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Apoptosis of Endothelial Cells Is Associated with Paracrine Induction of Adhesion Molecules

Evidence for an Interleukin-1β-Dependent Paracrine Loop

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Monocytic infiltration of the vessel wall is a hallmark of injury in a variety of vascular diseases. In the present study, we explored the relationship between endothelial apoptosis and hyperadhesiveness for monocytic cells. Apoptosis of human umbilical vein endothelial cells (HUVECs) was induced by either growth factor deprivation (GFD) for 24 hours or by incubation with mitomycin C (MMC) at 0.01 mg/ml for 24 hours and confirmed by light microscopy and DNA laddering. In parallel assessments of cell-cell adhesion, GFD and MMC induced hyperadhesiveness of HUVECs for the THP-1 monocytic cell line. Hyperadhesiveness developed in association with induction of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on HUVECs and was attenuated by monoclonal antibodies directed against these ligands. Culture medium conditioned by apoptotic HUVECs up-regulated the expression of adhesion molecules on normal HUVECs, suggesting that paracrine factors in the apoptotic milieu led to induction of adhesion molecules. Interleukin (IL)-1β was implicated as a putative mediator in this setting because 1) exogenous IL-1β up-regulates ICAM-1 and VCAM-1 with kinetics similar to those noted during endothelial cell apoptosis, 2) endothelial apoptosis was associated with increased expression of IL-1β converting enzyme, and 3) the adhesion-promoting actions of GFD and MMC were attenuated by an anti-IL-1β antibody. *(Am J Pathol 1998, 152:523–532)*

Atherosclerosis and various forms of chronic vascular injury are associated with adhesion of mononuclear leukocytes to the endothelium and transmigration to the intima, which in turn leads to formation of numerous growth factors and progression to a fibro-proliferative phase.[1] The leukocyte adhesion molecules intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 facilitate monocyte infiltration of the vessel wall,[1,2] but the mechanisms initiating adhesion molecule up-regulation in this setting are still being appreciated. Recently, factors associated with vascular injury and atherosclerosis, such as cholesterol oxide,[3] hyperglycemia,[4] and ischemia-reperfusion,[5] have been reported to induce apoptosis of endothelial cells *in vitro* and/or *in vivo*.

Apoptosis (or programmed cell death), represents a genetically programmed cellular death, first characterized by stereotypical morphological changes[6] and subsequently associated with internucleosomal cleavage of DNA.[7] In contrast with necrosis, an accidental form of cell death, the integrity of the cell membrane is preserved until late in the apoptotic process, preventing spillage of intracellular components into the extracellular milieu.[8–11] Therefore, apoptosis has been considered for decades a paradigm for a clean and noninflammatory cell death.[8–11] However, recent reports suggest that programmed cell death is not synonymous with noninflammatory cell death, as genes centrally implicated in the regulation of apoptosis, such as caspase-1/interleukin (IL)-1β converting enzyme (ICE), lead to activation of inflammatory mediators.[10,12–16] Moreover, induction of apoptosis *in vivo* in renal tubular cells has been recently found to be associated with immune cell infiltration at sites of programmed cell death.[17]

Taken together, these recent findings suggest a link between programmed cell death at the endothelial level and monocytic infiltration of the vessel wall. We therefore used two classical pro-apoptotic stimuli (growth factor

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deprivation (GFD) and DNA damage) to standardize a system of endothelial apoptosis and evaluate the basic relationship between programmed cell death and endothelial adhesiveness.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described[18–20] and used at passage levels 1 to 3. The cells were propagated on gelatin-coated (1%) tissue culture plates (Costar, Cambridge, MA) and cultured in RPMI 1640 plus glutamine cell culture medium (BioWhittaker, Walkersville, MD) supplemented with 15% bovine calf serum (BCS; Hyclone, Logan, UT), 15% NU-serum (Collaborative Biomedical Products, Bedford, MA), 50 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA), 8 U/ml heparin, 50 U/ml penicillin (BioWhittaker), and 50 μg/ml streptomycin (BioWhittaker) and maintained in a humidified atmosphere containing 5% CO_2/95% air at 37°C. Endothelial origin was confirmed by immunohistochemistry staining for von Willebrand factor and by determining up-regulation of E-selectin after stimulation with tumor necrosis factor (TNF)-α (10 ng/ml) for 4 hours. The THP-1 cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 plus glutamine cell culture medium (BioWhittaker) supplemented with 15% BCS (Hyclone), 50 U/ml penicillin (BioWhittaker), and 50 μg/ml streptomycin (BioWhittaker).

Assessment of Apoptosis by Light Microscopy and DNA Fragmentation Assay

For light microscopic examination, HUVECs were trypsinized gently, cytocentrifuged onto a slide, and stained with Wright-Giemsa. Endothelial cells were considered apoptotic when at least two of the following features were found: cell shrinkage, cytoplasmic condensation, or condensation of chromatin. These criteria represent readily identifiable morphological features shown to correlate with internucleosomal DNA fragmentation.[8] For DNA analysis, HUVECs were harvested and washed twice with phosphate-buffered saline (PBS) containing Ca and Mg (PBS+) at 4°C. DNA was isolated using a DNA extraction kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). In brief, HUVECs were resuspended in lysis buffer and incubated for 1 hour at 60°C with shaking. Nucleic acids were separated from proteins by centrifugation for 15 minutes at 2000 × *g* and collecting the supernatant. Ribonucleic acids were eliminated by incubating with RNAse for 15 minutes at 37°C. DNA was precipitated by adding 2 vol of 100% ethanol and incubating for 12 hours at −20°C. DNA precipitates were recovered by centrifugation at 2000 × *g* for 20 minutes. After drying, DNA was resuspended in 10 mmol/L Tris buffer, containing 0.1 mmol/L EDTA. DNA was electrophoresed in a 1.8% agarose gel at 60 V for 3 hours, stained with ethidium bromide, and viewed in ultraviolet light.

