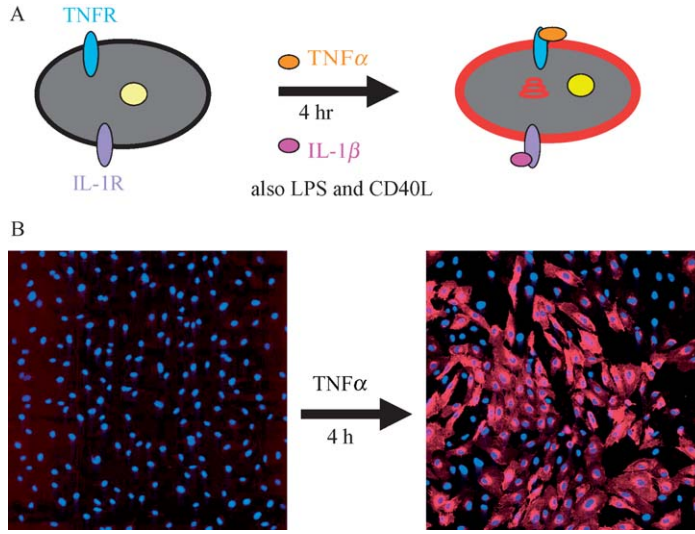

FIG. 5. Cell surface expression of E-selectin. (A) Schematic representation of the assay principle. After 4 h of stimulation with either TNFα or IL-1β, E-selectin accumulates at the cell surface. (B) HUVECs were stimulated with TNFα (10 ng/ml) for 4 h. To detect expression of E-selectin, cells were fixed and then incubated with monoclonal antibody against E-selectin and secondary antimouse IgG antibodies coupled to Alexa Fluor 647. Images of nonstimulated cells (left) and cells stimulated by TNFα for 4 h (right).

E-Selectin Expression Has Multiple Activators

Stimulation with IL-1β (Fig. 6B) results in a similar dose–response curve to that which is observed following stimulation with TNFα (Fig. 6A). A minimal response (5% positive cells) is observed after stimulation with a low cytokine concentration (30 ng/ml). This response increases with dose and plateaus at 50% positive cells following stimulation with 1 μg/ml IL-1β.

CD40L and LPS are also effective inducers of E-selectin expression but they are less potent than either TNFα or IL-1β. The dose–response relationships for CD40L and LPS are shown in Fig. 6C and D, respectively. In both cases, a smaller response is achieved than with cytokine stimulation; a maximum of 8% positive cells for CD40L and of 15% for LPS. Expression of E-selectin is dependent on IKK and proteasome activity, as demonstrated by the inhibitory effect of curcumin and RO106–9920 (Fig. 7).

Statistical analysis revealed a Z′ value of 0.77 for activation of the assay with TNFα.

