[36] Hemangioma Model for *in Vivo* Angiogenesis: Inducible Oxidative Stress and MCP-1 Expression in EOMA Cells

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Hemangioma: An *in Vivo* Angiogenesis Model

Hemangiomas are the most frequently occurring tumors of infancy, occurring in approximately 0.54/1000 live births.[1] These tumors of endothelial cell origin can occasionally threaten vision, airway, or life of affected patients. We felt this was an important model of angiogenesis to study for two reasons: the angiogenic processes are extremely potent with this tumor and it is commonly encountered in humans, providing important clinical relevance. Two known murine models of hemangioma are based on the subcutaneous inoculation of transformed endothelial cells. One cell line (EOMA) was derived from a spontaneously arising hemangioma in the 129/J strain[2] and the other from newborn mice directly infected with a retroviral vector expressing the oncogenic papovavirus polyoma middle T antigen.[3] Hemangiomas arise at the site of local virus injection, e.g., skin, thymus, or brain, and cells isolated from these tumors (e.g., skin, s.End cells; brain, b.End cells) can be maintained *in vitro* and inoculated *in vivo* to generate tumors. Both of these cell lines are characterized with regard to the expression of endothelial cell markers[4,5] and demonstrate a branching pattern on Matrigel *in vitro* similar to nontransformed endothelial cells. We have chosen to work with EOMA cells over End cells because EOMA have a clearly identified strain of origin that allows work on syngeneic animals available from commercial vendors. Experiments can be done with mice with an intact immune system rather than having to resort to nude or SCID mice models or tolerate MHC class II differences with the End cell model.

Rapidly proliferating hemangiomas are associated with macrophage infiltration,[1] and we have observed this with EOMA model as well (not shown). Clinical findings correlate the presence of tumor-associated macrophages with increased mortality and vascularity, implying that these cells facilitate the angiogenesis

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process.[6–8] We are particularly interested in the role of oxidants in regulating angiogenesis. The EOMA model is well suited to address this subject for the following major advantages: (1) injection of EOMA cells results in tumor production in 4 days with 100% efficiency, resulting in quick reliable data generation, (2) EOMA cells can be pharmacologically or genetically treated *in vitro* to study the effects of variable manipulation on endothelial cell behavior *in vitro,* and (3) manipulating these cells *in vitro* followed by injection *in vivo* provides a unique model for studying the influence of tumor cell-derived signals that regulate angiogenesis. Our focus on monocyte chemoattractant protein-1 (MCP-1) is based on the observation that MCP-1 is the primary stimulus for macrophage chemotaxis in numerous conditions.[9] Additionally, signal transduction mediators, NF-κB and AP-1, responsible for MCP-1 expression are redox responsive.[10,11]

Endothelial cells are capable of generating reactive oxygen species (ROS) via NADPH oxidase. A functional NADPH oxidase system is present in human and bovine endothelial cells.[12,13] Furthermore, in endothelial cells, NADPH oxidase appears to be the primary source of ROS with little input from mitochondrial or other sources.[13,14] The described methods associate changes in the redox state of endothelial cells with changes in inducible MCP-1 expression.

Generation of Hemangiomas

1. EOMA cells[2] are grown at 37° and in a 95% relative humidified atmosphere containing 5% CO_2 in 175-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml, Life Technologies, Inc., Carlsbad, CA). Cells are trypsinized when they reach ~85% confluency. The yield is approximately 5–6 million cells/flask.

2. Cells are washed three times in large volumes (e.g., 50 ml) of phosphate-buffered saline, pH 7.4 (PBS), counted, and resuspended in PBS at 5×10^7 cells/ml and kept on ice.

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FIG. 1. Tumor-bearing mouse 7 days after injection of EOMA cells.

3. The cell suspension is loaded into 1-cc insulin syringes with a 28-gauge needle (Becton Dickinson, Franklin Lakes, NJ) to a final volume of 100 μl per syringe.