Adhesion of Monocytic Cells to HUVECs

Adhesion of the THP-1 monocytic cells to HUVECs was assayed by slight modification of previously published methods.[21,22] Briefly, THP-1 cells were labeled with 40 mmol/L 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein penta-acetoxymethyl ester (BCECF-AM; Calbiochem, San Diego, CA) for 40 minutes at 37°C, washed in PBS+, and resuspended in PBS+ supplemented with 1% BCS. HUVECs were grown to confluence in gelatin-coated 96-well cell culture dishes. The culture medium was aspirated from each well, washed once with 100 μl of PBS+/1% BCS, and 100 μl of labeled monocytic cells (3×10^6/ml) were added per well (30 minutes at 37°C). For adhesion experiments of THP-1 to trypsinized HUVECs, the HUVEC monolayer was trypsinized for 2 minutes at 37°C, detachment of HUVECs was confirmed by light microscopy, the HUVECs were washed once with 100 μl of PBS+/1% BCS, and 100 μl of labeled monocytic cells (3×10^6/ml) were added per well (30 minutes at 37°C). After co-incubations, nonadherent monocytic cells were removed by aspiration of medium and washing gently with PBS+. Fluorescence was assessed in a Cytofluor 2300 fluorescence plate reader (Perceptive Biosystems, Framingham, MA), and the number of adherent monocytic cells was calculated from the fluorescence of the original cell suspension.

Flow Cytometry Analysis

The influence of an apoptotic milieu on VCAM-1 and ICAM-1 expression was assessed with confluent endothelial monolayers, grown on 24-well tissue culture plates (Costar), using a slight modification of a previously described method.[22] Briefly, HUVECs were trypsinized gently, washed once in PBS+, and incubated for 30 minutes on ice with either an anti-ICAM-1 monoclonal antibody (MAb; 10 μg/ml; gift of Dr. J. Neuringer, Boston, MA), an anti-VCAM-1 MAb (dilution, 1:100; gift of Dr. F. Luscinskas, Boston, MA), or control isotype-matched mouse immunoglobulins (Pharmingen, San Diego, CA), washed in

Table 1. Percentage of Apoptotic HUVECs after 4 and 24 Hours of Growth Factor Deprivation and Mitomycin C Treatment

	4 hours	24 hours
Normal medium	0.3 ± 0.3	4.9 ± 1.8
GFD	6.3 ± 1.2*	26.5 ± 3.1*
MMC	1.7 ± 0.7	23.8 ± 5.7*

HUVECs were grown to confluence in normal HUVEC medium and then incubated for an additional 4 or 24 hours in either normal medium, growth-factor-deficient medium, or normal medium supplemented with MMC (0.01 mg/ml). HUVECs were then trypsinized gently, cytocentrifuged onto a slide, and stained with Wright-Giemsa. A cell was considered apoptotic when at least two of the following criteria were found: cell shrinkage, cytoplasmic condensation, or condensation of chromatin. Data are means ± SEM of at least four experiments.

*$P < 0.05$ *versus* normal medium.