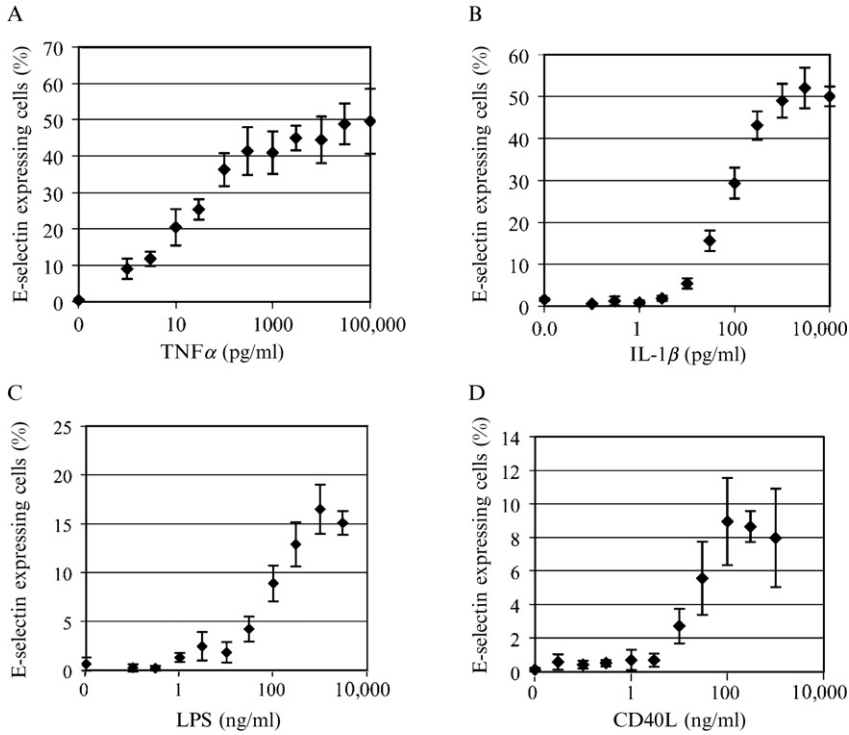

FIG. 6. Expression of E-selectin. (A) Stimulation with TNFα. HUVECs were treated with increasing amounts of TNFα. After 4 h, the cells were fixed and stained as described in Fig. 5B. Images of stained cells were analyzed using the "Object Intensity Module" of the IN Cell Analyzer software. The *y* axis shows the percentage of cells positive for E-selectin expression on the cell surface. The *x* axis shows concentration of TNFα. (B) Stimulation with IL-1β. HUVECs were treated with increasing amounts of IL-1β. After 4 h the cells were fixed and stained as described. Cells were analyzed as in A. The *x* axis shows concentration of IL-1β. (C) Stimulation with LPS. HUVECs were treated with increasing amounts of LPS. Cells were analyzed as in A. The *x* axis shows concentration of LPS. (D) Stimulation with CD40L. HUVECs were treated with increasing amounts of CD40L. Cells were analyzed as in A. The *x* axis shows concentration of CD40L.

VCAM-1 Assay

Assay Principle. Proinflammatory cytokines induce the transcription of proinflammatory genes (Li and Stark, 2002; Pober, 2002). One such protein, induced at late time points, is the vascular cell adhesion molecule-1, VCAM-1. The cell surface expression of VCAM-1 upon stimulation with cytokines in HUVECs therefore provides a model system that can be used

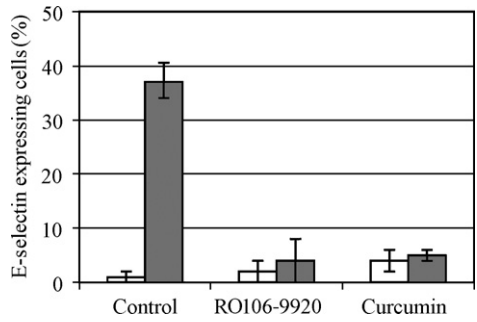

FIG. 7. E-Selectin expression is dependent on IKK and proteasome. HUVECs were treated with 10 ng/ml of TNFα and either 10 μM curcumin or 10 μM RO106-9920. After 4 h, the cells were analyzed as described in Fig. 6A.

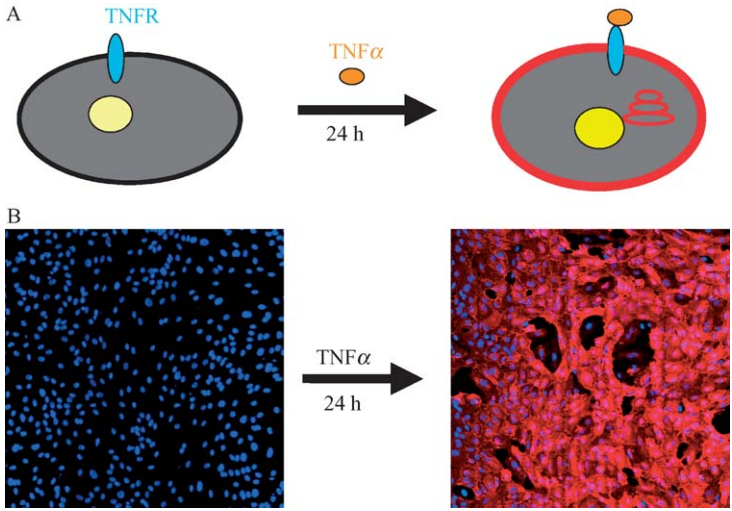

FIG. 8. Expression of VCAM-1. (A) HUVECs are stimulated with TNFα. After 24 h, VCAM-1 accumulates at the cell surface. (B) HUVECs were stimulated with TNFα for 24 h. To detect expression of VCAM-1, cells were fixed and incubated with a monoclonal antibody against VCAM-1 and secondary antimouse IgG antibodies coupled to Alexa Fluor 647. Images of nonstimulated cells (left) and cells stimulated with TNFα (right).

to study the effects of bioactive compounds on the responsiveness of endothelium to proinflammatory stimuli at late time points (Fig. 8A).

