28. A. O. Aliprantis *et al.*, *Science* **285**, 736 (1999).
29. THP-1 cells were stimulated with LPS, OspA, or d-OspA at 1 μg/ml (*31*). Supernatants were then assayed for IL-12 p40 by ELISA. LPS and OspA induced IL-12 p40 from THP-1 cells in a range of 1000 to 3500 pg/ml, whereas the amount of IL-12 p40 induced by d-OspA was below the level of detection.
30. HEK 293 cells not expressing human TLR-2 (detected with monoclonal anti–TLR-2 or reverse-transcriptase polymerase chain reaction) or HEK 293 clones stably transfected to express human TLR-2 were then further transfected with CD14 and reporter constructs as previously described (*19*). We plated 10^5 cells/well in six-well plates and transiently transfected them the following day with the NF-κB responsive E-selectin (ELAM) gene enhancer luciferase (pGL3) reporter gene (0.5 μg) and a β-galactosidase reporter plasmid (0.5 μg) as an internal control by the Superfect protocol at a 1:3 ratio of DNA (micrograms to Superfect (microliters). Cells were then incubated with the DNA-Superfect mixture for 2 hours and washed, and multiple transfectants were pooled and divided into separate wells for activation by a titration of LPS or lipoprotein stimuli (19-kD lipoprotein, Tp47, and OspA). Twenty-four hours later cells were stimulated for 6 hours then lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI), and 20 μl was used in the luciferase assay. HEK 293 control cells and TLR-2 stable clones were also cotransfected with a CD14 expression plasmid (*19*) (1 μg) or vector control (pCDNA3, 1 μg) with the same protocol.
31. T. J. Sellati *et al.*, *J. Immunol.* **160**, 5455 (1998); M. V. Norgard, B. S. Riley, J. A. Richardson, J. D. Radolf, *Infect. Immun.* **63**, 1507 (1995); M. V. Norgard *et al.*, *ibid.* **64**, 3845 (1996).
32. R. M. Wooten *et al.*, *J. Immunol.* **160**, 5485 (1998).
33. J. C. Chow, D. W. Young, D. T. Golenbock, W. J. Christ, F. Gusovsky, *J. Biol. Chem.* **274**, 10689 (1999); K. Hoshino *et al.*, *J. Immunol.* **162**, 3749 (1999); S. T. Qureshi *et al.*, *J. Exp. Med.* **189**, 615 (1999); S. N. Vogel *et al.*, *J. Immunol.* **162**, 5666 (1999); R. Shimazu *et al.*, *J. Exp. Med.* **189**, 1777 (1999); A. Poltorak *et al.*, *Science* **282**, 2085 (1998).
34. J. Chan, Y. Xing, R. S. Magliozzo, B. R. Bloom, *J. Exp. Med.* **175**, 1111 (1992); J. Chan, K. Tanaka, D. Carroll, J. Flynn, B. R. Bloom, *Infect. Immun.* **63**, 736 (1995).
35. J. D. MacMicking *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5243 (1997).
36. Q. W. Xie, R. Whisnant, C. Nathan, *J. Exp. Med.* **177**, 1779 (1993).
37. NO was measured as the concentration of NO_2^- in cell culture supernatants. We plated 10^5 RAW 264.7 cells in a 96-well plate in Dulbecco's modified Eagle medium (DMEM), 10% FCS. After a 1-hour incubation at 37°C, cells were stimulated with media or 19-kD lipoprotein (1 to 1000 ng/ml) for 24 hours. A 100-μl volume of the Greiss reagent (1:1 mix of 0.1% *N*-1-napthylethylenediamine-HCL and 1% sulfanilamide in 2.5% H_3PO_4; Sigma, St. Louis, MO) was added to 100 μl of cell culture supernatant and incubated for 10 min, and the absorbance was read at 560 nm. Sample nitrite concentrations were determined by comparison with a standard curve of $NaNO_2$ (Sigma) (*40*).
38. P. A. Sieling *et al.*, *J. Immunol.* **153**, 3639 (1994).
39. D. H. Libraty *et al.*, *J. Clin. Invest.* **99**, 336 (1997).
40. L. C. Green *et al.*, *Anal. Biochem.* **126**, 131 (1982).
41. Adherent monocytes were isolated as described previously (*14*). Cells were treated with no antibody, mouse anti–human TLR-2 neutralizing monoclonal antibody, or an isotype control mouse IgG1 (10 μg/ml) for 30 min before stimulation with LPS or 19-kD lipoprotein (50 ng/ml) and incubated for 16 hours. Supernatants were then harvested and assayed for IL-12 p40 by ELISA. CD40L- and interferon-γ (IFN-γ)–stimulated adherent monocytes were used to control for the anti–TLR-2 blocking of lipoprotein-induced IL-12. Adherent monocytes were treated with human IFN-γ (100 units/ml; Endogen, Cambridge, MA) for 16 hours to up-regulate CD40 expression. Cells were then treated with antibodies as stated above, then stimulated with soluble CD40L trimer (250 ng/ml; Immunex, Seattle, WA) for 16 hours. Neither antibody nor IFN-γ alone induced IL-12 production.
42. We thank P. Sieling, G. Cheng, M. Mark, C. Nathan, and H. Herschman for scientific discussion; M. Gately (Hoffman-La Roche) for POD-46D; and C. Nathan (Cornell Medical College, New York, NY) for the iNOS promoter. Supported by the NIH (to R.L.M., P.J.B., B.R.B., M.V.N.), the Howard Hughes Medical Institute (to B.R.B. and S.T.S.), the United Nations Development Programme/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases (IMMLEP), and the Dermatology Research Foundation of California, Incorporated.

