

INTERFERON  $\gamma$  INDUCES SYNTHESIS OF COMPLEMENT  
ALTERNATIVE PATHWAY PROTEINS BY HUMAN  
ENDOTHELIAL CELLS IN CULTURE

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Vascular endothelial cells, which form the interface between blood and solid tissues, interact with circulating leukocytes, including T lymphocytes, monocytes, and polymorphs and can mediate antigen presentation. Some of the factors involved in endothelial cell-leukocyte interaction or its regulation have been elucidated. These include ICAM-1 and the leukocyte adhesion family of molecules (1). The lymphokines IL-1, TNF, and IFN- $\gamma$  are known to stimulate differentially the interaction of various leukocyte types with endothelial cells in culture (2, 3). The involvement of the complement system and its receptors in mediating contact of other cell types with endothelia has not been widely investigated, although endothelial cells are known to express decay-accelerating factor (DAF) (4) and a C1q-binding activity (5). In this study, we present evidence that endothelial cells contain mRNA for complement factors H, I, and B, and that specific mRNA levels and factor H secretion are profoundly altered by IFN- $\gamma$ .

Materials and Methods

*Endothelial Cells.* Human umbilical vein endothelial cells (HUVEC) isolated according to Jaffe et al. (6) were established by Professor N. Kefalides at the Sir William Dunn School of Pathology, Oxford. Cells were grown in  $\alpha$ -MEM with 10% FCS, 20  $\mu$ g/ml endothelial cell growth supplement (No. E9005; Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, 90  $\mu$ g/ml heparin (Sigma Chemical Co.), 2.5  $\mu$ g/ml fungizone, 50  $\mu$ g/ml penicillin, 50  $\mu$ g/ml streptomycin. Cell culture reagents were from Gibco Laboratories, Paisley, Scotland or Flow Laboratories, Irvine, Scotland. Cells were grown to confluence in 75-cm<sup>2</sup> flasks coated with 1% gelatin. Confluent cells were subcultured using trypsin-EDTA, and cells in the fifth through tenth passage were used for experimental work. Cells were incubated with IFN- $\gamma$  (100 IU/ml), IL-1 (100 IU/ml), LPS (10  $\mu$ g/ml; from *Salmonella abortus equi*; Sigma Chemical Co.) or TNF (100 IU/ml) for 2 or 3 d before harvest. IFN- $\gamma$  and TNF were recombinant human proteins isolated from *Escherichia coli* produced by Genentech Inc. (San Francisco, CA) and supplied by Boehringer Ingelheim, Vienna, Austria. IL-1 was human rIL-1 $\alpha$  purified from *E. coli*, a gift from Dr. P. T. Lodemico, Hoffman-La Roche Inc., Nutley, NJ.

*Solubilization of Cells.* Confluent HUVEC were solubilized by either of two methods. For

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Western blotting and after biosynthetic labeling, cells were detached from the flask using a rubber "policeman" in the presence of lysis buffer (10 mM sodium phosphate, pH 7.5, containing 0.15 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 1% vol/vol NP-40, 100 µg/ml soybean trypsin inhibitor (Sigma Chemical Co.), 10 mM iodoacetamide (Sigma Chemical Co.), 2.5 mM diisopropylfluorophosphate (Sigma Chemical Co.), 2.5 µg/ml pepstatin A (Sigma Chemical Co.) and 20 µM 1,10 phenanthroline. The mixture was incubated 30 min at 0°C, centrifuged (20 min, 13,000 g), and the supernatant was frozen at -70°C. For Northern blot experiments cells were collected by trypsinization, washed four times with PBS at 4°C, then RNA was prepared as before (7). The procedure included a CsCl gradient step to separate RNA from protein.

*Proteins and Affinity Reagents.* Factor H was isolated as before (7). Polyclonal rabbit anti-factor H antibodies bound to CNBr-activated Sepharose were used in immunoadsorption.

