transcript. Introns 1, 2, and 3 in the human RasGRP1 gene are 4.6, 33.6, and 6.7 kb, respectively. Because RasGRP1 cDNAs that contain one or more large introns most likely would be missed in our cDNA selection and sequencing approach to identify new isoforms of RasGRP1, the number of abnormal isoforms of RasGRP1 in SLE patients almost certainly is greater than that noted in Fig. 2.

We failed to identify defective RasGRP1 transcripts that contained introns 1–10 (Fig. 2). Failure to remove any of these introns in the processed RasGRP1 transcript would result in a very early translation termination codon. Defective transcripts are rapidly degraded by the nonsense posttranscriptional pathway in yeast if the premature translation termination codon resides in the initial half of the coding domain of the transcript (35), as occurs in the defective RasGRP4 transcript in HMC-1 cells (22). Thus, our failure to identify improperly processed RasGRP1 transcripts in our SLE patients that contain introns 1 to 7 probably is the result of rapid catabolism of those defective RasGRP1 transcripts by the nonsense posttranscriptional pathway.

The reason why pre-RasGRP1 mRNA is improperly processed in a subgroup of our SLE patients was not deduced in our study. Nevertheless, we were able to rule out a number of possibilities. The most prevalent abnormal variant identified in the PBMCs, T cells, and B cells of our SLE patients is splice variant A, which lacks exon 11. We failed to identify a point mutation in the gene's exon 11 and its intron 10/exon 11 and exon 11/intron 11 splice junctions in the genomic DNA isolated from SLE patient 10, who had abundant exon 11-deleted isoforms. In addition, no mutation was found in the branch-point sequence of the human RasGRP1 gene, which is used to remove intron 10.

Fifteen of the introns (including intron 10) of the mouse, rat, and human RasGRP1 genes are U2-dependent. The exception in this gene is intron 3, which is U12-dependent (for review of U12-dependent introns, see Ref. 36). It has been estimated that only ~0.1% of all introns in the human genome are U12 dependent. Our analysis of the nucleotide sequences of the human RasGRP2, RasGRP3, and RasGRP4 genes revealed the unexpected finding that all four RasGRP genes contained a single U12-dependent intron, even though the size of this intron varies considerably. We previously showed that the RasGRP4 gene is transcribed in HMC-1 cells but that this mast cell line cannot remove intron 3 in its processed RasGRP4 transcript (22). Intron 3 is correctly removed from RasGRP1 splice variants A to M. Nevertheless, it is possible that some SLE patients have a defect in the processing of U12-dependent introns in their lymphocytes that adversely impacts the removal of downstream introns in the precursor RasGRP1 transcript. The B cells in PBMCs express both RasGRP1 and RasGRP3. As noted in Fig. 1*D*, we found no evidence of defective processing of the RasGRP3 transcript in these SLE patients. It therefore appears that SLE patients do not have a global defect in the U12-dependent processing of their transcripts and that the posttranscriptional defect we uncovered in RasGRP1 expression does not adversely affect the processing of the transcript that encodes another member of this family of signaling proteins in lymphocytes. We also discovered that the CD4 transcript is processed correctly in SLE patients, suggesting that the posttranslational defect is relatively specific to the RasGRP1 transcript in lymphocytes.

We considered the possibility that the aberrant splicing of the RasGRP1 precursor transcript in our SLE patients could be an indirect consequence of long-term PSL therapy. In this regard, SLE patient 8 had barely detectable levels of normal RasGRP1 transcripts (Fig. 1) and protein (Fig. 4). Because this patient had not been on PSL or any other therapeutic drug before his/her PBMCs were initially analyzed and because the patient continued to have aberrant RasGRP1 expression after he/she was treated with prednisolone and cyclophosphamide, the presence of defective RasGRP1 transcripts does not appear to be the primary consequence of the therapy used to treat our SLE patients. The data noted in Table II from the entire group of SLE patients support this conclusion. Nevertheless, SLE patient 3 did alter his/her expression of RasGRP1 after drug treatment to improve his/her clinical situation. Thus, there remains the possibility that RasGRP1 expression is affected by disease status or treatment in some SLE patients.