30 March 1999; accepted 2 July 1999

Cell Activation and Apoptosis by Bacterial Lipoproteins Through Toll-like Receptor-2

Antonios O. Aliprantis,[1]* Ruey-Bing Yang,[2]* Melanie R. Mark,[2] Shelly Suggett,[3] Brigitte Devaux,[3] Justin D. Radolf,[4] Gary R. Klimpel,[5] Paul Godowski,[2] Arturo Zychlinsky[1]†

Apoptosis is implicated in the generation and resolution of inflammation in response to bacterial pathogens. All bacterial pathogens produce lipoproteins (BLPs), which trigger the innate immune response. BLPs were found to induce apoptosis in THP-1 monocytic cells through human Toll-like receptor–2 (hTLR2). BLPs also initiated apoptosis in an epithelial cell line transfected with hTLR2. In addition, BLPs stimulated nuclear factor–κB, a transcriptional activator of multiple host defense genes, and activated the respiratory burst through hTLR2. Thus, hTLR2 is a molecular link between microbial products, apoptosis, and host defense mechanisms.

The innate immune system coordinates the inflammatory response to pathogens. To do so, cells of the innate immune system discriminate between self and nonself by receptors that identify molecules synthesized exclusively by microbes (*1*). These include lipopolysaccharide (LPS), peptidoglycans, lipotechoic acids, and BLPs (*2*). BLPs are characterized by a unique, NH_2-terminal lipo-amino acid, *N*-acyl-*S*-diacylglyceryl cysteine, and are ideal targets for innate immune surveillance because they are produced by all bacteria. Although BLPs are known to activate nuclear factor–κb (NF-κB) (*3*), cytokine production (*4*), and B cell expansion (*5*), it is unclear how the BLP signal is transduced into an intracellular message. Candidate BLP signal transducers are Toll receptors, which are characterized by an extracellular leucine-rich repeat domain and an interleukin-1 (IL-1) receptor type 1–like intracellular signaling domain (*6*). In *Drosophila*, Toll receptors are important for resistance to microbial pathogens (*7*). Toll and TLRs activate homologous signal transduction pathways leading to nuclear localization of NF-κB/Rel–type transcription factors (*8*). Both hTLR2 (*9*, *10*) and murine TLR4 (*11*) (mTLR4) are implicated in the innate response to LPS. mTLR4 does not appear to function analogously in LPS and BLP signaling. A mutation in mTLR4, which renders cells insensitive to LPS, does not abrogate BLP-induced responses in mice (*11*, *12*). Therefore, we investigated the role of hTLR2 in BLP signaling.

A population of human embryonic kidney 293 cells, which do not express hTLR2 (*10*, *13*), were stably transfected with an epitope-tagged hTLR2 (293hTLR2) and tested for the ability to respond to BLP in an NF-κB luciferase reporter gene assay (*14*). Synthetic lipoprotein analogs consisting of a palmitylated version of *N*-acyl-*S*-diacylglyceryl cysteine (Pam_3Cys) and a few COOH-terminal amino acids mimic the immunomodulatory effects of BLP (*15*). The synthetic bacterial lipopeptide, $Pam_3CysSerLys_4$ (sBLP), induced expression of the luciferase reporter gene in 293hTLR2 cells, but not in the parental line (Fig. 1A). Concentrations of sBLP or *Escherichia coli* murein lipoprotein (MLP) as low as 200 pM activated the reporter gene (Fig. 1B). The lipo–amino acid Pam_3Cys and a monoacylated derivative of sBLP (msBLP) generated by base hydrolysis (*16*) did not activate the luciferase reporter gene in 293 or 293hTLR2 cells (Fig. 1, A and B). This is consistent with previous observations that the acyl groups and peptide moieties of sBLP are critical for cell activation (*5*, *17*).