*cDNA Probes and Northern Blotting.* Northern blotting was done as before (8). RNA was denatured using formamide and formaldehyde, and electrophoresis was done on gels containing formaldehyde. cDNA probes used were as follows: B38-1, encoding the NH<sub>2</sub> terminal one-third of factor H (7); a C4bp probe (8); a full-length factor I-specific probe (9), kindly provided by Dr. C. Catterall, Celltech Ltd., Slough, England; the factor B probe pFB-3b (10); the CR3-β chain probe, J-9 (11), and the full-length C1q-B chain probe (12), provided respectively by Drs. R. D. Campbell, S. -K. A. Law and K. B. M. Reid, MRC Immunochemistry Unit, Oxford, England. Probes were labeled with <sup>32</sup>P by nick translation or random priming. Human liver mRNA, prepared as described (7), was used as a positive control in Northern blotting. Autoradiographs of blots were analyzed using an LKB Produkter (Bromma, Sweden) laser densitometer.

*SDS-PAGE and Western Blotting.* Samples of endothelial cell culture supernatants and solubilized cell extracts were analyzed on 7.5% SDS-polyacrylamide gels and blotted onto nitrocellulose (Hybond C; Amersham International, Amersham, UK). Blots were developed using rabbit polyclonal anti-factor H and alkaline phosphatase-conjugated second antibodies.

*Biosynthetic Labeling of Cells and Immunoadsorption.* HUVEC were grown to confluence, washed with cysteine-free medium, and incubated (30 min, 37°C) in the same medium. L-[<sup>35</sup>S]cysteine (sp act, 120 mCi/mmol; Amersham International) was added (250 µCi/75-cm<sup>2</sup> flask), and cultivation was continued for 12 h. Culture supernatants and cells were collected as described above, the cells were lysed, and culture supernatant (5 ml) or soluble cell extracts (5 ml) were incubated (16 h, 4°C) with 0.7 ml (packed volume) Sepharose-anti-factor H. The affinity resin was washed, and bound material was eluted and analyzed by SDS-PAGE as described previously (13). Cysteine-free medium was made using a MEM-Selectamine Kit (Gibco Laboratories) and dialyzed FCS.

## Results and Discussion

*Detection of Factor H and Factor H mRNA.* Northern blotting of endothelial cell total RNA (Fig. 1) with the factor H probe B38-1 showed that the characteristic 4.3-kb and 1.8-kb factor H mRNA species were detectable in HUVEC grown under standard conditions (Fig. 1, lane *b*). The presence of IFN-γ in the medium caused a six- to sevenfold increase (as judged by densitometry of the autoradiograph) in both mRNA species (Fig. 1, lane *c*). The quantity of the H-specific 4.3-kb mRNA was high in IFN-γ-stimulated cells, being ~20-25% of the level found in an equal weight of total liver mRNA. In contrast, factor H mRNA was not detectable in 20 µg of U937 cell poly(A)<sup>+</sup> mRNA, although these cells do express factor H (13).

The 4.3- and 1.8-kb mRNA species are of similar abundance in liver samples (Fig. 1, lane *a*; reference 7), whereas in HUVEC, the smaller mRNA is consistently present at only 10-20% of the level of the 4.3-kb species. The 1.8-kb mRNA is thought to arise by alternative splicing and encodes the first seven repeat units (~420 amino acids) of factor H, plus a short, unique COOH-terminal sequence (7). The differ-

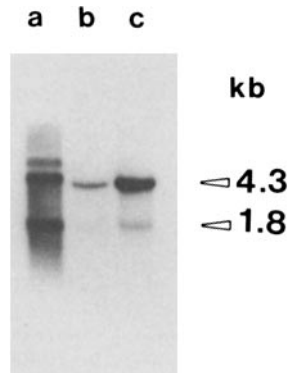


FIGURE 1. Expression of factor H by HUVEC: Northern blot analyses. Total RNA was run under denaturing conditions and blotted onto nitrocellulose. The filter was probed using the factor H probe B38-1. (Lane *a*) Total RNA (4  $\mu$ g) from human liver; (lane *b*) total RNA (20  $\mu$ g) from control HUVEC; (lane *c*) total RNA (20  $\mu$ g) from IFN- $\gamma$ -stimulated (100 IU/ml, 3 d) HUVEC. The 5.3-5.5-kb mRNA seen in liver RNA is thought to be a conformational artefact (7). Kilo-base markers shown.

ence in relative proportion of the two mRNAs between liver and HUVEC indicates a tissue-specific control mechanism, possibly influencing alternative splicing.