4. 129 P3 mice from Jackson Laboratories (Bar Harbor, ME) are syngeneic with EOMA cells. Six-week-old mice receive inhalation anesthesia and are injected with 100 μl of cell suspension for a total dose of 5×10^6 cells. The cells are injected subcutaneously and a large wheal is noted.

5. Visibly obvious tumor development is apparent 4 days after tumor injection (Figs. 1 and 2).

Note: A paper published in 1990,[4] which characterized the phenotype of EOMA cells, incorrectly identified these as MHC H-2^d, when in fact these cells are MHC H-2^b. The 129/J strain, which is the source of the EOMA, is originally from Jackson Laboratories and has been reclassified by them as 129 P3. They note an MHC background as H-2^b, which has been confirmed by flow cytometry A. VanBuskirk, personal communication. These tumors become very large in size, but do not metastasize. However, mice typically die 21–30 days after injection secondary to Kassabach–Merritt syndrome, which has been reported by others with this model as well.[15,16]

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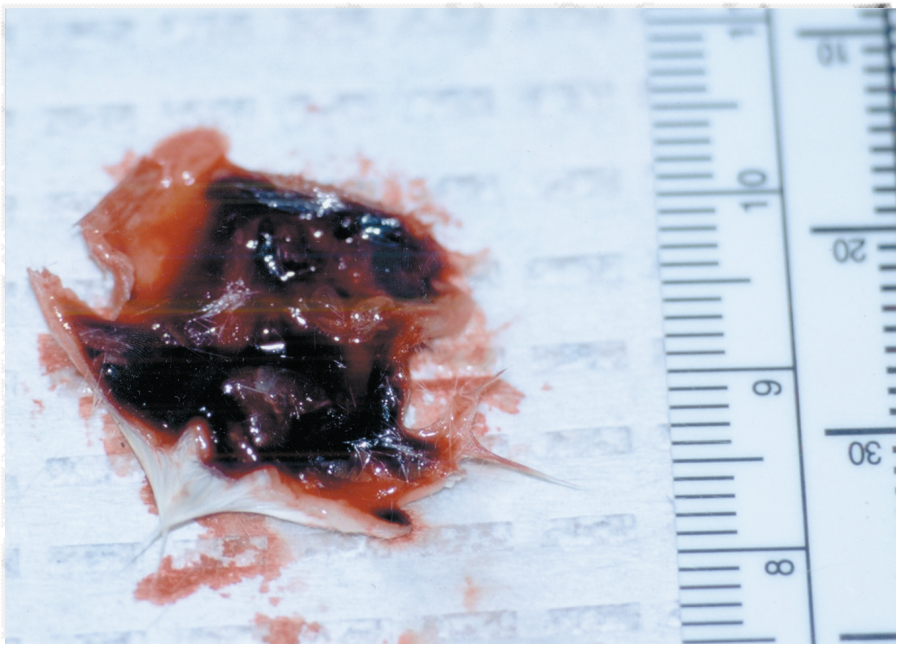

FIG. 2. Appearance of tumor at necropsy 7 days after injection of EOMA cells.

Western Blot Detection of Oxidized Proteins

Proteins are an important target for oxidative challenge. ROS modify amino acid side chains of proteins such as arginine, lysine, threonine, and proline residues to form protein carbonyls. Protein carbonyls can be detected in immunoassays by the reaction with dinitrophenyl hydrazine (DNPH) using monoclonal antidinitrophenyl (DNP) antibodies. This method has proven to be superior to the conventional spectrophotometric method because of its high sensitivity and reproducibility. Furthermore, in Western blot assays, relative oxidation of individual proteins can be determined.