[1]Skirball Institute and Department of Microbiology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA. [2]Department of Molecular Biology and [3]Antibody Technology Group, Genentech, South San Francisco, CA 94080, USA. [4]Center for Microbial Pathogenesis, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA. [5]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: zychlins@saturn.med.nyu.edu

The respiratory burst of phagocytes generates reactive oxygen species (ROS) that contribute to antimicrobial defense (*18*). We isolated a monoclonal antibody specific for hTLR2 (mAb 2392) (*19*). This antibody, but not an isotype control, completely abrogated the generation of ROS by peripheral blood leukocytes (PBLs) after exposure to sBLP (Fig. 1C), indicating that mAb 2392 is a blocking antibody. Although a role for other TLRs cannot be excluded, these data indicate that hTLR2 is critical for BLP responses and link Toll receptors to a transcription-independent, antimicrobial defense mechanism.

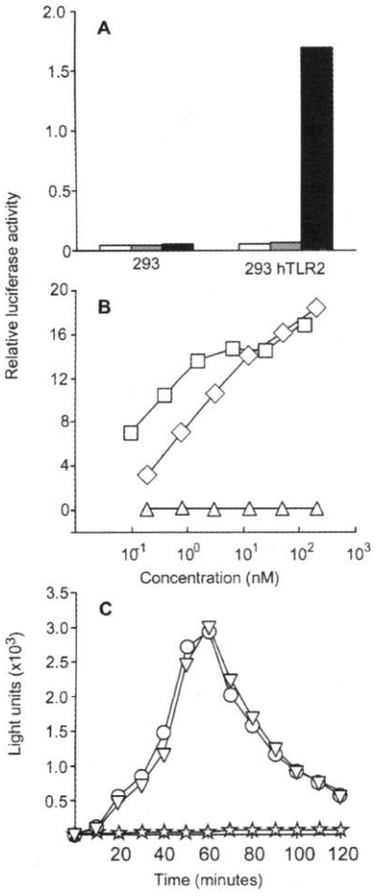

Fig. 1. hTLR2 mediates BLP-induced signaling. (**A**) An NF-κB reporter gene is activated in 293hTLR2 cells treated with sBLP. 293hTLR2 cells and parental 293 cells were transiently transfected with an NF-κB–regulated luciferase reporter construct and stimulated with sBLP (1000 ng/ml) (black bar), Pam_3Cys (1000 ng/ml) (gray bar), or an equivalent dilution of sBLP diluent (H_2O–0.05% HSA) (white bar). (**B**) Dose-response curve for NF-κB activation by BLP. 293hTLR2 cells were transiently transfected with an NF-κB–regulated luciferase reporter construct and incubated with sBLP (◇), MLP (□), or msBLP (△). (**C**) hTLR2-specific mAb 2392 blocks sBLP-mediated ROS production in PBLs. PBLs were preincubated with medium alone (○), mAb 2392 (25 μg/ml of) (☆), or an isotype control antibody (25 μg/ml) (▽) and exposed to sBLP (280 pg/ml). ROS production was measured by lucigenin-enhanced chemiluminescence every 10 min on a luminometer (*33*). Results are the average of two independent samples.

After incubation with sBLP, 293hTLR2 cells began to round up, bleb, and vacuolate (Fig. 2, E and F) (*20*), morphologic changes that are characteristic of apoptosis (*21*). The induction of cell death by sBLP was more pronounced in 293 cells coexpressing hTLR2 and human CD14 (293hTLR2/hCD14) (Fig. 2, G and H). CD14 is a membrane protein that potentiates LPS and BLP signaling (*22*). sBLP did not affect the morphology of parental 293 cells (Fig. 2, A and B) or cells expressing only hCD14 (293hCD14) (Fig. 2, C and D). After incubation with sBLP, 293hTLR2 cells and 293hTLR2/hCD14 cells showed a significant increase in apoptosis above background (Fig. 2I). As little as 10 ng/ml of either sBLP or MLP induced apoptosis in 293hTLR2 cells (Fig. 2L). Moreover, coexpression of hCD14 and hTLR2 augmented the total number of apoptotic cells, as well as sensitizing the cells to 10-fold lower doses of sBLP or MLP (Fig. 2M). Neither compound induced apoptosis in 293 or 293hCD14 cells (Fig. 2, I to K). Consistent with the NF-κB activation assays, neither msBLP nor Pam_3Cys induced apoptosis (Fig. 2, J to M). In comparison to sBLP, 10^3-fold higher concentrations of LPS were required to obtain similar numbers of apoptotic cells (Fig. 2, L and M). These assays were performed at serum concentrations (5%) sufficient to promote the interaction of LPS with CD14 (*23*). The LPS preparation was active because both sBLP and LPS induced a respiratory burst in PBLs at concentrations less than 1 ng/ml (*24*).