Unlike the RasGRP3 transcript and nearly all other transcripts in lymphocytes, the RasGRP1 transcript lacks a classical "AAUAAA" or "AUUAAA" polyadenylation signal sequence 10–30 nucleotides upstream of its poly(A) site. We did not determine the nucleotide sequences of the 3′ untranslated regions of the RasGRP1 transcripts in our SLE patients or healthy subjects. Nevertheless, it is unlikely that the unconventional polyadenylation site in the RasGRP1 gene is the problem, because reduced polyadenylation of eukaryotic pre-mRNA generally results in rapid catabolism of the transcript rather than defective splicing. A more likely explanation of our data is that a point mutation exists somewhere in the RasGRP1 gene in this subgroup of SLE patients that causes its precursor transcript to bind improperly to an undefined *cis*-acting factor in lymphocytes that ultimately controls the splicing of this transcript. In this regard, CD45/PTPRC is a tyrosine phosphatase found in T cells and other hemopoietic cells that has been linked to multiple sclerosis. Relevant to our study, two groups identified a polymorphism in exon 4 of the CD45 gene. This mutation does not result in a codon that encodes a new amino acid, but it somehow leads to aberrant splicing of the precursor transcript (37, 38). The latter studies provide a relevant example of how a translationally silent point mutation in a gene encoding a protein that participates in critical signaling events in T cells can lead to an autoimmune disorder.

If translated, all but splice variant M should encode RasGRP1 isoforms with an intact GEF domain. The major problem resides in the regulatory C-terminal half of the protein. The most prominent abnormal RasGRP1 isoform identified in our SLE patients was splice variant A, which lacks exon 11. Loss of this exon does not cause a frame-shift abnormality or a premature translation termination codon in the processed transcript. Nevertheless, if translated, the resulting abnormal RasGRP1 protein loses the 35-mer sequence of PLTPSKPPVVVDWASGVSPK PDPKTISKHVQRMVD that links the functional GEF domain to the regulatory DAG/phorbol ester- and calcium-binding domains. This amino acid sequence is present in human, chimpanzee, rhesus monkey, bovine, rat, and mouse RasGRP1. Because of its conservation throughout evolution, it is likely that loss of this 35-mer domain leads to a defective signaling protein in lymphocytes.

Nine of the other RasGRP1 splice variants identified in our study have a premature translation termination codon. If translated, these splice variants would encode truncated nonfunctional RasGRP1 isoforms that have lost as many as 624 of their C-terminal amino acids. Numerous studies have documented the biologic importance of the C-terminal half of RasGRP1 and its family members. The most thoroughly studied domain in this portion of RasGRP1 is its DAG/phorbol ester-binding sequence. Activation of T cells via their TCRs result in the rapid phospholipase γ1-dependent generation of DAG. DAG binds to the C1 domain in RasGRP1, thereby promoting translocation of the signaling protein to the inner leaflet of the cell's plasma membrane and then to the Golgi complex and endoplasmic reticulum. A naturally occurring RasGRP4 splice variant in the mast cells of the C3H/HeJ mouse

strain resembles the RasGRP1 splice variant L noted in Fig. 2. We previously showed that this RasGRP4 isoform is unresponsive to phorbol esters (24). Thus, even if translated, RasGRP1 splice variants C, D, G, H, I, K, L, and M should result in RasGRP1 isoforms that are unresponsive to DAG and phorbol esters. Okamura et al. (39) recently reported that the 127-mer amino acid sequence downstream of the DAG/phorbol ester-binding C1 domain in RasGRP3 also helps regulate the intracellular movement of this signaling protein inside cells. If the corresponding domain in RasGRP1 has a similar intracellular targeting function, splice variants B, E, and F also should not be fully functional in SLE patients even if translated.

