Evidence for generation of oxidants in early lesions includes (a) that the cell types present, endothelial cells, SMC, and macrophages, have all been shown to be capable of oxidizing lipids and lipoproteins in vitro,9,10 (b) lipid hydroperoxide constituents of oxidized LDL and lesions can promote per-oxidation of cellular lipids,11,12 and (c) inflammatory phago- cytes known to produce hydrogen peroxide reside in lesions.13,14 Various agents, including serum, thrombin, angiotensin II, and platelet-derived growth factor have been shown to induce tissue factor activity in SMC in vitro15; however, little is known about the effects of the lesion constituents, LDL, and reactive oxygen species on tissue factor expression in these cells. To understand further the regulation of tissue factor in SMC, we investigated the effects of LDL and hydrogen peroxide on the surface expression and activity of tissue factor.

Methods

Tissue Culture
SMC were prepared from explants of excised aortas of Sprague- Dawley rats and samples of human aorta as previously described.16 Cultures from passages 4 to 10 were used in these studies. Forty-
eight–well plates were seeded with ~25,000 cells per well in DMEM containing 10% fetal bovine serum (FBS). One day after seeding, the medium was changed to serum-free DMEM after being washed twice with PBS. All cells were in serum-free DMEM for 48 hours before the addition of the agonist of interest, as previously described.15

**Lipoprotein Isolation**

Human LDL was isolated from citrated plasma by differential ultracentrifugation between solvent density limits of 1.019 to 1.063 as previously described.12 EDTA was present (0.5 mmol/L) throughout the isolation procedure. Quality of all preparations was checked by assaying endotoxin level (<0.15 EU/mL) (Whittaker Bioproducts kit No. QCL-1000), electrophoretic mobility (Corning), and thioribarbituric acid reactivity.18,19 Preparations were assayed for total cholesterol (Boehringer Mannheim Diagnostics kit #236691) and total protein.20 Native LDL preparations were stored in 0.5 mmol/L cholesterol (Boehringer Mannheim Diagnostics kit #236691) and total protein.20 Native LDL preparations were stored in 0.5 mmol/L EDTA at 4°C until use to inhibit oxidation.

**Tissue Factor Assay**

Cell surface tissue factor activity was assessed with a 2-step amidolytic assay as previously described.16 After each well was washed twice with PBS, a reaction mixture containing 0.125 mL of phenol red-free M199, 25 mL of 2 mg/mL S-2222 (Pharmacia-ATPAR), and 20 mL containing 0.5 Factor VII units of Proplex T (Human Factor VII and Human Factor X. Baxter Biotech) was added to each well. Standards containing the same reaction mixture with varying amounts of rabbit brain thromboplastin (Sigma) were also prepared. One unit of tissue factor activity was defined as the amount of activity contained in 0.1 mL of resuspended rabbit brain thromboplastin. The reaction mixture remained on cell layers for ~20 minutes. Aliquots of the media were pipetted into 96-well plates and read, along with the standards, on a spectrophotometer at 405 nm. The tissue factor activity in each well was then calculated by use of the standard curve.

**Northern Hybridization**

Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method.21 Samples of total RNA (10 μg) were separated on a 1% agarose/2.2 mol/L formaldehyde gel and subsequently blotted onto Magna nylon membrane with 20 × SSC by capillary transfer according to previously published methods.22 The RNA was cross-linked to the membrane with an ultraviolet cross-linker (Stratagene). The blots were prehybridized for 2 to 6 hours at 42°C in 50% formamide, 1% SDS, 5 × SSC, 1 × Denhardt’s solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), 0.25 mg/mL denatured salmon sperm, and 50 μg/mL sodium phosphate (pH 6.5) and then hybridized with 2 × 106 cpm/mL of (α-32P) dCTP radiolabeled cDNA plasmid probe for rat tissue factor (generous gift from Dr Mark Taubman, Mount Sinai School of Medicine, New York, NY) at 42°C for 16 to 24 hours. After hybridization, blots were washed with 0.1% SDS, 2 × SSC for 30 minutes at 65°C, followed by 2 washes with 0.1% SDS, 0.1 × SSC for 30 minutes at 65°C. The blots were then exposed to XAR-5 x-ray film with intensifying screens at -70°C. Expression of glyceraldehyde 3-phosphate dehydrogenase mRNA was used as an internal control for the quantity of total mRNA on each lane of the gel, and this control was applied in all experiments.

