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[39] Determination of Cell-Cell Adhesion in Response to Oxidants and Antioxidants

By SASHWATI ROY, CHANDAN K. SEN, and LESTER PACKER

Introduction

Adhesion of leukocytes to endothelial cells is the earliest step in immune recognition process and is mediated by cell adhesion molecules (CAM).[1] In the early phases of cell adhesion, leukocytes transiently adhere to the vessel wall in a process termed "rolling." Rolling of leukocytes is mediated by a family of adhesive molecules called selectins, expressed both on the leukocyte and endothelial cell surface.[2] After "rolling," leukocytes firmly

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adhere to endothelial cells. This process is mediated by the binding of lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) and very late antigens-4 (VLA-4) expressed on leukocytes to intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD 106) present on the endothelial cell surface, respectively.[3] Platelet endothelial cell adhesion molecule-1 (PECAM-1) is one of the CAM that is involved in transendothelial migration of leukocytes through the vessel wall into inflamed foci.[4] CAM expression and adhesive properties of cells are greatly modified in several conditions such as cancer, atherosclerosis, diabetes, chronic inflammation, and ischemia–reperfusion injury.[5–10] Redox imbalances have been suggested to play a critical role in the etiology of the above-mentioned diseases.

The expression of CAM are known to be induced in response to several stimuli such as cytokines (tumor necrosis factor-α, TNF-α; interleukin-1α and -1β), phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide.[3] Oxidants and antioxidants have been shown to directly or indirectly influence the expression of CAM and other cell–cell adhesion processes.[11–14]

Significance and Overview of Cell–Cell Adhesion Assays

Cell–cell adhesion assay is a powerful tool for the study of interaction between leukocytes and endothelial cells during inflammation, immunological disorders, and other disorders where altered cell adhesion processes

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are implicated in the etiology of disease.[15] Cell–cell adhesion is expected to be mediated by a large number of CAM present on leukocytes and target cells; only some such molecules are characterized at present. Thus, although study of the expression of individual adhesion molecules is informative, prediction of actual cell–cell adhesion based on such data may not always be correct. A number of different methods have been used for quantifying cell–cell adhesion. Microscopic investigation of adhered cells and radioactive (^{51}Cr or ^{3}H) labeling of leukocytes in cell adhesion assays are some of the widely used methods.[16,17] Such assays are time consuming and require individual handling of samples. Several spectrophotometric assays such as biotin labeling of leukocytes have also been reported to study cell–cell interactions.[18] Most of these techniques include several steps, and therefore chances of error are high. Since the fluorescence microtiter plate reader became available, fluorescence based techniques have proven to be useful to study cell–cell interactions.[15,19] Here we describe a simple and highly sensitive fluorescent method using calcein to estimate cell–cell adhesion in response to oxidants and antioxidants. Among the various methods available for the determination of cell–cell adhesion, fluorescence labeling of adherent cells using calcein has several advantages, particularly in studies where the roles of oxidants and antioxidants are evaluated in cell adhesion processes. Several fluorescent probes are sensitive to changes in intracellular pH, Ca^{2+}, or redox state and thus may not be effectively used to study the effect of oxidants or antioxidants. Also, loading of some of these probes to live cells may interfere with several aspects of cell function. Calcein has proven to be safe in these respects.[19]

Cells and Culture Conditions

Human Jurkat T cells clone E6-1 (American Type Culture Collection, ATCC, Rockville, MD) are grown in RPMI 1640 medium (GIBCO-BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 110 mg/liter sodium pyruvate, and 2 m*M* L-glutamine (University of California, San Francisco, CA). ECV304 (ECV), a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal

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human umbilical cord, are obtained from ATCC (Bethesda, MD). ECV cells are grown in medium 199 (GIBCO-BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary cultures of human umbilical vein endothelial cells (HUVEC), are obtained from Cascade Biologics Inc. (Portland, OR). HUVEC cells are grown in medium 200 (Cascade Biologics Inc., Portland, OR) supplemented with low serum growth supplement (LSGS, Cascade Biologics Inc., Portland, OR). Cells are maintained in a standard culture incubator with humidified air containing 5% (v/v) CO_2 at 37°.

α-Lipoate (racemate mixture, ASTA Meica, Frankfurt, Germany) stock solution for ECV pretreatment is prepared fresh in basic phosphate-buffered saline (PBS) and pH is adjusted to 7.4. Hydrogen peroxide (H_2O_2, Sigma, St. Louis, MO) stock solution is prepared fresh in endotoxin-free type sterile distilled water. PMA (Sigma, St. Louis, MO) stock solution is prepared in sterile dimethyl sulfoxide (DMSO).