Cell Culture

To perform Western blot analysis on EOMA cells, they are seeded at 1.5×10^5 cells/ml density in 100-mm plates. Cells are incubated for 24 hr in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37° in a humidified atmosphere containing 5% CO_2. Following 24 hr of seeding, the culture medium is replaced with fresh medium and the cells are treated with redox-modulating agents for a particular time period. After the treatment period, cells are washed three times with ice-cold PBS, harvested by scraping, resuspended, and lysed in 50 m*M* Tris–HCl buffer containing 5 μM phenylmethylsulfonyl

fluoride (PMSF), 4 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotonin, and 2.5% sodium dodecyl sulfate (SDS). All reagents were of highest analytical grade and obtained from Sigma (St. Louis, MO). DNA is sheared by repeated passage through a 22-gauge needle. Cell lysates are centrifuged at 14,000*g*, and supernatants are stored at −80° overnight. All procedures are performed at 4°.

Extraction and Derivatization

Derivatization of protein-bound carbonyl groups with DNPH (Acros Organics, New Jersey) and immunodetection are performed according to the procedure of Shacter *et al.*[17]

1. Five microliters of 12% SDS (w/v) is added to a 5-μl aliquot of sample containing 20 μg of protein.
2. After the addition of 10 μl of 10 m*M* DNPH in 10% (v/v) trifluoroacetic acid (Acros Organics, New Jersey), the mixture is incubated for 15 min at room temperature.
3. The reaction mixture is neutralized by adding 7.5 μl of 2 *M* Tris base containing 30% (v/v) glycerol. Samples are treated with Laemmli loading buffer[18] containing 3% mercaptoethanol for 5 min at room temperature for reducing gels.

Immunodetection of Protein-Bound Carbonyl Groups

Proteins are separated by 10% SDS–PAGE under reducing conditions and are transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, San Francisco, CA) using a tank transfer system (Hoeffer mini VE, Amersham Pharmacia Biotech). Additionally, equal transfer is checked and quantified by reversible protein staining of the nitrocellulose membrane with Ponceau S (Sigma). After blocking with a 5% (w/v) fat-free milk solution at 37° for 1 hr, membranes are treated with rat monoclonal antibody to DNP (Zymed Laboratories, San Francisco, CA) at 1 : 1000 dilution overnight at 4° and washed 3× for 10 min with Tris-buffered saline containing 0.05% Tween 20 (TTBS). The secondary antibody horseradish peroxidase-conjugated mouse monoclonal antirat antibody (Zymed Laboratories) is used at 1 : 10,000 dilution for 1 hr at room temperature. After 6× for 10 min of washing with TTBS, immunoblots are visualized using Renaissance Western blot chemiluminescence reagent (NEN Life Science Products, Boston, MA) with exposure times between 2 and 5 min (Fig. 3).

Note: Precipitated proteins should be dissolved directly in 6% SDS and reacted with an equal amount of DNPH. If the samples already contain SDS, the