We generated RasGRP1-specific Abs to evaluate the consequences of defective RasGRP1 transcripts on the levels of normal RasGRP1 in T cells. After documenting the specificity of these anti-peptide Abs (Fig. 3), we used an immunoblot approach to monitor RasGRP1 protein levels in the T cells purified from SLE patients and healthy individuals. In confirmation of the RNA data noted in Fig. 1, SLE patients 8 and 9 contained very little, if any, ~95-kDa RasGRP1 protein in lysates of their T cells (Fig. 4). The only surprise was our failure to detect smaller sized proteins in the other SLE patients that would be expected to be derived from the relatively abundant splice variants A, B, or D. Based on this observation, we next transfected HEK-293 cells with expression constructs that encode normal RasGRP1 and its splice variants A, B, and D (Fig. 5). The appropriate RasGRP1 transcripts were found in all four transfectants. If anything, the level of the splice variant B transcript in its transfectant was ~3-fold higher than the level of the normal RasGRP1 transcript in its transfectant. Despite the high levels of splice variant B and D mRNA in their respective transfectants, no recombinant proteins were found in the lysates of these cells. Although it is possible that RasGRP1 spice variants B and D are metabolized in T cells and HEK-293 cells differently due to the fact that epithelial cells normally never express RasGRP1, our transfection/expression data and cell-free transcription:translation data suggest that many defective RasGRP1 transcripts are not efficiently translated in T cells by a control mechanism that remains to be identified. Whatever the mechanism, the net result is greatly diminished levels of functional RasGRP1 protein in the patient's T cells as noted in SLE patients 8 and 9 (Fig. 4*A*). Although the functional importance of having splice variants that encode abnormal isoforms of RasGRP1 remains to be determined, the T cells isolated from RasGRP1-defective SLE patient 8 were unable to produce significant amounts of IL-2 when activated (Fig. 4*E*). These preliminary data suggest that some alternatively spliced RasGRP1 isoforms and/or lower expression levels of RasGRP1 can profoundly alter T cell function. Considering the importance of RasGRP1 in lymphocyte development and function in the mouse, our data suggest that dysregulation of RasGRP1 expression is a contributing factor in the development of autoimmunity in a subset of SLE patients.

Disclosures

The authors have no financial conflict of interest.

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The Proinflammatory Cytokine Macrophage Migration Inhibitory Factor Regulates Glucose Metabolism during Systemic Inflammation[1]

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Inflammation provokes significant abnormalities in host metabolism that result from the systemic release of cytokines. An early response of the host is hyperglycemia and resistance to the action of insulin, which progresses over time to increased glucose uptake in peripheral tissue. Although the cytokine TNF-α has been shown to exert certain catabolic effects, recent studies suggest that the metabolic actions of TNF-α occur by the downstream regulation of additional mediators, such as macrophage migration inhibitory factor (MIF). We investigated the glycemic responses of endotoxemic mice genetically deficient in MIF ($MIF^{-/-}$). In contrast to wild-type mice, $MIF^{-/-}$ mice exhibit normal blood glucose and lactate responses following the administration of endotoxin, or TNF-α. $MIF^{-/-}$ mice also show markedly increased glucose uptake into white adipose tissue in vivo in the endotoxemic state. Treatment of adipocytes with MIF, or anti-MIF mAb, modulates insulin-mediated glucose transport and insulin receptor signal transduction; these effects include the phosphorylation of insulin receptor substrate-1, its association with the p85 regulatory subunit of PI3K, and the downstream phosphorylation of Akt. Genetic MIF deficiency also promotes adipogenesis, which is in accord with a downstream role for MIF in the action of TNF-α. These studies support an important role for MIF in host glucose metabolism during sepsis. *The Journal of Immunology,* **2007, 179: 5399–5406.**

Severe infection, trauma, or tissue invasion provoke metabolic abnormalities that contribute significantly to morbidity and mortality (1, 2). An early response of the host is hyperglycemia and resistance to the action of insulin. Tissue glycolysis continues despite decreased perfusion, and lactate production increases because of the incomplete oxidation of glycolytic end products. These metabolic derangements produce a catabolic state that compromises host immunity and tissue repair, and if unresolved, produce cachexia, progressive organ dysfunction, and death (1).

The development of a catabolic state in the setting of infection results from a combination of stress-induced hormonal responses and the production of proinflammatory mediators (1, 2). One postulated role for the cytokine TNF-α is to effect a redistribution of energy stores from adipose to peripheral tissue (1, 3). TNF-α has been shown to induce the synthesis of the allosteric stimulator of glycolysis, fructose 2,6-bisphosphate, leading to a depletion of plasma glucose and an accompanying elevation of glucose uptake into peripheral tissue. Indeed, TNF-α was cloned as the mediator "cachectin" because of its systemic effects on host metabolism, and an accumulation of data support a role for TNF-α in the development of insulin resistance (4). TNF-α also mediates insulin resistance and lipolysis in adipocytes, thus preventing the uptake and use of circulating glucose into this insulin sensitive tissue.