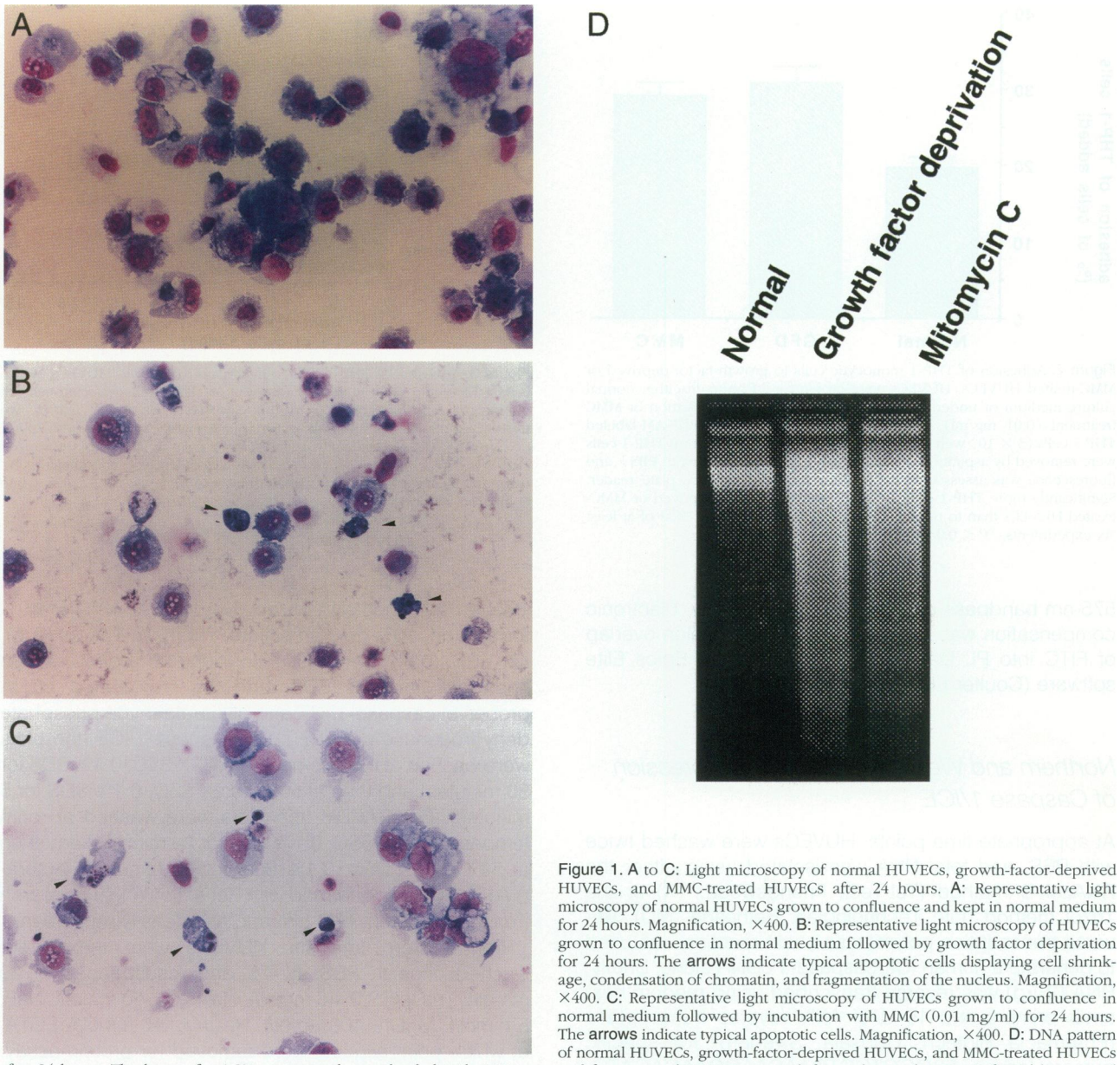

Figure 1. A to C: Light microscopy of normal HUVECs, growth-factor-deprived HUVECs, and MMC-treated HUVECs after 24 hours. A: Representative light microscopy of normal HUVECs grown to confluence and kept in normal medium for 24 hours. Magnification, ×400. B: Representative light microscopy of HUVECs grown to confluence in normal medium followed by growth factor deprivation for 24 hours. The arrows indicate typical apoptotic cells displaying cell shrinkage, condensation of chromatin, and fragmentation of the nucleus. Magnification, ×400. C: Representative light microscopy of HUVECs grown to confluence in normal medium followed by incubation with MMC (0.01 mg/ml) for 24 hours. The arrows indicate typical apoptotic cells. Magnification, ×400. D: DNA pattern of normal HUVECs, growth-factor-deprived HUVECs, and MMC-treated HUVECs after 24 hours. The lanes of a 1.8% agarose gel were loaded with DNA extracted from normal HUVECs, growth-factor-deprived HUVECs for 24 hours, and MMC-treated HUVECs (0.01 mg/ml for 24 hours), electrophoresed, stained with ethidium bromide, and viewed under ultraviolet light. A characteristic ladder pattern is present after growth factor deprivation and incubation with MMC but not in normal HUVECs. This gel is representative of at least three other experiments.

PBS+, and incubated for 30 minutes on ice with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Biosource International, Camarillo, CA). For concomitant assessment of apoptosis, staining with the two DNA dyes Hoechst 33342 (2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole (HT); Sigma Chemical Co, St. Louis, MO) and propidium iodide (PI) was undertaken as previously described.[22,23] In brief, HT (1 μg/ml) was added to the cell suspension (10 minutes at 37°C) and to prevent further uptake of HT, HUVECs were cooled to 4°C, centrifuged at 225 × *g* at 4°C, and resuspended in 1 ml of ice-cold PBS+. PI (Sigma) was added to each sample to a final concentration of 5 μg/ml immediately before flow cytometric analysis.

Analysis was performed on an Epics ESP flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an ultraviolet-enhanced argon ion laser and modified with the gated amplifier option that permits spatial beam separation resulting in minimal spectral overlap between HT, FITC, and PI fluorescence. HT fluorescence was accomplished by excitation with less than 5 mW of ultraviolet laser light (351 to 364 nm multiline); FITC and PI fluorescence was determined by excitation with 15 mW of 488-nm blue laser light. Detection of HT, FITC, and PI fluorescence was accomplished by using 405-, 525-, and

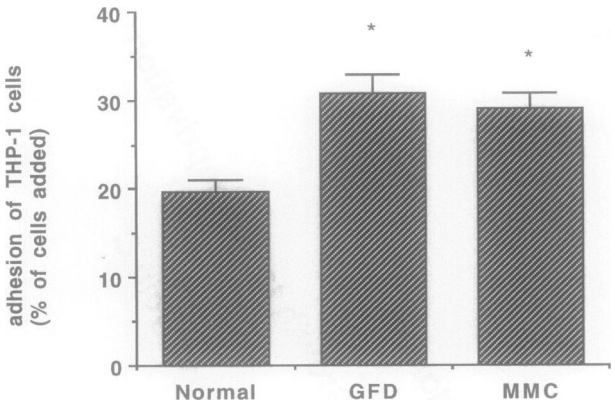

Figure 2. Adhesion of THP-1 monocytic cells to growth-factor-deprived or MMC-treated HUVECs. HUVECs were grown for 24 hours in either normal culture medium or under conditions of growth factor deprivation or MMC treatment (0.01 mg/ml) and then co-incubated with BCECF-AM-labeled THP-1 cells (3×10^5/well) for 30 minutes at 37°C. Nonadherent THP-1 cells were removed by aspiration of medium and washing gently with PBS+ and fluorescence was assessed in a Cytofluor 2300 fluorescence plate reader. Significantly more THP-1 cells adhered to growth-factor-deprived or MMC-treated HUVECs than to normal controls. Data are means ± SEM of at least six experiments. $^*P < 0.05$ when compared with normal.

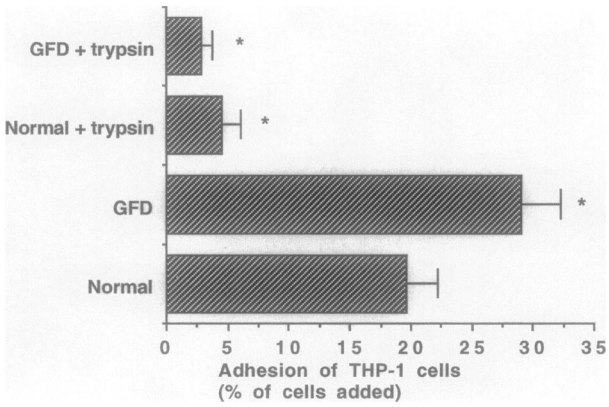

Figure 3. Adhesion of THP-1 cells to intact and trypsinized HUVECs monolayers. To confirm that increased adhesion of THP-1 cells reflected cell-cell interactions and not binding to the exposed extracellular matrix, we trypsinized the HUVECs monolayer for approximately 2 minutes with 200 μl of trypsin/EDTA. Significantly fewer THP-1 cells adhered to trypsinized HUVECs than to either normal or growth-factor-deprived endothelial cells. Data are means ± SEM of at least three experiments. $^*P < 0.05$ when compared with normal.

575-nm bandpass optical filters, respectively. Electronic compensation was used to correct for emission overlap of FITC into PI. Data were analyzed using Epics Elite software (Coulter Electronics).