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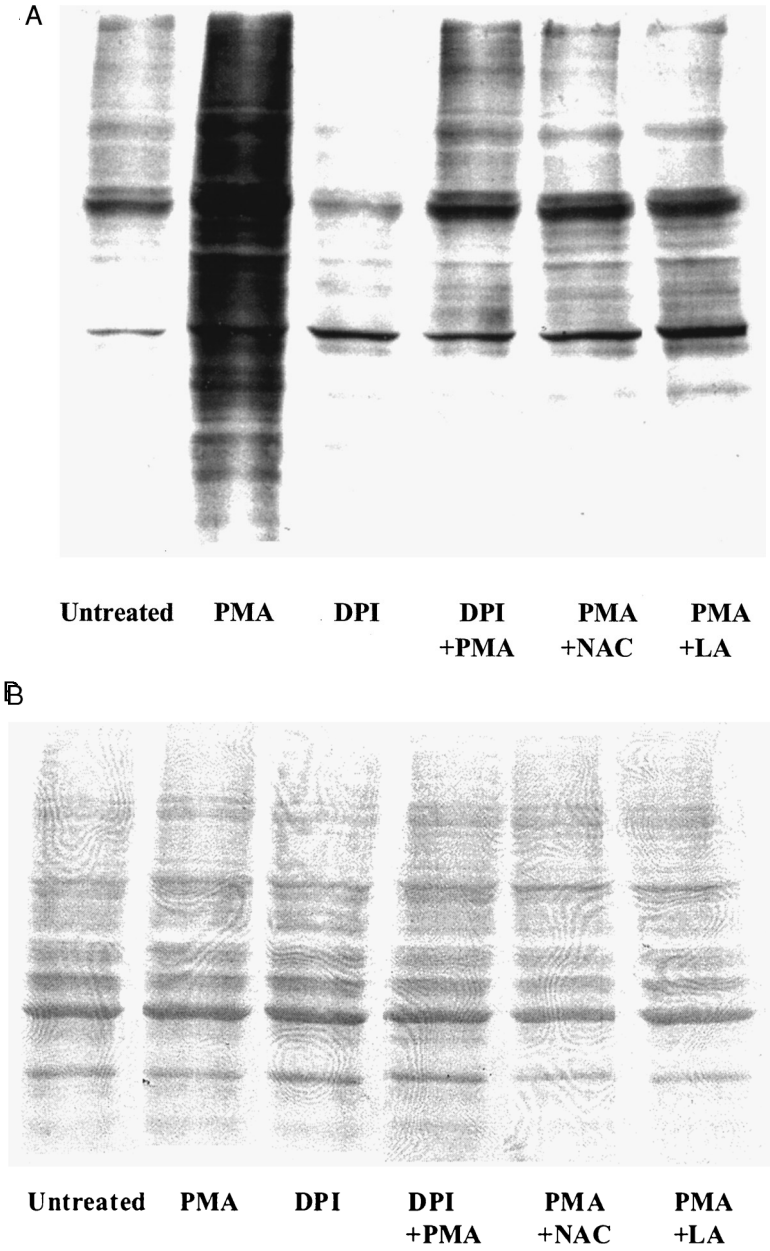

FIG. 3. (A) Protein oxidation in EOMA cells under varying degrees of oxidative stress documented by Western blot using an anti-DNP antibody to detect protein carbonyls. Cells are treated with diphenylene iodonium (DPI, 10 μM) for 1 hr prior to PMA stimulation. *N*-Acetylcysteine (NAC, 20 mM) and (*R*,*S*)-racemic lipoic acid (LA, 0.5 mM) are added at the same time as PMA. Cells are treated with PMA (100 nM) for 24 hr prior to harvesting. (B) Imaging of nitrocellulose membrane used in Western blot demonstrates equal transfer of proteins for all lanes of gel in (A).

amount of SDS in the samples should be taken into the account for the adjustment of the final SDS concentration. DNPH derivatization should be carried out at room temperature and should not exceed 30 min to avoid the formation of nonspecific reactions of unoxidized proteins. Derivatized samples are not boiled prior to SDS–PAGE. Samples without DNPH treatment are used as negative controls. For positive controls, cells are incubated with 200 μM hypochlorous acid (HOCl) for 30 min at 37° in a serum-free medium. The concentration of HOCl present in the diluted NaOCl solution (Fisher Scientific, Pittsburgh, PA) is determined spectrophotometrically using an extinction coefficient (350 M^{-1} cm^{-1}) at 290 nm as described previously.[19]

Footprints of Lipid Peroxidation

In this case, lipid peroxidation is detected using antiserum to 4-hydroxy-2-nonenal (HNE) protein adducts. A number of reactive aldehydes generated during lipid peroxidation process are potently cytotoxic and also have signaling functions[20] because of their stable nature and ability to diffuse within or from the cell. HNE, a major aldehyde produced during lipid peroxidation, has remarkable cytotoxic and mutagenic activities. One of the major mechanisms of HNE reactivity is the modification of sulfhydryl, histidine, and lysine residue of proteins. Although the method described is not suitable for the quantitative analysis, it provides direct comparisons of oxidative lipid damage in cells under varying conditions.