We investigated whether the induction of

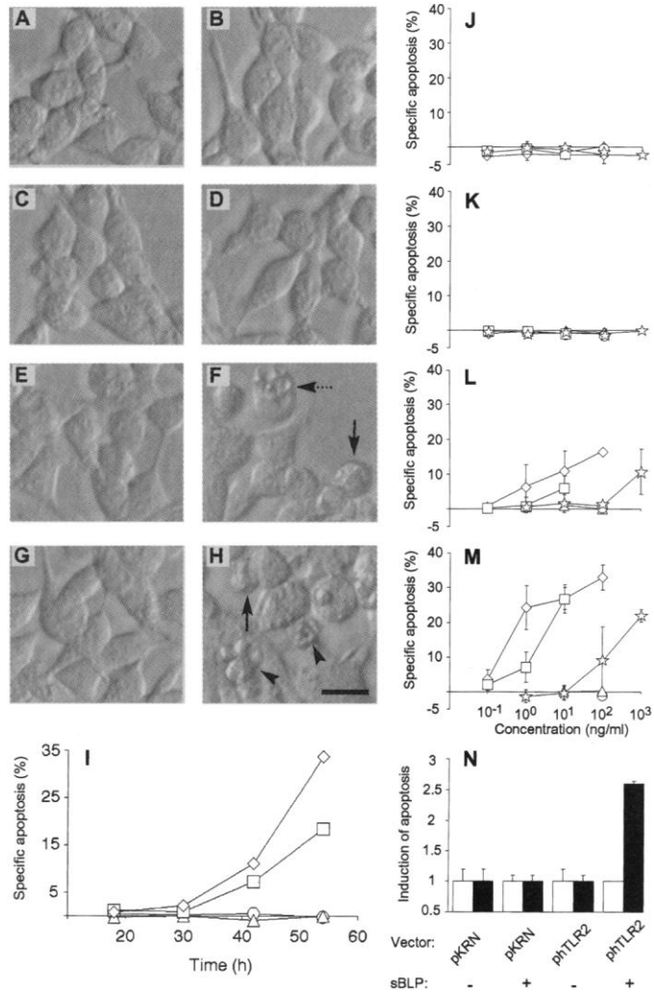

Fig. 2. BLPs induce apoptosis in 293 cells expressing hTLR2. (**A** to **H**) Morphology of 293 cells and stable transfectants exposed to sBLP. 293 (A and B), 293hCD14 (C and D), 293hTLR2 (E and F), and 293hTLR2/hCD14 (G and H) cells were incubated in medium alone (A, C, E, and G) or with sBLP (100 ng/ml) (B, D, F, and H). Cells with apoptotic morphology including rounding up (arrows), vacuolation (dashed arrow), and membrane blebbing (arrowheads) are indicated. Bar, 10 μm (A to H). (**I**) Time course of BLP-induced, hTLR2-mediated apoptosis. 293 (○), 293hCD14 (△), 293hTLR2 (□), and 293hTLR2/hCD14 (◇) cells were incubated in media only or media with sBLP (100 ng/ml), TUNEL stained, and analyzed by flow cytometry. (**J** to **M**) Dose-response curves for the induction of apoptosis by BLP. 293 (J), 293hCD14 (K), 293hTLR2 (L), and 293hTLR2/hCD14 (M) cells were incubated with sBLP (◇), MLP (□), msBLP (△), Pam_3Cys (○), or *Shigella flexneri* type 1A LPS (☆) for 60 hours, TUNEL stained, and analyzed by flow cytometry. (**N**) sBLP directly induces apoptosis in cells expressing hTLR2. 293 cells were transiently transfected with GFP and the indicated plasmid, incubated with sBLP (100 ng/ml), collected, stained for cell death with annexin V, and analyzed by flow cytometry. Results are presented as the fold induction of cell death in the GFP-negative (white bars) and GFP-positive (black bars) populations relative to cells transfected with control vector (pKRN) without the addition of sBLP. Data are the mean ± SD of two independent samples. Some SDs are within the limits of the data points.