Studies in endotoxemic mice suggest that certain of the metabolic actions of TNF-α may be mediated by the coordinate or downstream expression of macrophage migration inhibitory factor (MIF)[3] (5). MIF is expressed by many cell types, is released early in the activation response from preformed intracellular pools (5, 6), and its circulating levels correlate with the clinical severity of sepsis (7, 8). Notably, the immunoneutralization of MIF in endotoxemic mice normalizes glucose levels, prevents liver glycogen depletion, and decreases levels of fructose 2,6-bisphosphate in muscle. These effects also were observed in TNF-$\alpha^{-/-}$ mice treated with anti-MIF, thus verifying the intrinsic action of MIF in this inflammation-induced, catabolic response of liver and muscle (5).

The finding that cultured adipocytes secrete MIF in response to TNF-α (9) prompted us to investigate more closely MIF's action in glucose metabolism in adipocytes during endotexemia. In the present report, we present evidence that the action of TNF-α on adipose tissue during the systemic inflammatory response can be explained in large part by the autocrine/paracrine action of MIF.

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Received for publication August 6, 2007. Accepted for publication August 9, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants AI42310 (to R.B.), AR050498 (to R.B.), U24 DK-59635 (to J.K.K.), and ADA 1-04RA-47 (to J.K.K.) from the National Institutes of Health.

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[3] Abbreviations used in this paper: MIF, macrophage migration inhibitory factor; IRS-1, insulin receptor substrate 1; MEF, murine embryonic fibroblast; PPAR, peroxisome proliferator-activated receptor; 2-[^{14}C]DG, 2-deoxy-D-[1-^{14}C]glucose; WT, wild type.

Materials and Methods

Materials

Neutralizing anti-MIF mAb (NIH-III.D9) was previously described (7). Abs directed against the insulin receptor β subunit (C-19), TNFR-1, and cyclin D1 were obtained from Santa Cruz Biotechnology, and Abs against Akt and phospho-Akt (Ser^{473}) were obtained from Cell Signaling Technology. Abs directed against phosphotyrosine, insulin receptor substrate 1 (IRS-1), and the p85 regulatory subunit of the PI3K were purchased from Upstate Biotechnology. Recombinant mouse TNF-α and the mouse TNF-α ELISA kit were purchased from R&D Systems. MIF was measured by a murine-specific capture ELISA (7). Recombinant murine MIF was purified from an *Escherichia coli* expression system by FPLC and C8 chromatography to remove contaminating endotoxin (10), and contained <1 μg of endotoxin/gram of protein. *E. coli* LPS O111:B4 was obtained from Sigma-Aldrich, and troglitazone was a gift from Sankyo Company.

In vivo glucose and lactate metabolism in endotoxemic $MIF^{-/-}$ and $MIF^{+/+}$ mice

$MIF^{-/-}$ mice (11) were bred onto a pure BALB/c background (generation N10). BALB/c mice (wild type (WT), $MIF^{+/+}$) were obtained from Charles River Breeding Laboratories. Male mice (age 6–8 wk) were used, and all mice showed an equivalent increase in body weight with age. At least 4 days before in vivo experiments, the mice were anesthetized and surgery was performed to establish an indwelling catheter in the right internal jugular vein (12). Mice were fasted overnight (18 h) before experiments but had free access to water. At the indicated times, blood was collected from tail vessels to measure glucose levels. All studies were approved by the Yale Institutional Animal Care and Use Committee.

Organ-specific glucose metabolism in vivo was determined in awake mice using a modification of hyperinsulinemic-euglycemic clamp experiments as previously described (12). Briefly, a sublethal dose of LPS (16.6 μg/g) was i.p. injected into age-matched $MIF^{+/+}$ and $MIF^{-/-}$ mice (n = 7–8 for each group). At 30 min postinjection, a blood sample (40 μl) was collected for glucose and insulin measurements. This was followed by a continuous infusion of 3-[^{3}H]glucose (0.1 μCi/min) for 2 h to estimate whole body glucose turnover. To determine basal glucose uptake in individual organs, 2-deoxy-D-[1-^{14}C]glucose (2-[^{14}C]DG, 10 μCi) was i.v. administered as a bolus at 2 h postinjection. Blood samples were taken at 5-min intervals during the remaining 30 min for the measurement of plasma [^{3}H]glucose and 2-[^{14}C]DG concentrations. At the end of experiments (2.5 h postinjection with LPS), mice were anesthetized with sodium pentobarbital injection, and tissue samples (gastrocnemius, epididymal white adipose tissue, and intrascapular brown adipose tissue) were taken for biochemical analysis.