**Western Blot Analysis for Cell Surface Tissue Factor**

After exposure of cells to a given condition, cell layers were washed twice with PBS; rabbit anti-human tissue factor polyclonal antibody (American Diagnostics, Greenwich, Conn) at 0.5 μg/mL in DMEM was added to cell layers at 4°C for 2 hours to bind only cell surface tissue factor. Total cellular protein was extracted in ice-cold RIPA buffer containing protease inhibitors (leupeptin, phenylmethylsulfonylfluoride, pepstatin). Surface tissue factor was immunoprecipitated from 20 μg of total cellular protein (modified Lowry protocol20 by incubating with 20 μL of protein A sepharose-linked beads (Sigma) for 2 hours at room temperature. Protein A sepharose beads were washed twice with RIPA buffer, then incubated with 2×lamelli buffer with β-mercaptoethanol and heated for 3 minutes at 80°C. Supernatants from the beads were analyzed by SDS-PAGE with 10% acrylamide gels. Gels were blotted to membranes. Membranes were blocked with 5% milk powder and 0.1% Tween for 30 minutes, followed by 45 minutes of primary antibody (0.5 μg/mL rabbit anti-human tissue factor. American Diagnostics) in PBS containing 5% milk powder and 0.1% Tween and then washed 3 times in PBS and 0.1% Tween for 10 minutes. A 1:4000 dilution of a peroxidase-labeled secondary antibody (goat anti-rabbit IgG, Boehringer Mannheim, Indianapolis, Ind) was then added for 45 minutes. The blot was washed an additional 3 times and the signal was developed on film after reacting with ECL (Amersham, Buckinghamshire, England) with exposure to film for 10 to 30 seconds.

**Mutant Tissue Factor Preparation and Transfection**

For transfection of wild-type tissue factor cDNA, rat full-length cDNA in pBluescriptSK (Stratagene) was subcloned into PCDNA3 vector (Invitrogen). To obtain mutant tissue factor without the cytoplasmic tail, the coding for the cytoplasmic cysteine (residue 276) was mutated to a stop codon with the following oligonucleotides and polymerase chain reaction amplification of the full-length rat cDNA of tissue factor in pBluescript: forward: 5′-GGG GAT CCG ACA TGA CCT TCC CCA TGC GC-3′; reverse: 5′-GGG GAT CCT CAC AGA GAT ATG GTC AGC AG-3′. The polymerase chain reaction product was subcloned into the BamHI site of PCDNA3, and the orientation of the insert was determined by restriction analysis. The forward construct was in vitro translated to verify the product by translation into TnT-coupled reticulocyte lysate (Promega).

Transient transfections were carried out in quadruplicate by incubating cells with 1 μg/well of PCDNA3 construct (6-well plates) and 2 μL/mL of lipofectamine (Gibco-BRL) overnight. Cell layers were then washed with DMEM and incubated with DMEM containing 10% FBS for 24 hours. Cells were made quiescent by 48 hours in serum-free DMEM. Hydrogen peroxide (0.5 mmol/L) was then added to the appropriate wells for 2 hours before assay for surface tissue factor activity as outlined above.

To evaluate the efficiency of transfection 0.2 μg of pRSV β-galactosidase was cotransfected. The β-galactosidase activity in the cell layer was assayed with the use of Galactolight plus (Tropix) to normalize the measured tissue factor activity for transfection efficiency.

**Statistics**

Data are presented as mean±SD. All comparisons were made with the use of ANOVA and the statistical package SPSS 8.0 (SPSS Inc.).

**Results**

**Effects of LDL and Hydrogen Peroxide on Surface Tissue Factor Activity**

Quiescent rat aortic SMC were incubated with LDL (200 μg protein/mL), H2O2 (1 mmol/L), or FBS (10%, used here as a positive control) for 4 hours with the use of a protocol previously used to examine tissue factor induced by serum and other growth factors.15 FBS and hydrogen peroxide significantly increased surface tissue factor activity, but LDL did not (Figure 1). LDL also did not markedly increase tissue factor activity after exposures of up to 24 hours (data not shown).