apoptosis by BLPs through hTLR2 was direct or indirect (*25*). 293 cells were transiently cotransfected with expression vectors encoding hTLR2 and green fluorescent protein (GFP). After exposure to sBLP, an induction of apoptosis was detected only in the GFP-positive population (Fig. 2N). We did not detect cell death above background without the addition of sBLP or after transfection with the control vector. Thus, BLPs induce apoptosis directly through hTLR2 and not by stimulating the secretion of a soluble mediator of apoptosis.

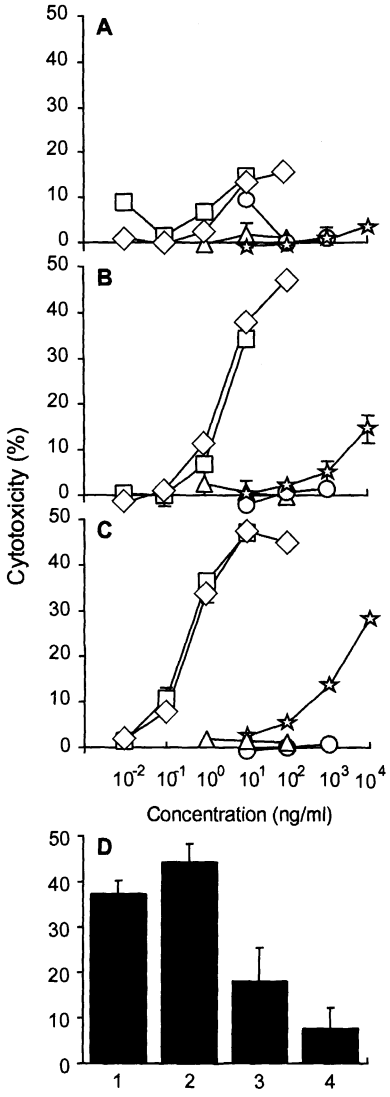

Fig. 3. BLPs are cytotoxic to the human monocytic cell line THP-1. (**A** to **C**) Dose-response curves for the induction of cell death by BLPs in THP-1 cells. THP-1 cells (A) or THP-1 cells initially treated with PMA (B) or cotreated with cycloheximide (C) were incubated with sBLP (◇), MLP (□), msBLP (△), Pam_3Cys (○), or *S. flexneri* type 1A LPS (☆). (**D**) hTLR2-specific mAb blocks sBLP-mediated cell death in THP-1 cells. THP-1 cells were initially incubated in medium (1), isotype control antibody (25 μg/ml) (2), mAb 2392 (10 μg/ml) (3), or mAb 2392 (25 μg/ml) (4) and treated with sBLP (625 pg/ml) and cycloheximide (50 μg/ml). Data are the mean ± SD of three samples. Some SDs are within the limits of the data points.

The transcription of *hTLR2* in THP-1 cells, a monocytic cell line that expresses hTLR2 endogenously (*10*), was confirmed by reverse transcriptase–polymerase chain reaction (*13*), and its surface expression by flow cytometric analysis with mAb 2392 (*24*). THP-1 cells exposed to sBLP or MLP (10 ng/ml) showed a modest increase in cytotoxicity (15%) (Fig. 3A) (*26*). However, initial treatment of the cells with the phorbol ester PMA (phorbol 12-myristate 13-acetate) or cotreatment with the translation inhibitor cycloheximide markedly increased cytotoxicity (Fig. 3, B and C). The cells displayed morphologic features of apoptosis, including cell shrinkage and blebbing. BLP-induced cell death occurred much more rapidly in THP-1 cells (6 hours) than in 293hTLR2 or 293hTLR2/hCD14 cells (>30 hours), suggesting that monocytes express a more appropriate pathway to execute hTLR2-mediated cell death. Neither msBLP nor Pam_3Cys were active in this assay (Fig. 3, A to C). One thousand–fold higher concentrations of LPS than sBLP were required to observe a cytotoxic effect in PMA- or cycloheximide-treated THP-1 cells (Fig. 3, B and C). Similar results were obtained in the presence of recombinant LPS binding protein (*24*). THP-1 cells were preincubated with mAb 2392 and exposed to sBLP. This antibody to hTLR2, but not an isotype control, attenuated the cell death response to sBLP (Fig. 3D). Therefore, the induction of apoptosis by sBLP requires hTLR2.