Northern and Western Analysis for Expression of Caspase 1/ICE

At appropriate time points, HUVECs were washed twice with PBS, and total RNA was isolated using either the RNAzol B method (Tel-Test, Friendswood, TX) or the Trizol method (Life Technologies, Bethesda, MD). Reverse transcription polymerase chain reaction was used to generate a human ICE/caspase-1 cDNA clone. Briefly, RNA (2 μg) was reverse transcribed using random hexamer primers and 15 U of AMV reverse transcriptase (Pharmacia Biotech, Uppsala, Sweden). ICE-specific primers (sense, 5′-GGGTGCTGAACAAGGAAGAGATG-3′; antisense, 5′-TGCAGATAATGAGAGCAAGACGTG-3′; Continental Laboratory Products, San Diego, CA) were used for polymerase chain reaction amplification using 5 μl of cDNA reaction mixture and 50 mmol/L KCL, 10 mmol/L Tris/HCl (pH 9.0), 1.5 mmol/L MgCl, 2.5 U of *Taq* polymerase (Pharmacia Biotech), 1 μmol/L of each primer, and 37.5 μl of water. The thermocycle protocol was as follows: 94°C for 5 minutes and 30 cycles at 66°C for 1 minute, 72°C for 2 minutes, and 94°C for 1 minute followed by one cycle at 72°C for 10 minutes. The amplified 410-bp cDNA fragment was separated of a 1.8% agarose gel, isolated, ligated into a TA-cloning plasmid (Invitrogen, San Diego, CA), cloned, digested with *Eco*RI (Life Technologies, Grand Island, NY), and labeled with ^{32}P for Northern analysis. To prepare the Northern blot, total RNA (5 μg) from HUVECs was fractionated on a 1% agarose, 0.7% formaldehyde denaturing gel and transferred overnight to a nylon membrane. Blots were prewashed and then hybridized overnight at 42°C in 40% formamide, 10% dextran sulfate, 7 mmol/L Tris (pH 7.6), 4X SSC, 0.8X Denhardt's solution, 20 μg/ml salmon sperm DNA, 0.5% SDS, and the ^{32}P-labeled cDNA probes of caspase-1/ICE and glyceraldehyde phosphate dehydrogenase (GAPDH). For caspase-1/ICE, the blots were washed at room temperature (2X SSC, 0.1% SDS for 30 minutes) and then at 50°C (0.2X SSC, 0.1% SDS for 5 minutes). For GAPDH, the blots were washed at room temperature (2X SSC, 0.1% SDS for 20 minutes) and then at 50°C (0.2X SSC, 0.1% SDS for 20 minutes). Autoradiography was performed with Reflection (NEN Research Products, Boston, MA) film and an intensifying screen.

For Western analysis, HUVECs were trypsinized, washed once in PBS at 4°C and resuspended in 20 mmol/L Tris, pH 8.0, 40 mmol/L $Na_4P_2O_7$, 50 mmol/L NaF, 5 mmol/L $MgCl_2$, 100 μmol/L Na_3VO_4, 10 mmol/L EDTA, at which point aliquots were removed for protein assay. The suspension was made to 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 3 mM phenylmethylsulfonyl fluoride, left at 4°C for 10 minutes, and centrifuged at 10,000 × *g* for 5 minutes. Samples containing 40 μg of proteins were separated on a 12% polyacrylamide gel and transferred to a nylon-supported nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with Tris-buffered saline/Tween 20 (TBST) plus 3% nonfat milk for 60 minutes, followed by overnight incubation with anti-ICE rabbit polyclonal antibody (Santa Cruz Biotechnology). The membrane was then washed three times with TBST, incubated with a horseradish-peroxidase-labeled secondary antibody for 1 hour (Upstate Biotechnology, Lake Placid, NY), and washed again three times in TBST. Signal was visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL) by exposing the membrane to film.

Table 2. Culture of HUVECs in an Apoptotic Milieu Is Associated with Induction of ICAM-1- and VCAM-1-Dependent Endothelial Hyperadhesiveness for THP-1 Cells

	Adherent Thp-1 cells (% of THP-1 cells added)	Adherent Thp-1 cells (% of THP-1 cells added)
	Isotype-matched Ig mouse	Anti-ICAM-1
Normal medium	16.4 ± 1.3%	12.7 ± 0.6%*
GFD	28.9 ± 1.1%	26.1 ± 1.0%†
MMC	24.9 ± 1.4%	20.7 ± 1.3%
	Isotype-matched Ig mouse	Anti-$VCAM_R1$
Normal medium	16.4 ± 1.1%	14.6 ± 1.1%
GFD	28.0 ± 1.0%	24.8 ± 1.4%†
MMC	25.3 ± 1.4%	25.1 ± 3.0%
	Isotype-matched Ig mouse	Anti-ICAM and anti-VCAM
Normal medium	18.1 ± 1.6%	13.3 ± 0.6%
GFD	35.2 ± 1.4%	26.0 ± 1.5%†
MMC	25.3 ± 1.5%	14.0 ± 0.6%‡

HUVECs were grown to confluence in normal medium and then incubated for an additional 24 hours in either normal medium or under conditions of either GFD or MMC treatment (0.01 mg/ml). The monolayers were then incubated with anti-ICAM-1 or anti-VCAM-1 or both anti-ICAM-1 and anti-VCAM-1 MAb or with an isotype-matched mouse immunoglobulin for 30 minutes before co-incubation with BCECF-AM-labeled THP-1 cells. Maximal inhibition of adhesion was achieved with anti-ICAM-1 and anti-VCAM-1 co-treatment. Data are means ± SEM of at least three experiments.

*$P < 0.05$ *versus* normal medium.

†$P < 0.05$ *versus* GFD.

‡$P < 0.05$ *versus* MMC.

IL-1β Levels

HUVECs at passage levels 1 to 3 were incubated for up to 24 hours in normal medium or normal medium plus mitomycin C (MMC; 0.01 mg/ml). The culture medium was then collected, and IL-1β levels were measured using an enzyme immunoassay specific for human interleukin-1β (Chemicon International, Temecula, CA).

Reagents

The IL-1β antibody was purchased from R&D Systems (Minneapolis, MN), and MMC was purchased from Sigma.

Statistical Analysis

Results were expressed as means ± SEM. Data were analyzed using either paired or unpaired Student's *t*-tests or analysis of variance, as appropriate. A P value of <0.05 was taken to represent a statistically significant difference between group means.

