Tumor Necrosis Factor-α Downregulates Protein S Secretion in Human Microvascular and Umbilical Vein Endothelial Cells But Not in the HepG-2 Hepatoma Cell Line

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Protein S deficiency, which is associated with thrombosis, can either be inherited or acquired. Recently, we reported that a decrease in free protein S was observed in 19 of 25 persons with HIV/AIDS. The proinflammatory cytokine, tumor necrosis factor-α (TNF-α), has been reported to be elevated in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients and has been shown to induce a procoagulant state on the surface of endothelial cells. We report here that recombinant TNF-α (rTNF-α) downregulated protein S synthesis in the SV-40T transfectant human microvascular endothelial cell line (HMEC-1) model system by approximately 70% and in primary human umbilical vein and dermal microvascular endothelial cell cultures by approximately 50%. Using the HMEC-1 model, Northern blot analysis showed a decrease in protein S RNA at 24 hours that was corroborated by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) quantitation. Evidence supporting the specificity of the TNF-α effect included the following: (1) TNF-α down-regulation of protein S was completely blocked by TNF neutralizing antibody; (2) the effect was transient, and protein S was restored to near normal levels after TNF was removed from cell cultures; (3) an antibody directed to the TNF RI (55-kD receptor) was shown to mimic the action of TNF-α on HMEC-1 cells; and (4) other proinflammatory cytokines, interleukin (IL)-1, IL-6, and TGF-β, had no effect on protein S secretion. However, TNF-α showed no regulatory control over protein S synthesis in the human hepatocellular carcinoma cell line HepG-2. We suggest that TNF-α downregulation of protein S may be a mechanism for localized procoagulant activity and thrombosis recently reported in some AIDS patients with associated protein S deficiency.

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HUMAN PROTEIN S is a 75-kD vitamin K--dependent plasma glycoprotein that acts as a cofactor for activated protein C (APC) in the anticoagulation cascade. Approximately 40% of the circulating protein S is physiologically active and is found free in the plasma, while the remainder is bound to the C4b binding protein. Even though hepatocytes are thought to be the major source of protein S production, vascular endothelial cells, megakaryocytes, osteoblasts, and neural-derived tissue synthesize significant amounts of protein S.

The physiologic relevance of protein S has been illustrated by many reports that have documented an association between recurrent thrombosis and inherited protein S deficiency. Genetic analysis of families with the inherited deficiency has shown that large DNA deletions of the protein S gene were present in some, but not all families. Other molecular studies have found point mutations in the protein S gene that have resulted in amino acid substitutions. These results suggest that any one of several DNA alterations may be responsible for an inherited functional or physical free protein S deficiency.

Inherited protein S deficiency can also be acquired. It is common in persons undergoing oral anticoagulant therapy with vitamin K antagonists, and has been reported to be associated with liver disease, pregnancy, oral contraceptive intake, infection-associated disseminated intravascular coagulation (DIC), and systemic lupus erythematosus. Although the association of acquired protein S deficiency with thrombosis is not as well documented as that of familial protein S deficiency, an increase in clinical evidence has made this association more apparent.

More recently, two reports have documented decreased protein S levels in some persons infected with the human immunodeficiency virus (HIV) and associated thrombosis. Although the mechanism(s) responsible for the decrease in protein S are currently not known, elevated plasma levels of some cytokines, e.g., tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6, are known to occur in some of the conditions associated with decreased levels of protein S such as HIV infection/acquired immunodeficiency syndrome (AIDS).

Cytokines are soluble effector proteins that are functionally involved in hematopoiesis, inflammation, hemorrhage, and the immune response. The effector function of cytokines is tightly controlled, and any dysfunction in their regulation could lead to inappropriate cytokine expression and contribute to disease pathogenesis. Because it has been suggested that cytokine production may be responsible for many of the symptoms associated with HIV, and it has been reported that some endothelial cell--derived proteins associated with coagulation and fibrinolytic pathways could be modulated in vitro by TNF and IL-1, our laboratory has begun to investigate the role of these cytokines in the regulation of protein S.