Hydrogen peroxide caused a marked increase in surface tissue factor activity in a time- and concentration-
dependent manner (Figure 2). No grossly apparent morphological changes suggestive of cell injury were noted by light microscopy at H2O2 concentrations of ≤1 mmol/L. The hydrogen peroxide–induced increase in surface tissue factor activity was maximal at 2 hours (Figure 2A). In contrast, tissue factor activity in response to FBS was maximal at 4 to 6 hours, consistent with previous reports by others.15,23 These distinct kinetics suggested that hydrogen peroxide and serum caused an increase in cell surface tissue factor activity by different mechanisms.

Effects of LDL and H2O2 on Tissue Factor mRNA and Surface Protein Expression

To understand further the mechanism of tissue factor induction by H2O2, Northern blot analysis of tissue factor mRNA was undertaken. FBS and LDL significantly increased steady-state tissue factor mRNA in rat aortic SMC at 90 minutes, but H2O2 did not (Figure 3), even at concentrations that markedly increased tissue factor activity. Furthermore, H2O2 did not increase tissue factor mRNA levels at 30 or 60 minutes (data not shown).

Antibody to rat tissue factor was not available. However, we were able to verify that human and rat SMC responded similarly in our system. H2O2 but not LDL enhanced cell surface activity in both species of SMC (data not shown). We therefore used human SMC to measure cell surface tissue factor protein by Western blot analysis after immunoprecipitation with anti-human tissue factor antibody (Figure 4). Tissue factor protein was found to be present on the surface of untreated SMC. Both FBS and LDL caused an increase in surface tissue factor protein expressed on SMC surface at 4 hours; H2O2 did not.

The above results suggested that LDL increased tissue factor gene expression, protein synthesis, and expression of inactive tissue factor protein on the cell surface. In contrast, H2O2 did not induce gene expression or increase tissue factor protein on the cell surface. Rather, H2O2 appeared to activate a latent form of surface tissue factor protein. To test this hypothesis, we treated cells with LDL for 4 hours to increase tissue factor surface protein and

**Figure 1.** Hydrogen peroxide but not LDL significantly increases cell surface tissue factor activity: Cell surface tissue factor activity in quiescent rat aortic SMC 4 hours after addition of LDL (200 μg protein/mL), FBS (10%), or hydrogen peroxide (1 mmol/L). Data represent mean±SD of 4 wells per treatment. *P<0.05 compared with no treatment (NT) or native LDL.

**Figure 2.** Hydrogen peroxide significantly increases surface tissue factor activity in a time- and concentration-dependent fashion. A. Quiescent cells were treated with hydrogen peroxide (0.5 mmol/L, ●) or FBS (10%, ○) for the specified periods of time before assessment of cell surface tissue factor activity. *P<0.05 compared with 0 hours. B. Hydrogen peroxide was added at specified concentrations and tissue factor surface activity was assayed 2 hours later. Concentrations >1 mmol/L (not shown) resulted in morphological changes in SMC suggestive of cell injury at 2 hours. Data represent mean±SD of 4 wells per treatment.

**Figure 3.** LDL but not hydrogen peroxide significantly increases tissue factor mRNA levels at 90 minutes: Northern blot analysis of tissue factor mRNA 90 minutes after addition of FBS (10%), LDL (200 μg protein/mL), or H2O2 (0.5 mmol/L) or in the absence of these treatments. Duplicate blots were hybridized for GAPDH to verify equal mRNA loading.

**Figure 4.** LDL but not hydrogen peroxide significantly increases surface tissue factor protein at 4 hours: Western blot analysis of immunoprecipitated human SMC tissue factor protein 2 hours after addition of H2O2 (0.5 mmol/L) or 4 hours after addition of FBS (10%) or LDL (200 μg protein/mL) or in the absence of these treatments (NT). Rabbit brain thromboplastin (RBT) is shown as a standard.
added H₂O₂ for the final 2 hours in an attempt to "activate" the LDL-enhanced level of latent tissue factor protein. The cells that received combined treatment with LDL and H₂O₂ had significantly more surface tissue factor activity than those treated with H₂O₂ alone (Figure 5). As expected, LDL alone did not increase activity.