Brightbill *et al.* report that BLPs activate NF-κB–dependent transcription through hTLR2 (*27*). We describe similar findings. However, we find that BLPs can also mediate apoptosis through hTLR2. Thus, hTLR2 signals for both cell activation and apoptosis. This dual signaling capacity is precedented by tumor necrosis factor receptor–1 (TNFR1), which stimulates parallel pathways to apoptosis and NF-κB activation (*28*).

Although many bacterial pathogens induce apoptosis in host cells (*29*), the implications of this phenomenon remain elusive. BLP-induced apoptosis could be important for (i) the initiation of inflammation (*30*), (ii) the resolution of inflammation (*31*), and (iii) generating the proper signals necessary for adaptive immune responses. The observation that sBLPs are excellent adjuvants supports the third hypothesis (*32*). The BLP-hTLR2 apoptotic pathway emerges as a mechanism potentially fulfilling multiple roles in the genesis and progression of innate and adaptive immune responses to bacteria.

References and Notes

1. R. Medzhitov and C. A. Janeway Jr., *Curr. Opin. Immunol.* **9**, 4 (1997).
2. B. Henderson, S. Poole, M. Wilson, *Microbiol. Rev.* **60**, 316 (1996).
3. M. V. Norgard *et al.*, *Infect. Immun.* **64**, 3845 (1996).
4. M. Kreutz *et al.*, *Immunology* **92**, 396 (1997).
5. W. G. Bessler *et al.*, *J. Immunol.* **135**, 1900 (1985).
6. F. L. Rock, G. Hardiman, J. C. Timans, R. A. Kastelein, J. F. Bazan, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 588 (1998).
7. B. Lemaitre, E. Nicolas, L. Michaut, J. M. Reichhart, J. A. Hoffmann, *Cell* **86**, 973 (1996); M. J. Williams, A. Rodriguez, D. A. Kimbrell, E. D. Eldon, *EMBO J.* **16**, 6120 (1997).
8. L. A. O'Neill and C. Greene, *J. Leukocyte Biol.* **63**, 650 (1998).
9. R. B. Yang *et al.*, *Nature* **395**, 284 (1998).
10. C. J. Kirschning, H. Wesche, T. Merrill Ayres, M. Rothe, *J. Exp. Med.* **188**, 2091 (1998).
11. A. Poltorak *et al.*, *Science* **282**, 2085 (1998).
12. H. Zhang, J. W. Peterson, D. W. Niesel, G. R. Klimpel, *J. Immunol.* **159**, 4868 (1997).
13. R.-B. Yang, M. R. Mark, P. Godowski, unpublished data.
14. Performed as in (*9*) with the following modifications. Cells were transfected in 15-cm dishes with pGL3-ELAM.tk and the *Renilla* luciferase reporter plasmid as an internal control, and after 1 hour were seeded in an opaque 96-well plate. After 24 hours, the indicated compounds were added in RPMI 1640, 0.05% human serum albumin (HSA) (Grifols, Miami, FL). Luciferase activity was determined after 6 hours. sBLP was from Boehringer Mannheim Biochemica, and Pam_3Cys was from Novabiochem. MLP (*12*) contained <25 pg of LPS per milligram of protein. Unless indicated, other reagents were from Sigma.
15. P. Hoffmann *et al.*, *Immunobiology* **177**, 158 (1988).
16. Base treatment was done as in V. Vidal *et al.*, *Nature Med.* **4**, 1416 (1998). The products were resolved by reversed-phase high-performance liquid chromatography with a C4 column (Vydac) and a linear acetonitrile gradient. Fractions were analyzed by electrospray ionization mass spectrometry. A fraction was isolated that contained (i) a compound that has lost two of the three palmitate moieties (molecular weight = 1032.6, major peak) and (ii) a compound that has lost the entire *S*-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl] moiety (molecular weight = 924.7, minor peak). The concentration was determined by ultraviolet absorption.
17. J. D. Radolf *et al.*, *J. Immunol.* **154**, 2866 (1995).
18. A. W. Segal and K. P. Shatwell, *Ann. N.Y. Acad. Sci.* **832**, 215 (1997).
19. mAb 2392 was generated by immunization of BALB/c mice with the purified, extracellular domain of hTLR2 (*9*). The specificity of mAb 2392 for hTLR2 was confirmed by the following methods: (i) An enzyme-linked immunosorbent assay which demonstrated that mAb 2392 reacted with hTLR2-Fc but not hTLR4-Fc. (ii) A flow cytometric analysis with 293 cells expressing gD epitope–tagged versions of hTLR2, hTLR4, and hTLR6. Although each cell line was labeled with mAb 5B6, which recognizes the gD epitope, only 293hTLR2 cells were labeled with mAb 2392. (iii) An immunoprecipitation (*24*), followed by protein immunoblotting with mAb 5B6, from the membrane extracts of 293 cells transiently transfected with gD epitope–tagged versions of hTLR2, hTLR1, hTLR4, and murine TLR2. mAb 2392 only precipitated hTLR2. However, equivalent amounts of each receptor were obtained when mAb 5B6 was used for the immunoprecipitation. Supplemental data illustrating the specificity of mAb 2392 can be seen at www.sciencemag.org/feature/data/1040361.shl.
20. The test compounds were added to the 293 stable cell lines (*9*) in serum-free Dulbecco's modified Eagle's medium, 0.05% HSA, 5% fetal bovine serum (FBS). Cells were either photographed or stained with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) by using the Apoptosis Detection System (Promega) and analyzed by flow cytometry. At least 5000 events were counted per sample. Results are presented as specific apoptosis (%) determined as [(% TUNEL-positive cells in experimental − % TUNEL-positive cells in control)/(100 − % TUNEL-positive cells in control] × 100. The % TUNEL-positive cells in controls was always <5%.
21. J. Kerr, A. Wyllie, A. Currie, *Br. J. Cancer* **26**, 239 (1972).