Calcein Labeling of Cells

Calcein acetoxymethyl ester (calcein-AM, Molecular Probes, Eugene, OR) is used to fluorescently label Jurkat T cells. Nonfluorescent calcein-AM is lipophilic and is cleaved by intracellular esterases to yield highly charged fluorescent calcein that is retained by viable cells. The fluorescence labeling of Jurkat T cells is achieved by incubating cells (1×10^7 cells/ml) with 5 μM calcein-AM in RPMI 1640 for 30 min at 37°. Calcein-AM is prepared as a 1 mM stock in DMSO and stored in aliquots at −20°. After loading of calcein-AM, cells are washed three times with phosphate-buffered saline (pH 7.4, PBS) to remove excess dye. Cell viability, as detected by trypan blue exclusion, is >95% up to 3 hr after loading. The cells are finally resuspended in phenol red free RPMI 1640 containing 10% fetal calf serum at a density of 2×10^6 cells/ml.

Cell–Cell Adhesion Assay

Monolayers of HUVEC or ECV are seeded at a density of 10^4 cells/well in 96 well tissue culture plates (Falcon 3072, Becton Dickinson, Franklin Lakes, NJ). After 24 hr of seeding, the cells are pretreated with α-lipoate for 48 hr and then activated with H_2O_2 (100 μM, 1 hr), TNF-α (50 ng/ml, 6 hr), or PMA (100 nM, 24 hr). The relative contributions of ICAM-1 and VCAM-1 to the adherence of Jurkat T cells to ECV may be investigated by treating ECV with anti-human ICAM-1 (10 μg/ml) or anti-human VCAM-1 (10 μg/ml) antibodies for 30 min before coculture of ECV with

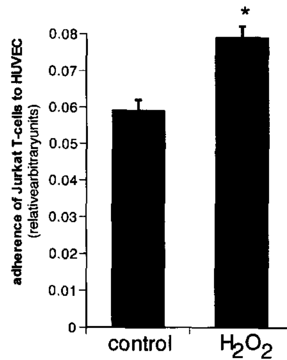

FIG. 1. Hydrogen peroxide (H_2O_2) increases adhesion of human Jurkat T cells to HUVEC. HUVEC were activated with 0.1 m*M* H_2O_2 for 1 hr. Following 1 hr of activation, cells were washed three times with PBS and then cocultured with calcein-AM labeled Jurkat T cells for 1 hr. Data are mean ±SD of at least five experiments. *, $p < 0.01$ (Student's *t*-test) when compared with nonactivated HUVEC.

Jurkat T cells. Confluent cultures of HUVEC/ECV should be used to avoid any nonspecific attachment of T cells to the plastic bed of the 96-well plate. Before the assay of cell–cell adhesion, the HUVEC or ECV monolayers are washed three times with PBS. Calcein-AM labeled Jurkat T cells (2×10^5 cells/well) are cocultured with HUVEC or ECV monolayer for 1 hr in a culture incubator with humidified air containing 5% CO_2 at 37°. Blank wells with HUVEC or ECV monolayer alone are maintained in final Jurkat T-cell suspension medium. After the coculture period, the nonadherent Jurkat T cells are removed from monolayers by washing each well four times with D (Dulbecco's)-PBS using a multichannel pipette with wide open tips.

Fluorescence Microtiter Plate Reader

The fluorescence intensity of each well is measured using a fluorescence plate reader (CS-9301, Shimadzu, Corporation, Columbia, MD). The excitation and emission wavelengths for the calcein molecule are 480 and 530 nm, respectively. Data from the plate reader are collected and processed using a CS-9301PC software (Shimadzu Corporation, Columbia, MD).