Biochemical analysis and calculations

Plasma glucose concentration was determined by the glucose oxidase method using the Beckman Glucose Analyzer 2, and plasma insulin concentration was measured by radioimmunoassay (Linco Research). Plasma concentrations of 3-[^{3}H]glucose and 2-[^{14}C]DG were determined after deproteinization of plasma samples as previously described (12). For the determination of tissue 2-[^{14}C]DG-6-phosphate content, tissue samples were homogenized and the supernatants subjected to ion-exchange chromatography to separate 2-[^{14}C]DG-6-phosphate from 2-[^{14}C]DG.

The rates of basal whole body glucose turnover and hepatic glucose production were determined as the ratio of the [^{3}H]glucose infusion rate to the specific activity of plasma glucose during the final 30 min of the experiments. Glucose uptake in individual tissues was calculated from the plasma 2-[^{14}C]DG profile, which was fitted with a double exponential or linear curve using mathematical modeling MLAB software (Civilized Software) and tissue 2-[^{14}C]DG-6-phosphate content (12).

Immunoblotting studies

Cells were lysed in ice-cold buffer (20 mM Tris-HCl, 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM sodium orthovanadate, and 50 mM NaF) that included a protease inhibitor mixture (Roche), and disrupted by aspiration through a 21-gauge needle. After incubation on ice for 30 min and microcentrifugation for 10 min, the supernatants were collected and equal amounts of cellular proteins were fractionated on 10% SDS gels, transferred to polyvinylidine difluoride membranes, and analyzed by Western blotting.

Immunoprecipitation

Equal amounts of cell lysates were incubated with anti-p85 Ab, and the samples were mixed by rotation overnight at 4°C. A total of 20 μl of protein A/G coupled-beads were then added for an additional 1 h of mixing at 4°C, and the pellets were collected by centrifugation and washed twice with 1 ml of ice-cold lysis. The pellets finally were resuspended in 50 μl of 2× sample buffer, boiled for 5 min, and analyzed by SDS-PAGE and Western blotting.

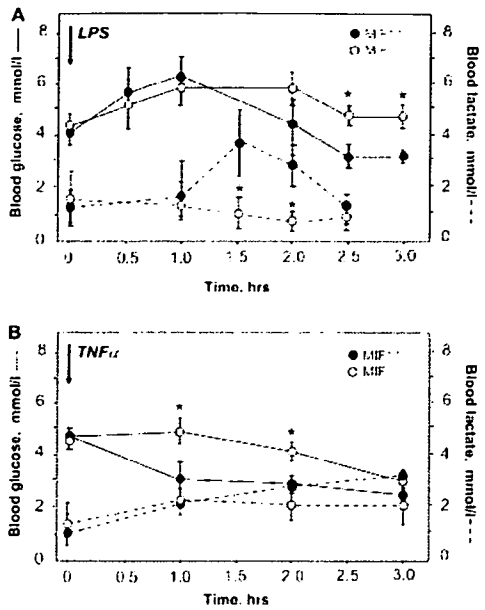

FIGURE 1. Glycemic and plasma lactate responses to endotoxemia. *A*, Blood glucose (solid line) and lactate (dotted line) levels in $MIF^{-/-}$ and WT ($MIF^{+/+}$) mice after injection of sublethal LPS (16.6 μg/g). *B*, Blood glucose (solid line) and lactate (dotted line) levels in $MIF^{-/-}$ and $MIF^{+/+}$ mice after a single i.p. injection of TNF-α (160 μg/kg). Results are the calculated mean glucose level ± SD for four mice per time point. *, $p <$ 0.05 for the $MIF^{-/-}$ vs the $MIF^{+/+}$ mice. For the blood glucose response in the $MIF^{+/+}$ mice, the 2.5-h and 3-h values differed significantly from the 0.5-h and 1-h values ($p < 0.05$). For the blood lactate response in the $MIF^{+/+}$ mice, the 1.5-h and 2-h values differed significantly from the 1-h and 2.5-h values ($p < 0.05$). The experiments shown are representative of three independent studies.