Role of Cytoplasmic Domain of Tissue Factor on H₂O₂ Activation

In an initial step to explore the molecular mechanism underlying H₂O₂ activation of surface tissue factor protein, we evaluated the role of the cytoplasmic tail of the tissue factor molecule. That the cytoplasmic tail would contain sites for H₂O₂ regulation was suggested by reports that the cytoplasmic tail contains a cysteine residue near the inner leaflet of the plasma membrane that is palmitoylated²⁴,²⁵ In addition, the cytoplasmic tail has been shown to contain at least 3 phosphorylation sites,²⁶ the significance of which are unknown. We therefore tested whether hydrogen peroxide activation required regulatory elements in the cytoplasmic tail. We made and expressed in bovine aortic endothelial cells (BAEC) and bovine aortic SMC (BASMC) cDNA encoding wild-type human tissue factor and a mutant tissue factor TF(1-244), which lacked the cytoplasmic domain from cysteine 245 (residues 245 to 263). We chose bovine cells because it has previously been demonstrated that human factors VII and X, used in our activity assay, are poor substrates for bovine tissue factor.²⁷ We confirmed that BASMC and BAEC had minimal detectable basal and no detectable FBS-inducible or hydrogen peroxide-inducible surface tissue factor activity (Figure 6). BASMC transfected with cDNA for the wild-type or the mutant tissue factor yielded significant tissue factor activity (Figure 7). Cells transfected with cDNA for the mutant in the reverse orientation did not. Transfectants expressing either wild-type or the mutant cDNA lacking the cytoplasmic domain of the tissue factor molecule exhibited enhanced tissue factor activity with H₂O₂ treatment, demonstrating that activation does not require the cytoplasmic tail. Similar results were obtained in BAEC (Figure 7).

Discussion

Tissue factor is expressed on SMC and macrophages in human atherosclerotic lesions,¹³–⁵ and it has been proposed that the resulting increase in local thrombin production could play a pivotal role in the development of thrombus in acute coronary syndrome⁵ or promote smooth
muscle proliferation in atherosclerotic lesion growth or in restenosis after angioplasty. The goals of this study were to evaluate the effects of LDL and hydrogen peroxide on tissue factor gene expression and cell surface activity in aortic SMC. The concentration of LDL used to induce tissue factor mRNA and surface protein levels in our studies was well within a range that would be available in the interstitial fluid of an early lesion site in the arterial intima. Although the local vascular tissue concentration of hydrogen peroxide at the site of production by macrophages is unknown, the concentration range over which we observed tissue factor activation is similar to the range used by others to induce other biological functions in SMC, such as proliferation and alterations in calcium homeostasis.

Northern and Western blot analysis revealed that incubation of SMC with LDL increased the steady-state level of tissue factor mRNA and cell surface protein. Surprisingly, however, LDL did not markedly increase SMC surface tissue factor activity. In contrast, incubation of SMC with H_2O_2 increased surface tissue factor activity but caused no change in tissue factor mRNA or protein. It appears, therefore, that tissue factor may exist on the cell surface in an inactive state and that its activation may represent a separate, regulatable event. These findings have led us to propose a novel, 2-step pathway by which SMC tissue factor surface activity can be increased, a pathway that may have pathological consequences in early vascular lesion sites or vascular injury sites. The first step begins at the mRNA level, with LDL causing an increase in the accumulation of tissue factor mRNA leading to increased tissue factor protein synthesis and expression on the cell surface. The second step involves an H_2O_2-mediated posttranslational modification that activates the latent cell surface tissue factor. In this proposed pathway, tissue factor may exist on the cell surface in an inactive state and require activation before participation in the initiation of the extrinsic pathway.

The concept that tissue factor can exist in an inactive form has been proposed previously in a variety of contexts. Suggestions to explain the observations have included (a) that cellular pools of cryptic tissue factor exist, for example, in caveolae; (b) that activity is controlled by tissue factor interactions with tissue factor pathway inhibitor (TFPI); or (c) that activation of latent tissue factor can be regulated by changes in tissue factor quaternary structure. Several instances of cell perturbation, including mild trypsin treatment, sublytic detergent treatment, and apoptosis-related cell changes have been reported to enhance the activity of apparently latent tissue factor in various cell systems. Our results reveal that H_2O_2 also has the capacity to enhance activity of a preexisting inactive pool of cell surface tissue factor.