Results

Induction of Endothelial Cell Apoptosis Is Associated with Increased Adhesiveness for Monocytic Cells

Deprivation of growth factors and induction of DNA damage are two well defined stimuli for apoptosis of diverse cell types.[10] In the present study, endothelial cell apoptosis was induced either by GFD (HUVECs grown in RPMI 1640 with glutamine solely) or by incubation with the DNA-damaging agent MMC (0.01 mg/ml) for up to 24 hours to assess the influence of an apoptotic milieu on endothelial adhesiveness for monocytic cell lines. Apoptosis was evaluated by light microscopy, by dual-laser flow cytometry using HT and PI, and by DNA fragmentation analysis. The proportion of HUVECs displaying apoptotic features increased significantly with time in response to GFD or MMC treatment (Table 1 and Figure 1). A characteristic DNA ladder pattern was observed with HUVECs grown in pro-apoptotic conditions for 24 hours but not with HUVECs of similar passage level grown in normal conditions (Figure 1). In parallel fluorescence-based assessments of cell-cell adhesion, hyperadhesiveness of HUVECs for THP-1 monocytic cells was observed after either GFD or MMC administration (Figure 2). At least 24 hours of exposure to an apoptotic milieu was required for hyperadhesiveness to develop (data not shown). Light microscopy analysis indicated that increased adhesion of THP-1 cells was due to interaction with endothelial cells and not due to adhesion to matrix components after endothelial retraction or detachment. To explore further the latter possibility, the extracellular matrix was exposed by gentle trypsinization before assessing adhesion of THP-1 cells. Significantly fewer THP-1 cells adhered to trypsinized HUVECs than to either normal or growth-factor-deprived endothelial monolayer (Figure 3), ruling out a contribution from cell matrix interactions.

The Hyperadhesiveness of HUVECs Grown under Pro-Apoptotic Conditions Is ICAM-1 and VCAM-1 Dependent

To define the ligands responsible for endothelial hyperadhesiveness after induction of apoptosis, MAbs against ICAM-1 and VCAM-1 were used. Anti-VCAM-1 was associated with a significant but small decrease in the hyperadhesiveness of GFD-treated HUVECs. Anti-ICAM-1 led to a marginal effect in GFD-treated HUVECs.

Table 3. Percentage of HUVECs Expressing ICAM-1 or VCAM-1 after 24 Hours of Growth Factor Deprivation or Incubation with Mitomycin

	ICAM-1	VCAM-1
Normal medium	7.4 ± 0.7	1.0 ± 0.2
MMC	28.3 ± 8.2*	2.5 ± 0.9
GFD	17.2 ± 2.2*	4.8 ± 1.2*

HUVECs were grown to confluence in normal medium and then incubated for an additional 24 hours in either normal medium, growth-factor-deficient medium, or normal medium supplemented with MMC (0.01 mg/ml). HUVECs were then trypsinized gently and labeled with an anti-ICAM-1 MAb, anti-VCAM-1 MAb, or control isotype-matched antibody, followed by incubation with a FITC-labeled goat anti-mouse secondary antibody. Levels of ICAM-1 and VCAM-1 expression were evaluated by flow cytometry. Data are means ± SEM of at least four experiments.

*$P < 0.05$ *versus* normal medium.

However, co-treatment with both antibodies was associated with significant (albeit incomplete) inhibition of hyperadhesiveness for both GFD- and MMC-treated HUVECs (Table 2). In agreement with these functional studies, both GFD and MMC treatment (0.01 mg/ml) for 24 hours were associated with increased HUVEC surface expression of ICAM-1 and, to a lesser degree, VCAM-1 as determined by flow cytometry (Table 3 and Figure 4). Using a FITC-labeled isotype-matched mouse immunoglobulin, similar fluorescence patterns were found between normal and GFD- and MMC-treated HUVECs, thereby suggesting that induction of adhesion molecules was a specific finding.

Furthermore, we used three-color flow cytometry to define the population of HUVECs that expressed ICAM-1 concomitantly with quantitation of the level of apoptosis. After 24 hours of pro-apoptotic conditioning, HUVECs were labeled with an anti-ICAM-1 MAb or control isotype-matched antibody, followed by incubation with a FITC-labeled goat anti-mouse secondary antibody and staining with HT and PI for concomitant assessment of apoptosis.[22,23] Interestingly, up-regulation of ICAM-1 occurred preferentially in nonapoptotic cells (low HT/low PI; data not shown). Together these data raise the possibility that mediators released by apoptotic HUVECs may act in a paracrine fashion to induce adhesion molecule expression by adjacent viable cells.