22. T. J. Sellati *et al.*, *J. Immunol.* **160**, 5455 (1998).
23. D. Heumann *et al.*, *ibid.* **148**, 3505 (1992).
24. A. O. Aliprantis and A. Zychlinsky, unpublished data.
25. Plasmids pKRN and phTLR2 (*9*) were prepared with the Endotoxin-free Plasmid Maxi kit (Qiagen). 293 cells were transfected with the Effectene Transfection reagent (Qiagen). Thirty hours after transfection, an equal volume of diluent or diluent containing sBLP (200 ng/ml) was added. Thirty-six hours later the cells were stained with Annexin V–biotin (Pharmingen) and Streptavidin Tri-Color (Caltag) and analyzed by flow cytometry.
26. THP-1 cells (American Type Culture Collection) were maintained in RPMI 1640, 10% FBS, penicillin, streptomycin, and 2 mM L-glutamine. Six hours after seeding THP-1 cells in RPMI 1640, the indicated compounds diluted in RPMI 1640, 0.05% HSA were added. Six hours later, cell death was quantified by a lactate dehydrogenase release assay (Promega). Where indicated, cells were first treated with 32 nM PMA for 6 hours or cotreated with cycloheximide (50 μg/ml). PMA and cylcoheximide diluents (dimethyl sulfoxide and ethanol) were not cytotoxic.
27. H. D. Brightbill *et al.*, *Science* **285**, 732 (1999).
28. A. Ashkenazi and V. M. Dixit, *ibid.* **281**, 1305 (1998).
29. J. E. Moss, A. O. Aliprantis, A. Zychlinsky, *Int. Rev. Cytol.* **187**, 203 (1999).
30. A. Zychlinsky and P. Sansonetti, *J. Clin. Invest.* **100**, 493 (1997).
31. J. Savill, *J. Leukocyte Biol.* **61**, 375 (1997).
32. W. G. Bessler *et al.*, *Int. J. Immunopharmacol.* **19**, 547 (1997).
33. N. M. Iovine, P. Elsbach, J. Weiss, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10973 (1997).
34. We thank D. Littman, V. Dixit, C. Scharff, and J. Moss for critical reading of the manuscript, M. Garabedian for discussions, and T. Neubert for mass spectrometry analysis. pCDNA3 and pGFP were a gift of the Littman Lab. A.O.A. was supported by a grant from the Life and Health Insurance Fund. This work was supported by grants from the National Institute of Allergy and Infectious Diseases (AI 37720-04 to A.Z. and AI-38894 to J.D.R.).

25 March 1999; accepted 2 July 1999

Genetic Mechanisms of Age Regulation of Human Blood Coagulation Factor IX

Sumiko Kurachi, Yoshihiro Deyashiki, Junko Takeshita, Kotoku Kurachi*

Blood coagulation capacity increases with age in healthy individuals. Through extensive longitudinal analyses of human factor IX gene expression in transgenic mice, two essential age-regulatory elements, AE5′ and AE3′, have been identified. These elements are required and together are sufficient for normal age regulation of factor IX expression. AE5′, a PEA-3 related element present in the 5′ upstream region of the gene encoding factor IX, is responsible for age-stable expression of the gene. AE3′, in the middle of the 3′ untranslated region, is responsible for age-associated elevation in messenger RNA levels. In a concerted manner, AE5′ and AE3′ recapitulate natural patterns of the advancing age–associated increase in factor IX gene expression.