Glucose transport assay

Glucose uptake into cells was measured by an established protocol (13). Briefly, differentiated 3T3-L1 adipocytes were washed twice with transport solution (140 nM NaCl, 20 mM HEPES/Na, 5 mM KCl, 2.5 mM $MgSO_4$, and 1 mM $CaCl_2$) and incubated for 10 min with 0.5 μCi/ml of 2-deoxy-[^{3}H]glucose. The cells then were washed three times and lysed with 50 mM NaOH. The lysates were collected and radioactivity was measured. Cytochalasin B (10 μM) was used for the determination of noncarrier-mediated deoxyglucose uptake.

Preparation of murine adipocytes

Mouse primary adipocytes were prepared from the epididymal fat pads of $MIF^{-/-}$ and $MIF^{+/+}$ mice. Adipose tissue was removed, minced, and digested with 1 mg/ml collagenase in DMEM and 1% fatty acid-free BSA (14). Cells were filtered through nylon mesh, washed, and incubated overnight with or without TNF-α.

For differentiation studies, murine embryonic fibroblasts (MEF) from $MIF^{-/-}$ and $MIF^{+/+}$ were prepared from day 14.5 embryos (15). The cells were cultured in DMEM/10% FBS in either 6-well plates or in chamber slides. Adipocyte differentiation of primary murine fibroblasts was induced by DMEM/10% FBS with 0.5 mM isobutylmethylxanthine, 1.0 μM dexamethasone, and 10 μg/ml insulin for 48 h. The medium then was replaced with DMEM supplemented with 10% FBS (16). Adipocyte differentiation in different experiments was induced 48 h after confluence by addition of the differentiation medium indicated in *Results*. The differentiation medium was replaced every 48 h.

Oil red O staining

Cultured cells were washed with PBS and fixed with buffered formalin, and cytoplasmic lipid accumulation was analyzed by staining with Oil Red O

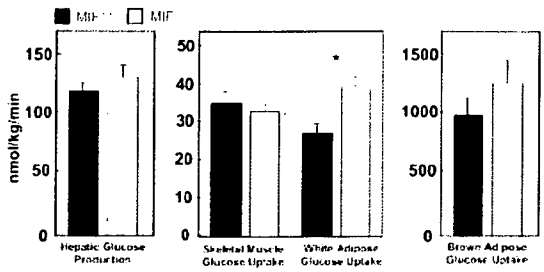

FIGURE 2. In vivo glucose metabolism in endotoxemic $MIF^{-/-}$ and $MIF^{+/+}$ mice. Seven to eight mice per group were pretreated with LPS, and organ-specific glucose metabolism was measured using labeled glucose in awake mice as described in the *Materials and Methods*. *, $p < 0.03$.

(16). The excess stain was removed and the cells were washed several times with water before microscopic examination and color quantification by NIH Image Analysis software.

Statistical analysis

Data are presented as mean ± SD. The statistical comparisons between groups were conducted using Student's t test. Values for $p < 0.05$ were considered significant.

Results

$MIF^{-/-}$ mice have an altered glycemic and plasma lactate response to endotoxin

Gram-negative endotoxin (LPS) is a powerful stimulus for the release of TNF-α and other proinflammatory cytokines, and LPS induces an acute hypoglycemic response when administered to experimental animals or human subjects (17). Prior studies using a neutralizing Ab approach demonstrated that anti-MIF inhibits the characteristic development of hypoglycemia in LPS-injected mice (5). We sought to verify these findings by administering a sublethal dose of LPS (16.6 μg/g) to $MIF^{-/-}$ mice and WT controls ($MIF^{+/+}$), and sampling their blood for measuring plasma glucose and lactate levels. $MIF^{-/-}$ mice showed a preservation of their glycemic and plasma lactate responses when compared with the WT mice (Fig. 1*A*). These differences were transient but most evident for lactate at 1.5 h and glucose at 2.5 h, which reflects the time necessary for systemic production of proinflammatory cytokines such as TNF-α and MIF (18). Plasma glucose levels were markedly lower (at 2.5 and 3 h), and plasma lactate levels were correspondingly higher (at 1.5 and 2 h) in the $MIF^{+/+}$ mice than in the $MIF^{-/-}$ mice. Although MIF has been shown to influence β-cell function (19), we observed no significant changes in plasma insulin levels, either at baseline (data not shown) or after LPS (2.5 h: $MIF^{+/+}$, 79 ± 12 pM, $MIF^{-/-}$, 69 ± 11 pM, p = NS; n = 5 mice per group). We also measured serum corticosterone, which may mediate increased glycemia as part of a systemic stress response. Notably, $MIF^{-/-}$ mice showed a decrease in circulating corticosterone levels when compared with $MIF^{+/+}$ mice, suggesting that glucocorticoids did not account for the increase in blood sugar during MIF deficiency (2.5 h: $MIF^{+/+}$, 0.42 ± 0.07 μM, $MIF^{-/-}$, 0.16 ± 0.04 μM, $p < 0.05$). There also were no differences in $MIF^{+/+}$ vs $MIF^{-/-}$ mice in circulating adiponectin, TNF-α, or