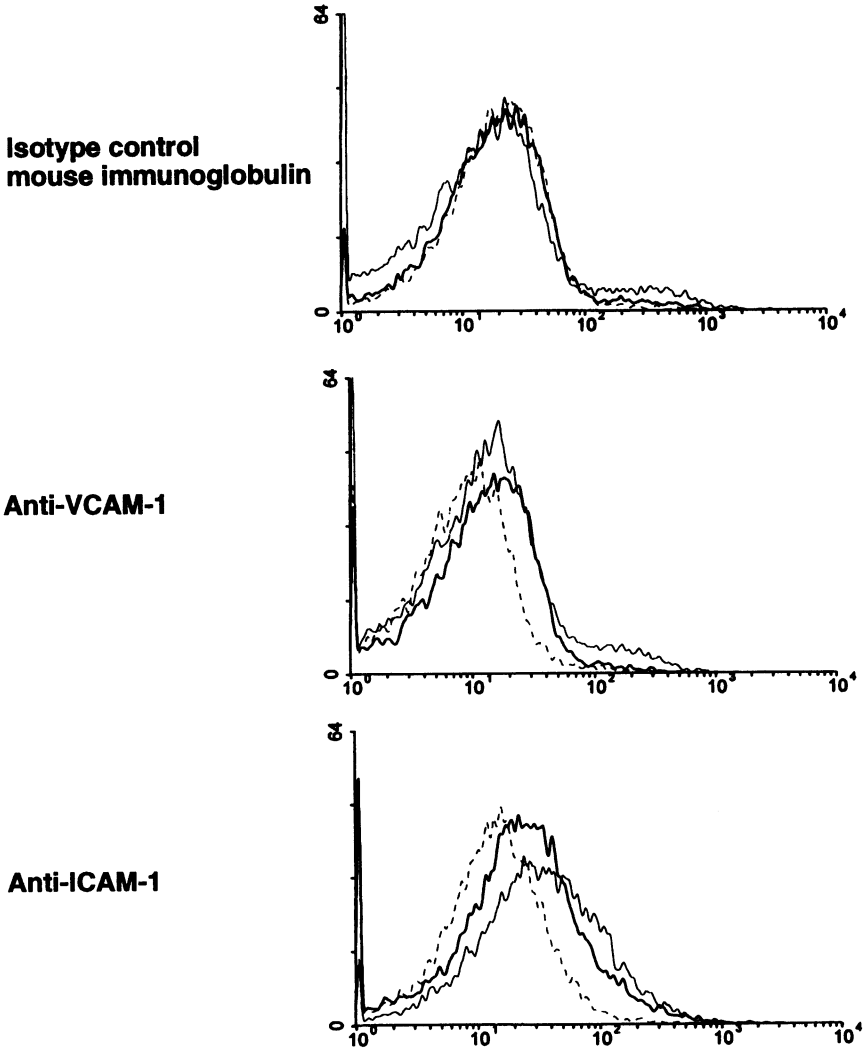

Figure 4. Up-regulation of ICAM-1 after growth factor deprivation or treatment with MMC. HUVECs were grown to confluence in normal medium and then incubated for an additional 24 hours in either normal medium, growth-factor-deficient medium, or normal medium supplemented with MMC (0.01 mg/ml). HUVECs were then labeled with an anti-VCAM-1 MAb, anti-ICAM-1 MAb followed by incubation with a FITC-labeled goat anti-mouse secondary antibody or with a control isotype-matched FITC-labeled mouse immunoglobulin. In each graph, normal HUVECs are represented by the **dashed line**, growth-factor-deprived HUVECs by the **thick line**, and MMC-treated HUVECs by the **thin line**. VCAM-1 expression (**middle graph**) and ICAM-1 expression (**bottom graph**) are increased in HUVECs treated with either growth factor deprivation or MMC. No difference in fluorescence level can be seen with the control antibody (**top graph**), showing that the increased expression of VCAM-1 and ICAM-1 are specific.

Table 4. Evidence for ICAM-1 Up-Regulation through Paracine Signaling

	% of HUVECs expressing ICAM-1
(1) Normal medium	7.8 ± 0.4
(2) Normal medium conditioned by normal HUVECs	8.8 ± 0.6
(3) Normal medium conditioned by apoptotic HUVECs	16.0 ± 2.7*
(4) Growth-factor-deprived medium	18.6 ± 4.2*
(5) Growth-factor-deprived medium conditioned by apoptotic HUVECs	15.0 ± 5.0*
(6) Normal medium conditioned by necrotic HUVECs	4.9 ± 0.4

Normal HUVECs were incubated for 24 hours in either (1) normal medium, (2) normal medium that had been used to grow normal HUVECs for 24 hours, (3) normal medium reconstituted from RPMI plus glutamine used to serum starve HUVECs for 24 hours, (4) RPMI plus glutamine alone, (5) RPMI plus glutamine used to serum starve HUVECs for 24 hours, (6) normal medium reconstituted from RPMI plus glutamine used to induce necrosis in HUVECs (cells heated at 56 °C for 30 minutes). ICAM-1 expression was evaluated by FACS analysis as described in Materials and Methods. Data are means ± SEM of at least three experiments.

*$P < 0.05$ *versus* normal medium.

Paracrine Signaling from the Endothelial Apoptotic Milieu Leads to Hyperadhesiveness

To assess the role of paracrine signals as stimuli for adhesion molecule up-regulation within an apoptotic milieu, we focused on the mechanism of induction of ICAM-1 by GFD. RPMI 1640 plus glutamine conditioned for 24 hours by apoptotic HUVECs induced ICAM-1 expression when transferred to a healthy HUVEC monolayer (Table 4). Induction of ICAM-1 was observed whether or not the conditioned medium was repleted with growth factors before addition to the new monolayer. The level of ICAM-1 induced under these circumstances was similar to that induced by GFD alone (Table 4). In contrast, ICAM-1 expression was not augmented when HUVECs were exposed to medium conditioned by cells undergoing primary necrosis and replenished with growth factors. This suggests that the paracrine mediator inducing ICAM-1 expression is specific to apoptosis and is not a general phenomenon associated with any type of cell death. In aggregate, these studies suggested that endothelial cells undergoing programmed cell death elaborate a soluble mediator(s) that can induce adhesion molecule expression in surrounding cells.

Recent reports suggest that the caspase family of cysteine proteases is involved in the molecular regulation of programmed cell death in various cell types, including endothelial cells.[13–16,24] Induction of apoptosis in mammalian cells is associated with activation of caspase-1/ICE, leading to cleavage of the inactive 31-kd IL-1β precursor to yield the active cytokine.[14–16] To explore the possible role of IL-1β as the paracrine inducer of hyperadhesiveness in our system, we evaluated the expression of ICE during apoptosis of endothelial cells. Both GFD and MMC treatment of endothelial cells were associated with increased expression of ICE, both at the gene and protein level (Figure 5). The level of IL-1β was measured in normal and MMC-treated HUVECs (0.01 mg/ml for 24 hours). The level of IL-1β was slightly but significantly higher in MMC-treated HUVECs (normal, 1.0 ± 1.0 pg/ml, *versus* MMC, 16.0 ± 3.0 pg/ml; $P < 0.05$). To assess the functional importance of this result on apoptosis-associated hyperadhesiveness, a blocking anti-IL-1β antibody (final concentration, 600 ng/ml) was added to HUVECs concomitantly with induction of apoptosis (GFDor MMC). After 24 hours, adhesion of THP-1 to HUVECs was assessed. In both GFD- and MMC-treated HUVECs the percentage of adherent THP-1 cells was significantly decreased by IL-1β blockade (Figure 6).