Oxidant effects on tissue factor have been previously reported; however, these are distinct from the sequence of events that we have observed. For example, Golino et al. reported tissue factor induction by xanthine:xanthine oxidase in rabbit endothelial cells. However, their studies showed that reactive oxygen species induced synthesis of new tissue factor protein, distinguishing this from the oxidant pathway that we observed in SMC. In addition, copper treatment of human monocyctic THP-1 cells yielded an antioxidant-inhibitable increase in tissue factor mRNA, protein, and activity. These studies, too, are distinct from the effects we have observed, in that in our SMC cultures the oxidant H_2O_2 did not increase mRNA over a wide range of time and concentration.

To begin to define a mechanism for the H_2O_2-mediated activation, we hypothesized that the increased activity was due to the oxidant influencing regulatory elements of the cytoplasmic tail, for example, one of the phosphorylation sites or the palmitoylated cysteine. Others have demonstrated that the cytoplasmic tail of tissue factor can be phosphorylated and that these sites are highly conserved among diverse species. The cysteine residue can be palmitoylated and is a potential site for regulating molecular interactions between tissue factor and the plasma membrane. Our results confirmed that the cytoplasmic tail is not required for tissue factor activity, but they further demonstrated that the cytoplasmic tail is not necessary for the hydrogen peroxide–mediated increase in surface tissue factor activity.

This negative finding is important because it confines H_2O_2 regulation of tissue factor activity to the transmembrane or, more likely, the extracellular domain. Hydrogen peroxide may act, for example, by altering the factor VII or factor X binding sites or by altering the local membrane milieu. The activity of tissue factor is known to be affected by the negative charge of the local phospholipid composition in the membrane. Phospholipids with a negative charge, such as phosphatidylserine and phosphatidylethanolamine, are thought to increase the delivery of factor X to the tissue factor:factor VIIa complex. Certain oxysterols have been shown to increase tissue factor activity in monocyctic THP-1 cells by increasing the amount of phosphatidylserine in the outer leaflet. However, hydrogen peroxide treatment has been shown not to increase outer membrane phosphatidylserine content in at least one cell system, the red blood cell, casting doubt on whether our observations are a result of such a membrane alteration.

TFPI is a glycoprotein known to bind to tissue factor and block its interaction with factors VII and X. It is possible that LDL, known to contain TFPI, could deliver TFPI to the SMC, blocking the activity of tissue factor, and that H_2O_2 may cause dissociation of surface tissue factor from TFPI. Oxidation of LDL in free solution has been reported to decrease TFPI activity. However, in our studies, H_2O_2 enhanced tissue factor surface activity even in the absence of added LDL (Figure 2), indicating that activation by H_2O_2 can occur in the absence of exogenously delivered TFPI. It is also possible that there is endogenous TFPI on the surface of SMC. TFPI has been reported on SMC surfaces and the kidney mesangial cell, a related mesenchymal cell, has been shown to produce TFPI. Further studies will be necessary to elucidate whether H_2O_2-mediated disruption of tissue factor–TFPI interactions on the SMC surface is responsible for our observations;
however, such an outcome would be consistent with our observations that activation is an extracellular event.

We have made 2 novel observations in SMC related to the expression of tissue factor. We have shown that LDL increases tissue factor protein production and expression on the surfaces of both rat and human aortic SMC without an increase in tissue factor activity. We have also demonstrated for the first time that the extrinsic pathway of blood coagulation can be regulated at the cell surface by hydroperoxide. These findings may help to explain the observations that significant tissue factor expression is found in human atherosclerotic lesions, in which SMC, LDL, and H$_2$O$_2$-producing macrophages are all present in abundance. Furthermore, the activation of latent tissue factor by reactive oxygen species may help to explain procoagulant events in reperfusion and coronary ischemia.

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Marc S. Penn, Chandrashekhar V. Patel, Mei-Zhen Cui, Paul E. DiCorleto and Guy M. Chisolm

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