MATERIALS AND METHODS

Cell culture. The HepG-2 hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). The establishment of an SV-40T transfectant human microvascular endothelial cell line, HMEC-1, has been previously described. Both cell lines were maintained by the Biological Products Branch, Scientific Resources Program of The Centers for Disease Control and Prevention. Human umbilical vein endothelial cells (HUVEC, Clonetics, San Diego, CA) and human dermal microvascular endo-
thelial cells (HDMEC, Cell Systems Corp, Kirkland, WA) were obtained commercially and propagated using the manufacturer’s medium and accompanying instructions. Experiments performed with HepG-2 and HMEC-1 used cells at passage levels 18 to 28 and 8 to 16, respectively, while HUVEC and HDMEC were used at passage levels 3 to 7. Cells, propagated as monolayer cultures, were initially plated at a density of 0.5 to 1 x 10^5 cells/cm^2 in 2.0-cm^2 24-well plates (Costar, Cambridge, MA) or for RNA harvest, a 75-cm^2 culture flask. Once confluence was established, culture fluids were removed by aspiration and fresh media with or without added cytokine was dispensed over the cell monolayers (zero time). Cells and culture fluids were harvested at scheduled intervals for protein S RNA analysis by Northern blot and for antigen quantification by enzyme-linked immunosorbent assay (ELISA) and Western blot. Cell viability and proliferation was determined by neutral red and naphthol blue black assays as described previously. Data presented here represent measurement averages (mean ± 1 SD) from four to six replicate culture well fluids from at least two separate experimental determinations.

Cytokines. Recombinant human TNF-α, TNF-β, IL-1α, and IL-6, and highly purified TGF-β were purchased from R & D Systems, Inc. (Minneapolis, MN). The manufacturer had determined endotoxin levels to be less than 0.1 ng per 1 µg of cytokine. Unless stated otherwise, TNF and TGF-β additions to cell cultures were at 10 ng/mL of culture media. IL-1α and IL-6 were used at 5 ng/mL of culture media.

ELISA. The ELISA used to quantify protein S in cell culture fluids has been previously described. Briefly, goat antiprotein S antiserum (American Diagnostica, Greenwich, CT) fractionated on DEAE-Sephadex A50 (fall through after equilibration in 0.1 mol/L citrate-phosphate buffer, pH 5.0 containing H2O2 (0.00133%). The coated ELISA plate was blocked with 7% (wt/vol) normal goat serum, and detected with horseradish peroxidase (HRP)-conjugated rabbit antigoat IgG (Cappel, West Chester, PA) and a chemiluminescent detection system (Amersham). Bound antibody was detected with HRP-conjugated rabbit antirabbit IgG (Amersham, Arlington Heights, IL) diluted 1/1,500 in the same buffer. HRP activity was measured against o-phenylenediamine (Sigma) dissolved at 0.4 mg/mL in 0.05 mol/L citrate-phosphate buffer, pH 5.0 containing H2O2 (0.00133%). Absorbance at 490 nm was determined with an EL312e Microplate Reader (Biotek, Winooski, VT). Data reduction used the KinetiCalc software package (Biotek). Purified protein S used for calibration and as a positive control was obtained from American Diagnostica and has been previously characterized.

Western blot. A nonreducing 8% gel was charged with 30 µL of media from cell cultures that had been incubated without and with TNF for the time periods indicated. Following electrophoresis, the gel was transblotted onto ECL nitrocellulose paper (Amersham) by using BioRad (Richmond, CA) gel and transblot apparatus, and incubated with goat antiprotein S antiserum (1:1,000; American Diagnostica). Bound antibody was detected with HRP-conjugated rabbit antigoat IgG (Cappel, West Chester, PA) and a chemiluminescent detection system (Amersham).

Northern blot. Total cellular RNA was isolated using the TRI Reagent Kit (Molecular Research Center, Inc, Cincinnati, OH) according to the manufacturer’s protocol. Following electrophoresis in 1% agarose gel containing 0.67 mol/L formaldehyde, RNA was transferred and cross-linked to nylon membranes (MSI, Westbord, MA). An 810-bp polymerase chain reaction generated protein S fragment was 32P-labeled by random priming (Pharmacia, Piscataway, NJ). The labeled probe was subsequently purified by being passed through a Sephadex G-50 column. Hybridization and posthybridization washes were performed as previously described.