A third type of factor H mRNA (1.2-1.5 kb) occurs in liver (7) but is detectable only with 3' cDNA probes, and was not investigated here.

Incubation of HUVEC with TNF did not affect factor H mRNA levels, while IL-1 and LPS had a small suppressive effect (10-50% decrease in the level of both H mRNA species; results not shown). Cells incubated with both IL-1 (100 IU/ml) and IFN- $\gamma$  (100 IU/ml) had similar H mRNA levels to control cells, which indicates that IL-1 suppresses the effect of IFN- $\gamma$ .

The synthesis and secretion of factor H by HUVEC was confirmed by biosynthetic labeling with [ $^{35}$ S]cysteine followed by immunoadsorption with solid-phase anti-factor H antibodies (Fig. 2 *A*). Radiolabeled protein comigrating on SDS-PAGE with factor H in both reducing and nonreducing conditions was observed. The quantity of labeled protein detectable was increased when cells were incubated with [ $^{35}$ S]cysteine in the presence of 100 IU/ml IFN- $\gamma$ . Factor H was also detectable in unconcentrated culture supernatants by Western blotting (Fig. 2 *B*), and again the quantity detectable was greater in supernatants from IFN- $\gamma$ -treated cells. Factor H was detectable in detergent-solubilized cell extracts by both biosynthetic labeling and Western blotting (not shown), but it was not established whether this was cell surface-associated factor H (13), or an internal pool destined for export.

**Detection of mRNA for Other Complement Components.** Since factor H mRNA was readily detectable, and C3 secretion by HUVEC has been reported by others (14), mRNA for other alternative pathway-associated proteins was sought. Factor I mRNA was detectable in HUVEC grown under standard conditions (Fig. 3 *A*). IFN- $\gamma$  treatment increased the factor I mRNA level >10-fold. Factor B mRNA was detected in IFN- $\gamma$ -treated HUVEC, but not in unstimulated cells (Fig. 3 *B*). The level of B mRNA in IFN- $\gamma$ -stimulated cells was low, relative to the liver control (1-1.5% of the B mRNA present in an equal weight of total liver mRNA). mRNA for C1q, C4bp, and the  $\beta$  chain of CR3, p150, 95, and LFA-1 was not detected in either unstimulated or IFN- $\gamma$ -treated cells. The presence of mRNA for H, I, and B, together with demonstration of C3 secretion (14), implies that HUVEC may be able to assemble the complete alternative complement pathway. Absence of C1q and C4bp

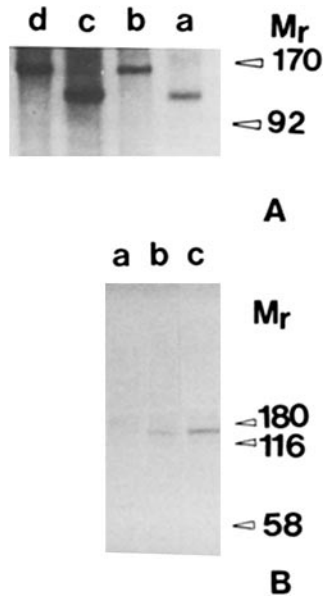


FIGURE 2. Expression of factor H by HUVEC: biosynthetic labeling and Western blot analyses. (A) HUVEC were labeled with [<sup>35</sup>S]cysteine for 12 h at confluence in the presence or absence of IFN- $\gamma$  (100 IU/ml), and the supernatant was immunoadsorbed with polyclonal anti-factor H antibodies. Specifically immunoadsorbed material was analyzed by SDS-PAGE under nonreducing and reducing conditions as described in Materials and Methods. (Lane a) Control HUVEC, nonreducing conditions; (lane b), control HUVEC, reducing conditions; (lane c), IFN- $\gamma$ -stimulated HUVEC, nonreducing conditions; (lane d) IFN- $\gamma$ -stimulated HUVEC, reducing conditions. (B) HUVEC were maintained at confluence in the presence or absence of IFN- $\gamma$  (100 IU/ml) for 3 d. The culture supernatants (100  $\mu$ l) were then analyzed for the presence of factor H by Western blotting using polyclonal anti-factor H antibodies as described in Materials and Methods. (Lane a) Medium only; (lane b) control HUVEC; (lane c) IFN- $\gamma$ -stimulated HUVEC. The gels were run under nonreducing conditions.

mRNA may indicate general lack, or a low level, of synthesis of classical pathway components and regulators.