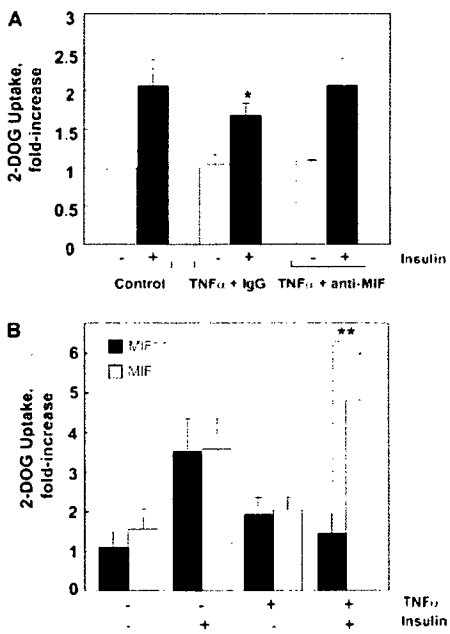

FIGURE 3. TNF-α has stimulatory action in glucose uptake into adipocytes. *A*, MIF neutralization reverses TNF-α-mediated reduction in adipocyte glucose transport. Cultured WT adipocytes were treated with TNF-α (20 ng/ml) together with an anti-MIF mAb or isotype control (each at 80 μg/ml). Basal and insulin-stimulated (100 nM, 5 min) uptake of 2-deoxyglucose (2-DOG) were performed as described in *Materials and Methods*. *B*, Primary mouse adipocytes were prepared from $MIF^{-/-}$ and $MIF^{+/+}$ mice and incubated overnight with or without TNF-α (20 ng/ml) for glucose transport studies. Data are means ± SD (n = 4 cultures). Data shown are representative of three independent experiments. *, $p < 0.05$ vs control; **, $p < 0.005$.

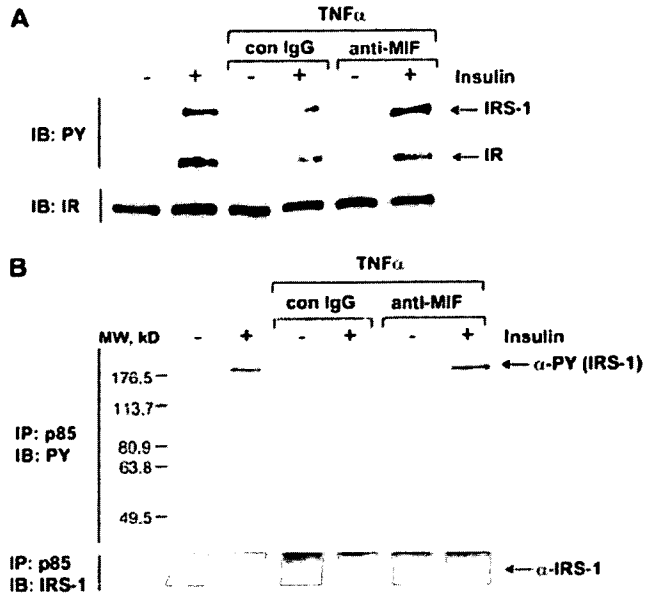

FIGURE 4. TNF-α action on the signal transduction events of the insulin receptor. *A*, Anti-MIF reduces TNF-α inhibition of the phosphorylation of IRS-1. 3T3-L1 adipocytes were stimulated with 20 ng/ml TNF-α for 6 h together with anti-MIF mAb or an isotypic control (IgG1) (each at 80 μg/ml) followed by stimulation with insulin (100 nM, 5 min). Cells were lysed, and Western blot analysis was performed with an anti-phosphotyrosine Ab (α-PY). The blots were stripped and reprobed with an insulin receptor-specific Ab (α-IRS-1). *B*, Anti-MIF mAb reduces the TNF-α-mediated decrease in the association of IRS-1 and PI3K subunit, p85. Cultured 3T3-L1 adipocytes were treated with 20 ng/ml TNF-α for 6 h together with anti-MIF mAb or an isotype control (each at 80 μg/ml), followed by stimulation with 100 nM insulin for 5 min. Whole cell lysates were immunoprecipitated with an anti-p85 Ab followed by Western blot analysis with an anti-phosphotyrosine Ab (α-PY). The blots were stripped and reprobed with an anti-IRS-1 Ab (α-IRS-1).