Discussion

Adhesion of mononuclear leukocytes to the endothelium and transmigration to the intima are central events in the pathophysiology of atherosclerosis and other forms of chronic vascular injury.[1,2,25] There is compelling evi-

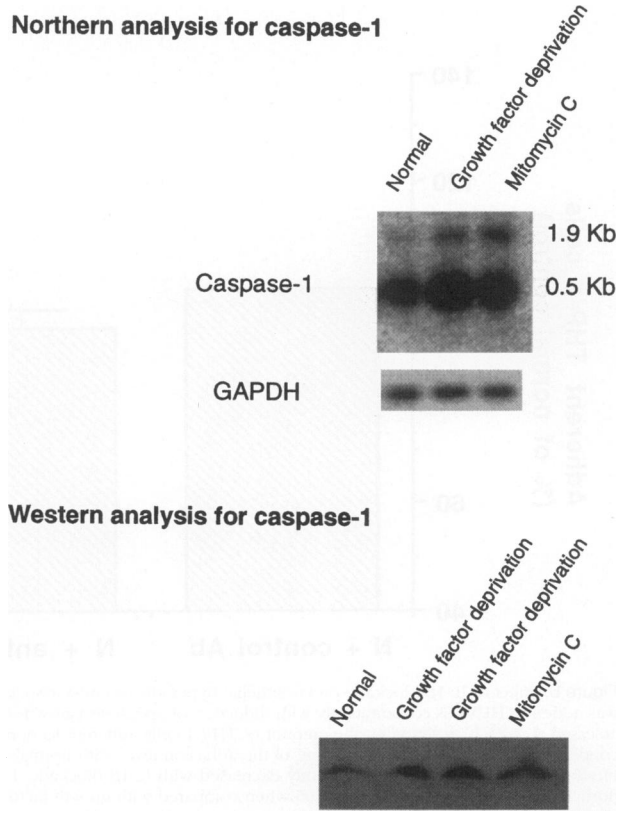

Figure 5. Gene and protein expression of caspase-1/ICE during induction of apoptosis of endothelial cells. HUVECs were grown for 24 hours in either normal culture medium or growth factor deprivation or in the presence of MMC (0.01 mg/ml). The expression of caspase-1/ICE was evaluated by Northern and Western analysis as described in Materials and Methods. Both growth factor deprivation and incubation with MMC were associated with significant induction of the 1.9- and 0.5-kb alternatively spliced products of caspase-1/ICE (**top**) and of the caspase-1/ICE protein (**bottom**).

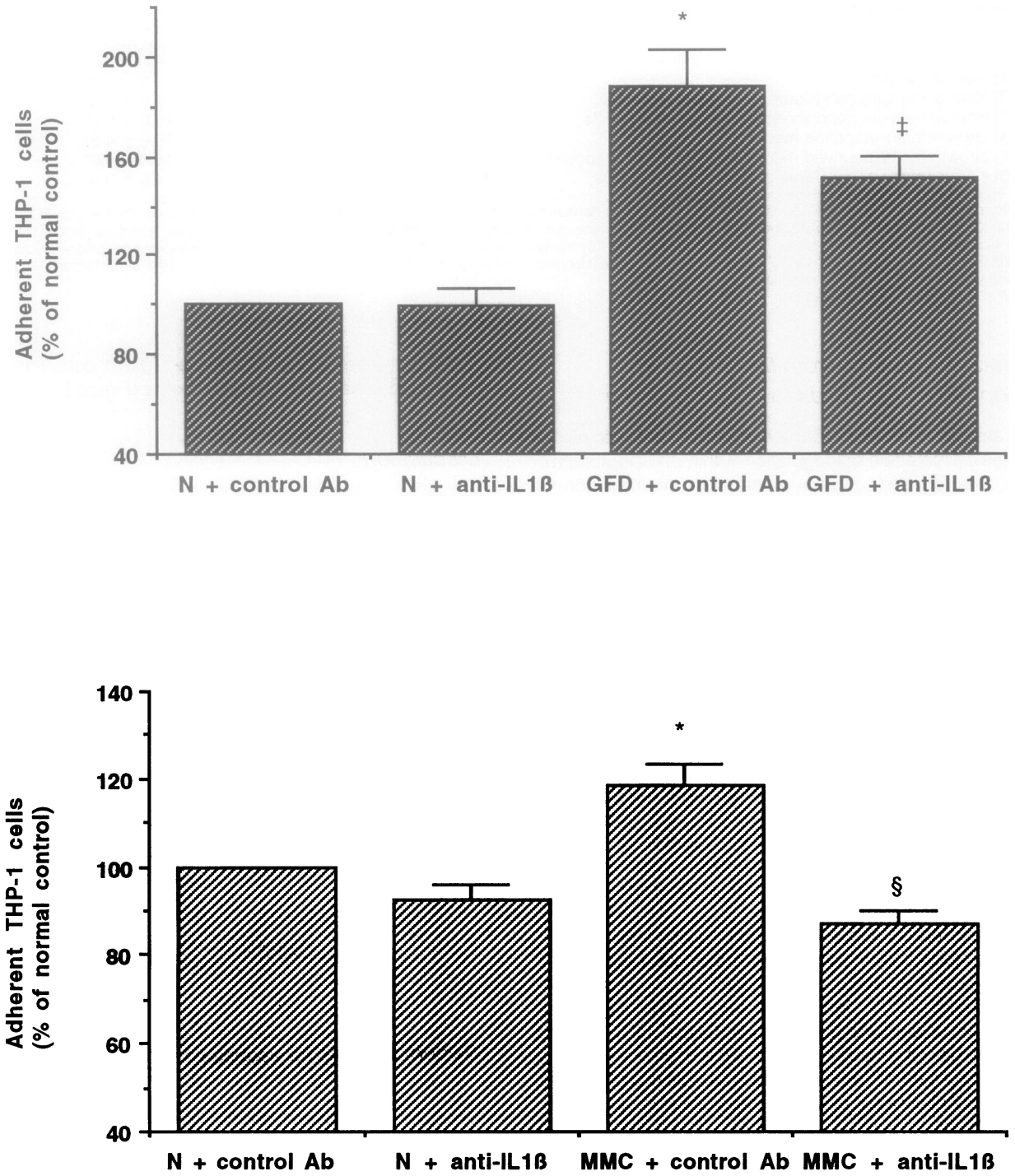

Figure 6. Effect of IL-1β blockade on endothelial hyperadhesiveness associated with induction of apoptosis. A blocking anti-IL-1β antibody or control antibody was added to HUVECs concomitantly with induction of apoptosis (growth factor deprivation or MMC). After 24 hours, adhesion of THP-1 cells to HUVECs was assessed. For each experiment, the amount of THP-1 cells adherent to normal HUVECs was taken as 100%; the level of adhesion in the various experimental conditions is expressed as a percentage of the adhesion level with normal HUVECs. In both growth-factor-deprived and MMC-treated HUVECs the percentage of adherent THP-1 cells was significantly decreased with IL-1β blockade. Data are means ± SEM of at least five experiments. $^{*}P < 0.05$ when compared with normal plus control antibody; $^{\ddagger}P < 0.05$ when compared with growth factor deprivation plus control antibody; $^{\S}P < 0.05$ when compared with MMC treatment plus control antibody.