Blood coagulation potential in humans, as well as in other mammals, reaches the young adult level around the age of weaning (*1*), followed by a gradual increase during young adulthood and almost doubling by old age (*2*). This age-associated increase in coagulation potential takes place in healthy centenarians (*3*), indicating that the increase is a normal age-associated phenomenon. It may also contribute to the development and progression of age-associated cardiovascular and thrombotic disorders (*4*). This increase in blood coagulation potential coincides with increases in plasma levels of procoagulant factors such as factor IX, whereas plasma levels of anticoagulation factors (such as antithrombin III and protein C) or of factors involved in fibrinolysis are only marginally affected (*5*).

Blood coagulation factor IX (FIX), a plasma protease precursor, occupies a key position in the blood coagulation cascade where the intrinsic and extrinsic pathways merge (*6*). FIX is synthesized in the liver with strict tissue specificity, and its deficiency results in the bleeding disorder hemophilia B. Like human FIX (hFIX), mouse FIX (mFIX) plasma activity increases with age. Mouse FIX plasma activity is also directly correlated with an increase in the level of liver mFIX mRNA (*2*).

To explore the molecular mechanisms that underlie the age-related increase in FIX activity, we constructed a series of hFIX minigene expression vectors (*7*). We first analyzed them in vitro with HepG2 cells, a human hepatoma cell line, and then generated transgenic mice with the hFIX minigene vectors and carried out longitudinal analyses of hFIX expression in vivo for the entire lifespans of founders and successive generations of transgenic mice. The use of hFIX minigene expression vectors, which can produce high levels of plasma hFIX in vitro and in vivo, provided both a reliable animal assay system and multiple unexpected critical insights into the regulatory mechanisms of the hFIX gene. These minigenes consisted of the hFIX homologous components, including promoter sequences of various lengths spanning up to nucleotide (nt) −2231 in the 5′ flanking region, the coding region with a middle-portion truncated first intron, and the

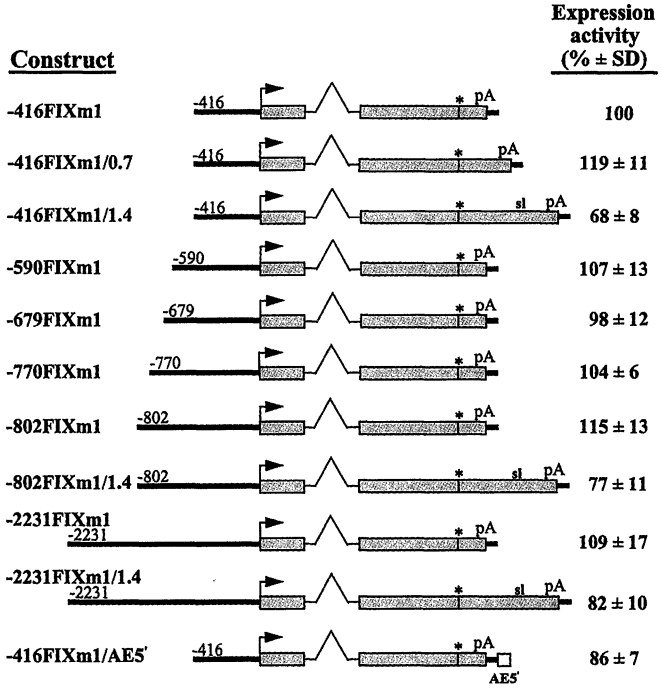

Fig. 1. Human FIX minigene expression constructs and in vitro transient expression activities. The structure is depicted with the pro-moter regions (thick horizontal lines at left) with the 5′ terminal nt number. Transcribed hFIX regions (gray rectangles, with thin peaked lines representing the shortened first intron) are followed by 3′ flanking sequence regions (thick horizontal lines at right). Transient expression activities are expressed relative to that of −416FIXm1 (~50 ng per 10^6 cells per 48 hours in four to five independent assays). Activities were normalized to the size of the minigenes used. Arrow, transcription start site; asterisk, translation stop codon; pA, polyadenylation; sl, potential stem-loop forming dinucleotide repeats.

Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109–0618, USA. E-mail: kkurachi@umich.edu

*To whom correspondence should be addressed.