HUVECs were stimulated with TNFα for 24 h, and expression of VCAM-1 was monitored. Fixed cells were incubated with a monoclonal antibody against VCAM-1 and secondary antimouse IgG antibodies

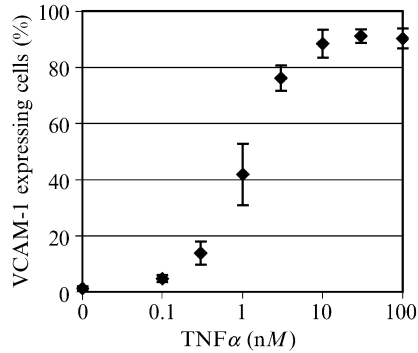

FIG. 9. TNFα induces VCAM-1 expression in a dose–response manner. HUVECs were stimulated for 24 h with the indicated concentrations of TNFα. After 4 h cells were fixed and stained as described. Images of stained cells were analyzed using the "object intensity module" of the IN Cell Analyzer. The percentage of cells showing expression of VCAM-1 was measured.

coupled to Alexa Fluor 647 (Fig. 8B). The left side of Fig. 8 shows images of nonstimulated cells, and the right side of Fig. 8 shows images of cells stimulated with TNFα.

The induction of VCAM-1 with TNFα was a robust and highly reproducible result, with a Z' value calculated at 0.8.

A dose-response curve, shown in Fig. 9, indicates that TNFα induces VCAM-1 expression in a dose-dependent manner with an EC_{50} of 2 ng/ml. The response plateaus at 5 ng TNFα with over 80% of the cells expressing VCAM-1.

The VCAM-1 Assay Can be Used to Screen for Bioactive Compounds

The expression of VCAM-1 is dependent on IKK and NF-κB translocation as tested by IKK inhibitors (data not shown). VCAM-1 expression is strongly dependent on active p38 MAP kinase activity, as tested with the compound SB202190 (a p38 MAP kinase inhibitor). SB202190 blocked VCAM-1 expression with an IC_{50} of approximately 2 μM (Fig. 10). Taken together, these results also validate our assay, as VCAM-1 induction is known to be dependent on both IKK and p38 MAP kinase pathways.

Concluding Remarks and Future Perspectives

We have shown that by using immunofluorescent probes in combination with high-speed automated microscopy and automated image analysis, one can develop robust assays in nontransformed human cells. As shown

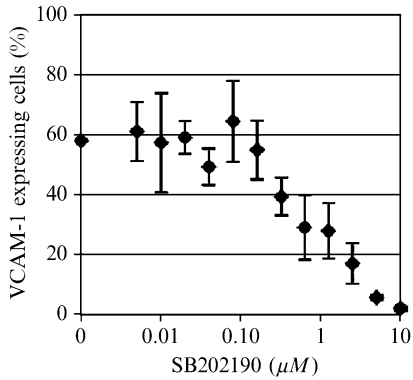

FIG. 10. p38 MAPK inhibitor SB202190 inhibits VCAM-1 expression after TNFα stimulation. HUVECs were incubated with TNFα (10 ng/ml) and increasing amounts of the MAP kinase inhibitor SB202190. After 24 h cells were fixed and stained as described. Images of stained cells were analyzed using the ''object intensity module'' of the IN Cell Analyzer. The *y* axis shows the percentage of cells positive for VCAM-1 expression.

earlier, antibodies can be applied to monitor nuclear translocation (NF-κB assay) or changes in the expression profile of target proteins (VCAM-1 and E-selectin assay).

Posttranslational modifications are often indicators of the activity status of a protein. Antibodies specific for these modifications, including Ser/Thr phosphorylation, Tyr phosphorylation, and Lys acetylation, are available for a growing number of proteins and are widely used to study signaling in mammalian cells. The challenge is to develop assays based on these antibodies that are robust enough for automated screening. Once these assays are developed they can directly monitor the activity of a target protein. Such assays will allow us to identify new small molecule-based drugs and research probes that modulate the targets activity.

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