dence that the leukocyte adhesion molecules ICAM-1 and VCAM-1 facilitate monocyte infiltration of the vessel wall in this setting[1,2]; however, the mechanisms by which these adhesion molecules are initially induced in various forms of vascular injury are still being appreciated. Recently, various factors implicated in the pathophysiology of atherosclerosis, such as cholesterol oxides,[3] high glucose,[4] and ischemia[5] were found to induce apoptosis of

endothelial cells. Here, we present evidence that stimulation of endothelial cell apoptosis leads to the activation of pro-inflammatory genes and to the formation of a pro-inflammatory milieu that acts in a paracrine fashion to induce ICAM-1 and VCAM-1 expression on neighboring endothelial cells and thereby increases their adhesiveness for monocytes.

Deprivation of growth factors and induction of DNA damage were used to standardize a model of endothelial cell apoptosis because 1) they represent classical pro-apoptotic stimuli in a wide variety of cells[10] and 2) in contrast with other pro-apoptotic factors for the endothelium (TNF[26,27] and bacterial endotoxin[28,29]), they have no known direct effect on adhesion molecules, therefore allowing us to study the role of apoptosis per se on changes in adhesiveness. Induction of endothelial cell apoptosis by either deprivation of growth factors or mitomycin-induced DNA damage was associated with hyperadhesiveness of HUVEC monolayers for monocytic cells. The observed increase in monocyte adhesion to HUVEC monolayers was due to cell-cell interactions and not due to monocyte adhesion to exposed matrix elements after endothelial cell retraction or detachment. The hyperadhesiveness of HUVECs for monocytes appeared due, at least in part, to induction of endothelial ICAM-1 and VCAM-1 as determined by assessment of endothelial expression of those ligands and in functional assays using adhesion-blocking MAbs. However, as ICAM-1 and VCAM-1 blockade did not completely abrogate the hyperadhesive state associated with either GFD or MMC treatment, other ligands, yet to be defined, are also contributing to endothelial hyperadhesiveness. In this regard, phosphatidylserine expression by apoptotic endothelial cells and recognition by phosphatidylserine receptors on THP-1 cells could represent other candidates for increased adhesiveness of endothelial cells grown in pro-apoptotic conditions[30–33] and will be studied.

Using three-color flow cytometry for concomitant identification of apoptotic cells and ICAM-1 expression, we found that up-regulation of adhesion molecules occurred preferentially on surviving HUVECs. The aforementioned experiment suggested that induction of endothelial cell apoptosis is associated with the release of factors that act in a paracrine fashion to up-regulate adhesion molecule expression by adjacent surviving cells. Although programmed cell death was initially postulated to represent a noninflammatory pathway for cellular death, recent reports suggest that apoptosis can be associated with induction of pro-inflammatory genes[14–16,34] and release of pro-inflammatory cytokines[12,35] and growth factors.[36] To explore this possibility in our system, we evaluated the effect of media conditioned by apoptotic HUVECs on ICAM-1 cell surface expression by normal HUVECs. Complete medium reconstituted with RPMI plus glutamine conditioned by apoptotic HUVECs induced expression of ICAM-1 whereas complete medium reconstituted with RPMI plus glutamine and conditioned by either normal HUVECs or HUVECs undergoing primary necrosis did not modulate the expression of adhesion molecules.

Caspase-1/ICE and other members of the caspase gene family play a central role in the execution of apoptosis in many mammalian cells,[13–16] including endothelial cells.[24] In keeping with this function, we found that caspase-1/ICE expression was significantly increased in GFD and MMC-treated HUVECs in association with induction of apoptosis. A second consequence of induction of caspase-1/ICE expression is cleavage of pro-IL-1β and release of the active pro-inflammatory cytokine. In the present study, induction of apoptosis with MMC was associated with significantly higher levels of IL-1β as compared with normal medium. Although the overall increase in IL-1β levels was small, it is possible that HUVECs surviving in the vicinity of apoptotic cells are exposed to much higher concentrations of IL-1β. Therefore, to further evaluate the functional role of IL-1β in our system, a blocking anti-IL-1β antibody was used concomitantly with induction of apoptosis. IL-1β blockade was associated with a significant decrease in endothelial hyperadhesiveness, both in GFD- and MMC-treated HUVECs. This strongly suggested that IL-1β appeared to serve as an important paracrine mediator responsible for induction of HUVEC hyperadhesiveness for monocytes. However, as the hyperadhesiveness of endothelial cells grown in pro-apoptotic conditions was not completely abrogated by IL-1β blockade, other factors yet to be defined also contribute to the creation of a pro-adhesive milieu. The recent finding that ICE-deficient mice display impaired production of IL-1α, TNF-α, IL-6,[37,38] and active interferon-γ,[34] in addition to IL-1β, may suggest other likely inflammatory candidates for endothelial hyperadhesiveness. The role of these inflammatory mediators in our system is actually being tested.

In summary, we have shown that apoptosis of endothelial cells *in vitro* is associated with the establishment of a pro-inflammatory milieu leading to paracrine induction of ICAM-1 and VCAM-1 and, in turn, increased adhesion of monocytic cells. Our results raise the possibility that programmed cell death of endothelial cells could represent the initiating event leading to adhesion and transmigration of monocytic cells into the vessel wall, a feature common to various forms of endothelial injury *in vivo*. Moreover, it raises the possibility that blockade of IL-1β may prevent adhesion of mononuclear leukocytes and subsequent vascular remodeling and scarring in vascular diseases.

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