Immunodetection of 4-Hydroxy-2-nonenal Protein Adducts

For the detection of HNE protein adducts, cell lysates are treated with Laemmli sample buffer containing 3% mercaptoethanol for 5 min at 100°. Protein (20 μg) is run on 10% SDS–PAGE and is transferred to a nitrocellulose membrane. After blocking as described earlier for carbonyl detection, the membrane is incubated overnight with antiserum to HNE–protein adducts (Alexis, San Diego, CA) at a dilution of 1 : 500. After washing 3× for 10 min with TTBS containing 0.1% Tween 20, the membrane is incubated for 1 hr at room temperature with a 1 : 10,000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody (Upstate Biotechnology, Lake Placid, NY). The membrane is then washed 6× for 10 min with TTBS containing 0.1% Tween 20, and visualization is performed as described earlier. Equal protein loading is tested and quantified by probing the membrane for mouse β-actin using an antibody against mouse β-actin (Sigma) at

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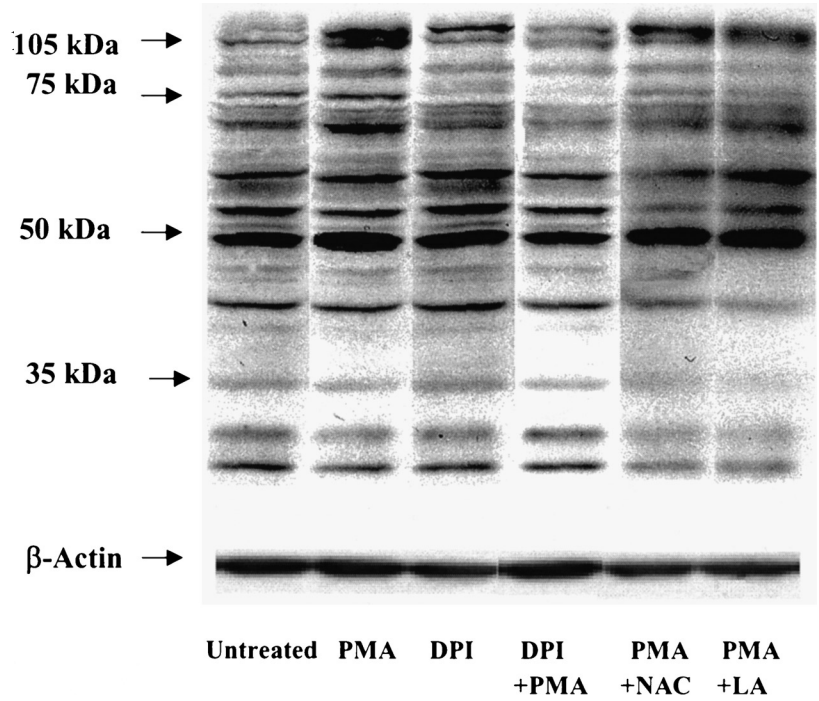

FIG. 4. Lipid peroxidation in EOMA cells under varying degrees of oxidative stress documented by Western blot using antibody to HNE–protein adducts. β-Actin imaging documents equal transfer of cellular proteins for all lanes.

a 1 : 5000 dilution and a 1 : 10,000 dilution of goat anti-mouse secondary antibody (NEN Life Science Products, Boston, MA) (Fig. 4).

Notes: Various protein bands show immunoreactivity with HNE–protein adducts. Specificity and sensitivity of the reactivity of the antiserum are tested using positive controls (50 μM 4-HNE-treated cells for 2 hr at 37°). Cells treated with phorbol 12-myristate 13-acetate (PMA) exhibited a strong HNE positive band at 48 kDa, which is consistent with previous reports.[21]

PMA-Induced Oxidation in EOMA Cells

Phorbol 12-myristate 13-acetate is a potent but nonspecific stimulus of oxidant production that is used to induce oxidant generation in endothelial cells[22] (Fig. 5).