RESULTS

Protein S was constitutively synthesized and released by the three different endothelial cell cultures, and the increase was almost linear with time. Figure 1 shows typical relative...
DOWNREGULATION OF PROTEIN S BY TNF

Fig 2. Specificity of TNF-α downregulation effect on endothelial cell (HMEC-1) protein S production. (A) TNF-α downregulated protein S in a dose-dependent manner (0 ng/mL; ○; 0.1; △; 1.0; ▽; 5.0; ▼; 10; □; 50; △); and was not cytotoxic to the HMEC-1 cell line (●), and TNF downregulation was (B) abrogated with the addition of anti-TNF-α neutralizing antibody (untreated; ○; TNF; △; 2 μg antibody + TNF, ▽; 50 μg antibody + TNF, ▼). (C) The TNF-α effect on protein S levels in HMEC-1 culture fluids was not observed with other inflammatory cytokines. IL-1; △; IL-6; ▽; TGF-β; ▼. TNF-β (△) was observed to downregulate protein S, but not to the same extent as TNF-α (●).

amounts of antigen accumulation in culture fluids in nanograms per 10^6 cells. In the majority of experiments, data were collected over a 96-hour incubation period and protein S concentration expressed as nanograms per milliliter of culture fluid.

The extent of TNF-α-induced downregulation of protein S in HMEC-1 is illustrated in Fig 2. Dose-response experiments (Fig 2A) demonstrated that a TNF-α concentration as low as 0.1 ng/mL could reduce protein S levels. However, at the concentration of 0.1 ng/mL, the reduction in protein S antigen was 20% less than control cultures at 72 and 96 hours, whereas at higher concentrations marked reductions were present by 48 hours. Maximal downregulation of protein S (~70%) was at 72 hours when the TNF-α concentration was between 5 and 10 ng/mL. A TNF-α concentration of 50 ng/mL had no further effect. Cell viability (Fig 2A) and proliferation (data not shown) were not affected by any concentration of TNF-α used. Antibody neutralization experiments (Fig 2B) demonstrated the specificity of TNF-α in the downregulation of protein S. The anti-TNF neutralizing antibody preparation at 2 μg/mL abolished the inhibitory effects of TNF-α and had no adverse effects on the cell culture when used alone at 50 μg/mL. Functionally related cytokines, IL-1α and IL-6, had no effect on protein S production in the HMEC-1 cell line (Fig 2C). Another multifunctional cytokine, TGF-β, had no effect on protein S secretion. Although TNF-β or lymphotoxin did exhibit an inhibitory effect on protein S production, this inhibition was about 50% of that experienced with TNF-α (Fig 2C). The combination of TNF-α and TNF-β did not result in either additive or synergistic inhibition (data not shown), and the inhibition observed was the same as that with TNF-α alone. TNF-β downregulation of protein S was also dose-dependent; the effect was seen maximally at 5 to 10 ng/mL.
translated, while RNA for the low-density lipoprotein receptor was upregulated (Fig 5). Similar observations have previously been reported.43,44

Western blot analysis of HMEC-1 protein S synthesis demonstrated a successive decrease in the 75-kD protein S antigen as a function of TNF-α treatment over time (Fig 6). The decrease in protein S relative to that in the control was striking at 48 hours and persistent at 72 and 96 hours. The decrease in protein S antigen seen in Western blot and ELISA was corroborated by the decrease of protein S RNA in Northern blot analysis (Fig 7). Analysis of RNA levels showed a biphasic pattern of expression in which TNF-α treatment induced an increase in transcript levels at 30 minutes and at 6 hours as compared with those in the control. This increase was followed by a marked decrease of protein S message at 24 and 72 hours (Fig 7A). This biphasic increase was not due to unequal RNA loading because ethidium bromide staining demonstrated equal amounts in each lane (Fig 7B).

Wash-out experiments documented that the continuous presence of TNF-α was required to suppress production of protein S. Protein S levels rebounded after TNF-α was washed out after 24 hours and fresh medium was added (Fig 8). Similar results were seen when TNF-α was washed out after 12, 48, and 72 hours (data not shown).

An antibody (AB225-PB) against the 55-kD TNF receptor, described by the manufacturer to have agonist activity on the human A549 cell line, was found to act as a TNF agonist on HMEC-1 (Fig 9). The antibody-mediated down-
regulation of protein S was nearly identical to the cytokine-induced inhibition. No additive or synergistic effects were observed when TNF-α and the antibody agonist were combined (data not shown). The TNF agonist had no effect on the HepG-2 cell line.