Oxidant-Induced Adherence of Human Jurkat T Cells to HUVEC

We observed a significant ~25% ($p < 0.01$) increase in the adherence of Jurkat T cells to HUVEC following activation of HUVEC with 0.1 m*M* H_2O_2 for 1 hr (Fig. 1). Reactive oxygen species (ROS) generated by hypo-

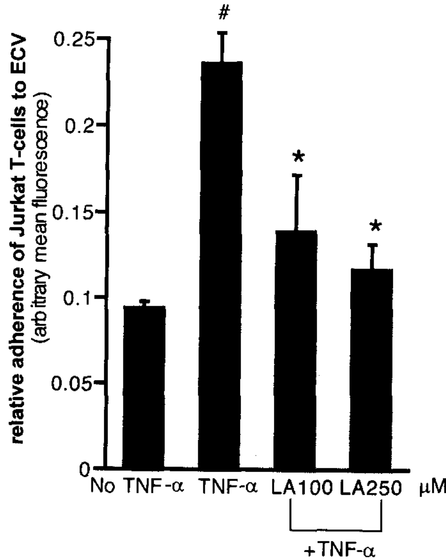

FIG. 2. TNF-α induced adhesion of human Jurkat T cell to ECV cell is inhibited by α-lipoate pretreatment. ECV cells were pretreated with α-lipoate (100 or 250 μM) for 72 hr and then activated with 50 ng/ml TNF-α for 6 hr. Cells were washed three times with PBS and then co-cultured with calcein-AM labeled Jurkat T cells for 1 hr. Data are mean ± SD of at least five experiments. #, $p < 0.01$ (Student's t-test) when compared with control (no TNF-α) cells. *, $p < 0.01$ (Student's t-test) when compared with LA nontreated and TNF-α-treated cells.

xanthine (HX)-xanthine oxidase (XO) have been previously reported to increase the adherence of polymorphonuclear neutrophil adherence to HX-XO treated HUVEC both immediately and after 2 hours.[11] We and others[11] have observed that such increased binding of leukocytes to oxidant-exposed HUVEC involves ICAM-1, but is independent of its upregulation.

Antioxidant Regulation of TNF-α Induced Adhesion of Human Jurkat T Cell

To investigate the role of antioxidants on cytokine-induced lymphocyte–endothelial cell adhesion, ECV cells are pretreated with thiol antioxidant α-lipoate and then activated with TNF-α (50 ng/ml) for 6 hr. Treatment of ECV cells markedly increased (1.5-fold) Jurkat T cell adhesion to ECV compared to cells that were not treated with TNF-α. Pretreatment of ECV cells with α-lipoate (100 or 250 μM) for 72 hr effectively downregulated TNF-α induced ICAM-1 expression compared to cells that were not pretreated with α-lipoate (Fig. 2).

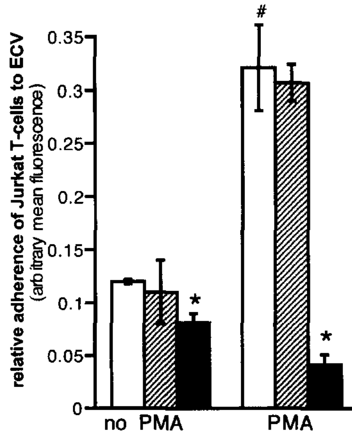

FIG. 3. PMA-induced adhesion of Jurkat T-cells to ECV cells and relative contributions of ICAM-1 and VCAM-1 molecules in such adhesion. ECV cells were treated with or without 100 n*M* PMA (open bars) for 24 hr. Following activation the cells were treated with anti-human VCAM-1 (10 μg/ml, hatched bars) or anti-human ICAM-1 (10 μg/ml, solid bars) antibodies for 30 min prior to coculture with Jurkat T cells. Adhesion assay was performed with calcein-AM labeled Jurkat T cells for 1 hr as described in the text. Data are mean $\pm$ SD of at least five experiments. #, $p < 0.01$ (Student's *t*-test) when compared with control (no PMA) cells. *, $p < 0.01$ (Student's *t*-test) when compared PMA-treated cells.

Effect of Anti-ICAM-1 and Anti-VCAM-1 on PMA-Induced Cell–Cell Adhesion

The relative contributions of ICAM-1 and VCAM-1 in Jurkat T cells' adherence to ECV cells was characterized following treatment of PMA-activated or nonactivated ECV cells with anti-human ICAM-1 and anti-human VCAM-1 antibody. Treatment of ECV cells with anti-ICAM-1 markedly (85–90%) blocked PMA-induced Jurkat T-cell adhesion to ECV cells. Anti-human VCAM-1 antibody treatment of ECV cells had no significant effect on such cell–cell adhesion, suggesting that ICAM-1, but not VCAM-1, plays a major role in agonist induced adhesion of Jurkat T cells to ECV cells (Fig. 3). ICAM-1 antibody also decreased (~35%) the adherence of Jurkat T cells to nonactivated ECV. Again, anti-human VCAM-1 antibody treatment of ECV cells had no significant effect on such cell–cell adhesion.