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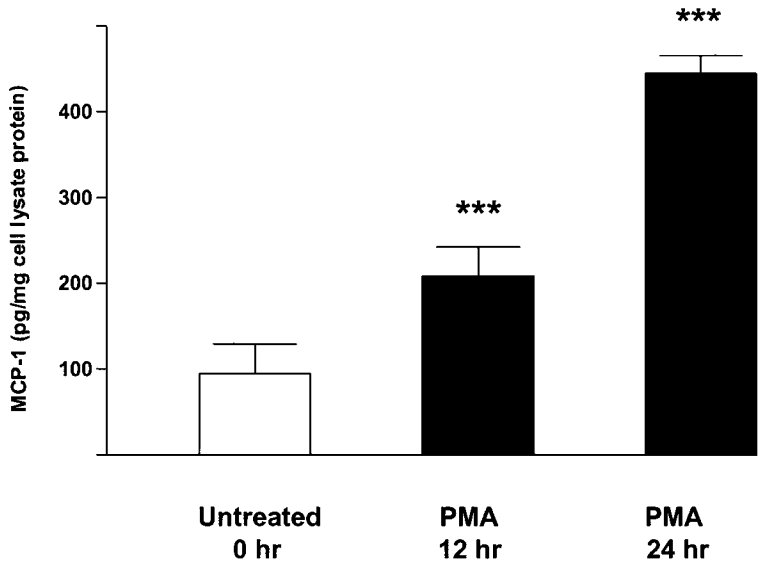

FIG. 5. Kinetics of PMA-induced MCP-1 expression. Selection of PMA concentration based on dose–response curve (data not shown). One-way analysis of variance (ANOVA) was used to compare the effects of PMA and the effects of antioxidants on MCP-1 expression versus untreated control cells. Results are log transformed before statistical analyses to correct for skewing and are presented as mean ± standard deviation (SD). Differences are compared to PMA-untreated cells: $^{***}p < 0.001$.

Western blots document the presence of oxidant footprints in untreated EOMA cells. PMA treatment significantly increased protein oxidation and lipid peroxidation (Figs. 3 and 4). Thus, EOMA cells can generate ROS in response to PMA treatment. PMA-induced oxidation in EOMA cells is sensitive to diphenylene iodonium (DPI) (Calbiochem, San Diego, California), a nonspecific inhibitor of NADPH oxidase.[23] PMA-induced oxidative events in EOMA cells are also inhibited by thiol-based antioxidants *N*-acetylcysteine (NAC) (Sigma) and (*R*,*S*)-racemic lipoic acid (LA, ASTA Medica, Frankfurt, Germany).[24]

Association of PMA-Induced Oxidation with Changes in MCP-1 Protein Expression

MCP-1 protein is detected using ELISA (Fig. 6). DPI and NAC, as well as LA treatments, suppressed PMA-induced MCP-1 expression, suggesting the involvment of redox-sensitive steps in the pathway of PMA-induced MCP-1 expression.

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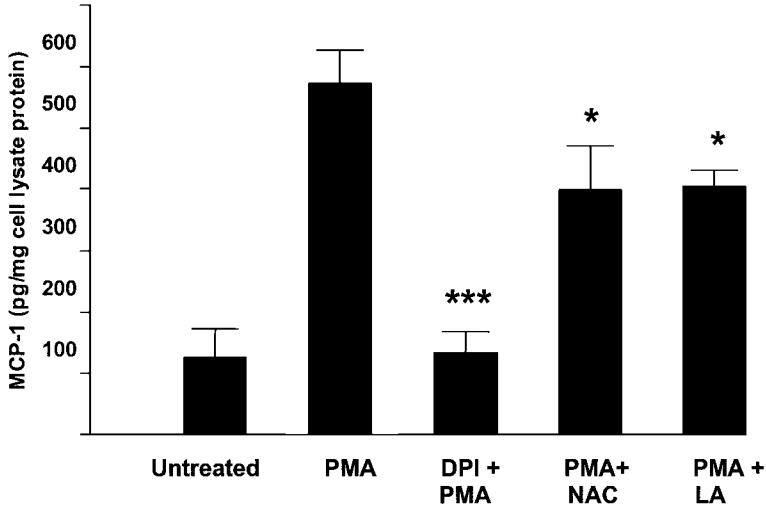