Addition of vitamin K, alone or in combination with TNF, did not alter the levels of protein S production from that measured in untreated or TNF-treated HMEC-1 cells.

DISCUSSION

This study has demonstrated that the proinflammatory cytokine TNF-α can negatively regulate the anticoagulant co-factor, protein S, in the SV-40T-transfected human microvascular endothelial cell line, HMEC-1. This downregulation was seen at the transcriptional and posttranscriptional levels. The negative regulation of protein S appeared to be TNF-specific; other functionally related cytokines, IL-1α and IL-6, had no effect. Another multifunctional cytokine, TGF-β, also had no effect on protein S production in the HMEC-1 cell line. Furthermore, neutralizing anti-TNF antibodies...
abolished the inhibitory effect of TNF-α. That TNF-β or lymphotixin also inhibited protein S secretion was not surprising, because TNF-α and TNF-β are known to share the same receptors (TNF-R55 and TNF-R75) and to have similar affinity. This decrease was approximately 50% of that seen with TNF-α and the difference in inhibition can perhaps be explained by postreceptor signaling pathways that are unique to each receptor and cytokine/receptor interaction. The TNF-α effect was not permanent; by removing TNF-α, protein S secretion returned to near-normal levels. When expanded from the HMEC-1 model, TNF was shown to significantly downregulate protein S synthesis in low-passage, non-transfected HUVEC and HDMEC cultures, indicating that this observation of TNF dysregulation of protein S synthesis may represent a more general phenomenon with implications for in vivo vascular procoagulant activity.

In contrast to its effect in the HMEC-1 cell line and primary endothelial cell cultures, TNF-α had no measurable effect on protein S production in the HepG-2 hepatoma cell line. However, the HepG-2 cells were responsive to TNF-α, as demonstrated by its effect on the LDL-receptor and its own TNF-R55-kD receptor.

Earlier studies have shown that antibodies against the TNF-α receptors (55 kD and 75 kD) can mimic the actions of TNF-α. Our results have demonstrated that an antibody against the 55-kD TNF-receptor was similar to TNF-α in downregulating protein S. This observation is of potential significance because it provides another TNF-related mechanism for the downregulation of protein S in the absence of TNF. Furthermore, this antibody-mediated event may have a more prolonged physiologic effect, since its half-life and stability may be greater than those of TNF.

Serum levels of TNF and IL-1 have been found to be elevated in patients with either bacterial or viral infections. Current evidence suggests that these cytokines are not only a consequence of infection, but also play a role in disease progression. Not surprisingly, increased serum levels of TNF and IL-1 have been documented in many patients infected with HIV. Two recent studies have shown that plasma levels of total protein S are also decreased in a number of individuals with HIV/AIDS. One study found that out of 25 HIV-positive patients 19 had decreased protein S levels and these of 19 had a history of thrombosis following the onset of HIV positivity. This coagulation abnormality was reportedly associated with an overall decrease in free and total protein S rather than to changes in the normal physiologic distribution of free and bound protein S, which may occur in response to elevated levels of the acute-phase reactant C-reactive protein.

A known source of protein S, the endothelium is responsive to TNF and IL-1 exposure and investigators have shown that these cytokines can modulate endothelial cell derived proteins of the coagulation cascades in nearly identical fashion. However, we did not see a similar effect on protein S production with IL-1 in the HMEC-1 cell line. The lack of an IL-1 response was not due to the absence of functional IL-1 receptors, since we have determined in other studies that PAI-1, leukemia inhibitory factor, and IL-6 RNA were induced by IL-1 in the HMEC-1 cells (unpublished data), as is the case with primary endothelial cell cultures.

Our studies are the first, that we are aware of, to show a direct correlation between TNF and the downregulation of the anticoagulant cofactor protein S in an endothelial cell line and primary HUVEC and HDMEC cultures. These results may indicate a possible mechanism for the decreased protein S levels and associated thrombosis seen in some AIDS patients. Furthermore, the HMEC-1 cells may serve as a convenient model for the study of regulatory mechanisms for protein S and other endothelial cell-derived proteins.

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