Expression of complement components and modulation of this expression by IFN- $\gamma$  is a further characteristic common to both endothelial cells and cells of monocyte/macrophage lineage. The apparently high quantity of factor H mRNA relative to factor B mRNA in stimulated or unstimulated HUVEC may suggest that complement secretion by these cells is principally protective. High levels of factor H, together with DAF (4), may influence resistance of endothelial cells to complement attack or may reduce immune complex deposition on blood vessel walls. Complement components secreted by HUVEC may participate in local C3b deposition on material destined for uptake by phagocytes, or may be involved in C3 deposition on endothelial cells themselves. Similar phenomena of covalent C3b deposition on macrophage and monocytes have been described, and may be involved in cell-cell

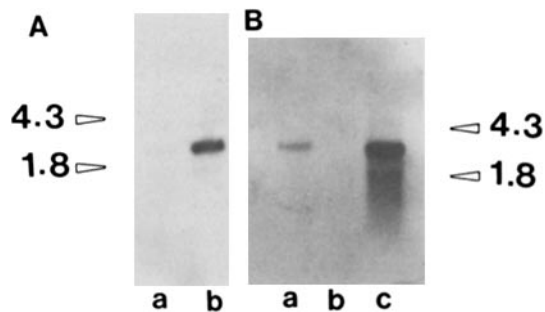


FIGURE 3. Expression of complement factor I and factor B by HUVEC: Northern blot analyses. Total RNA was run under denaturing conditions and blotted onto nitrocellulose. (A) The filter was probed using the factor I-specific cDNA probe. (a) total RNA (20  $\mu$ g) from control HUVEC; (b) total RNA (20  $\mu$ g) from IFN- $\gamma$ -stimulated HUVEC. (B) The filter was probed using a factor B-specific cDNA probe. (a) total RNA (20  $\mu$ g) from IFN- $\gamma$ -stimulated HUVEC (100 IU/ml, 3 d); (b) total RNA (20  $\mu$ g) from control HUVEC; (c) total RNA (4  $\mu$ g) from human liver.

interaction processes or in antigen processing (15). C3b, if deposited on endothelial cells, would be rapidly converted to iC3b by the action of factors H and I, and would serve as a local ligand for interaction of endothelia with cells bearing CR3 or p150,95. Recent work on inflammatory cell recruitment in vivo indicates a key role for CR3 at the stage of adhesion to endothelium (16). CR3 also plays a role in T cell-dependent recruitment of myelomonocytic cells (Rosen, H., G. Milon, and S. Gordon, unpublished results). T cell-derived IFN- $\gamma$  may induce an appropriate ligand by its effect on complement component secretion by endothelial cells and therefore may have a role in adhesion of myelomonocytic cells.

### Summary

Human umbilical vein endothelial cells grown in vitro under standard conditions contain a high level of mRNA specific for the complement regulatory factors H and I. An additional 1.8-kb mRNA encoding a truncated form of factor H is also present. IFN- $\gamma$  stimulation of the cells causes a 6-7-fold increase in both factor H mRNA species, and a >10-fold increase in factor I mRNA. IL-1 and LPS slightly suppressed factor H mRNA, while TNF had no effect. mRNA for factor B is also detectable in IFN- $\gamma$ -stimulated cells, but messengers for C1q, C4bp, and CR3  $\beta$  chain were not found. Secretion of factor H protein was also stimulated by IFN- $\gamma$ . The presence of mRNA for factors H, B, and I, together with C3 secretion, demonstrated by others, suggests that endothelial cells can assemble the complete alternative complement pathway. Endothelial cell complement may be involved in leukocyte-endothelium interactions mediated by leukocyte C3 receptors.

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