FIG. 6. Downregulation of PMA-induced MCP-1 expression by modulators of the cellular redox state. EOMA cells are treated with DPI (10 μM) for 1 hr prior to PMA stimulation. NAC (20 mM) and LA (0.5 mM) are added at the same time as PMA. Cells are treated with PMA (100 nM) for 24 hr prior to harvesting. When inhibitors are added to PMA, DPI results in a marked inhibition of MCP-1 expression (***$p < 0.001$), whereas NAC and LA treatments cause a significant but smaller inhibition of MCP-1 induction (*$p < 0.05$).

ELISA

To perform ELISA, EOMA cells are cultured in 100-mm plates as described earlier. At the end of the experiment, plates are washed three times with ice-cold PBS and are harvested by scraping. Harvested cells are centrifuged at 1000g for 10 min at 4° and are resuspended in 250 μl of extraction buffer [PBS, pH 7.4, containing 5 μM PMSF, 4 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotonin]. Cells are disrupted by sonication (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) on ice with three 15-sec bursts. Sonicates are centrifuged, and supernatants are stored at −80° overnight. All procedures are performed at 4°. ELISA plates are coated with 100 μl/well of affinity-purified anti-mouse JE/MCP-1 capture antibody (R&D Systems, Minneapolis, MN) at 0.4 μg/ml in PBS. Sealed plates are incubated overnight at room temperature. After incubation, wells are washed four times with 0.05% Tween 20 in PBS and blocked with 300 μl PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3. Plates are sealed and incubated at room temperature for a minimum of 1 hr. After four washes, 100 μl of standards (recombinant mouse JE/CCL2, R&D Systems), controls, or samples is diluted in dilution buffer (1% BSA in PBS) and added to each well. Standards are used in the range of 20–250 pg/ml. Following an overnight incubation at 4°, wells are washed four times, and 100 μl of the biotinylated anti-mouse JE antibody (R&D Systems) at a dilution of 0.1 μg/ml in dilution buffer is added to each well.

Sealed plates are incubated for 2 hr at room temperature. After washing the plate four times, 100 μl of streptavidin–HRP conjugate (Chemicon, Temecula, CA) at a dilution of 1 μg/ml in dilution buffer is added to each well. Covered plates are incubated for 30 min at room temperature. After six washes, 100 μl of HRP substrate solution (R&D Systems) is added, and plates are incubated for 20 min, protected from light, at room temperature. Finally, 50 μl of stop solution (1 *M* H_2SO_4) is added to the wells and mixed gently. Optical density is determined using a multiple well plate reader at 450 nm. Wavelength correction is done by a reference reading at 560 nm. ELISA results are normalized to total protein concentration using a BCA protein assay kit (Pierce, Rockford, IL).

Acknowledgments

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[37] Redox Aspects of Vascular Response to Injury

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Introduction

The concerted alterations in vascular structure and function that occur in response to a mechanical injury represent more than a model to explore interactions among the endothelium, platelets, and smooth muscle cells.[1] With the development of invasive endovascular strategies for atherosclerosis therapy, the vascular repair reaction has taken an important clinical dimension as a major component of restenosis postangioplasty.[2] In addition, vascular response to injury displays all biological processes typical of vascular pathophysiology (Table I) and thus constitutes a relevant model for many vascular diseases.

The involvement of redox processes as mediators of the vascular repair reaction has been postulated on the basis of their increasingly evident role as signaling mediators of cell proliferation, differentiation, senescence, and apoptosis.[3–5] In

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