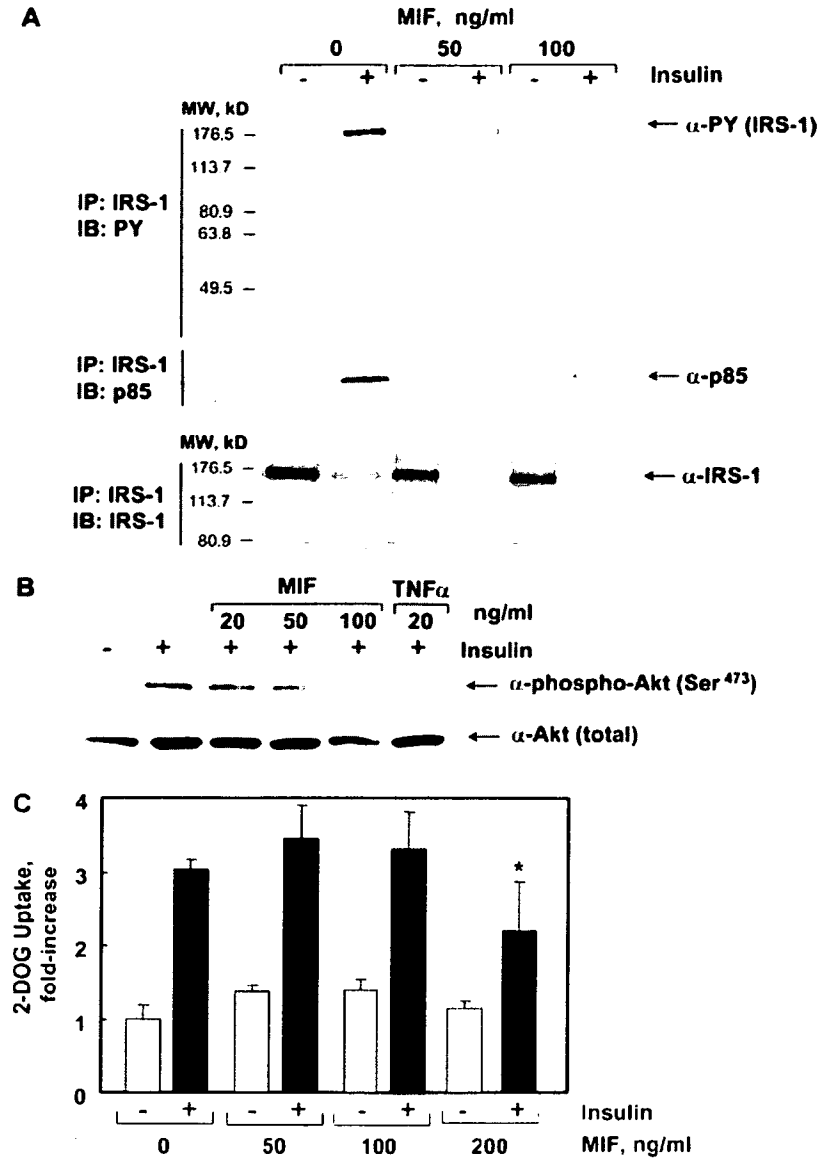

FIGURE 5. MIF-induced action on insulin signal transduction in adipocytes. *A*, MIF inhibits the insulin-induced tyrosine phosphorylation of IRS-1. Adipocytes were treated with increasing concentrations of recombinant MIF followed by insulin (100 nM) for 5 min. Immunoprecipitates were prepared from whole cell lysates using an anti-IRS-1 Ab followed by blotting analysis with anti-phosphotyrosine Ab (α-PY) (see *Materials and Methods*). The membrane was stripped and then reprobed with an anti-p85 Ab (α-p85) or an anti-IRS-1 Ab (α-IRS-1). *B*, MIF inhibits insulin-mediated phosphorylation of Akt. Cultured adipocytes were pretreated with MIF followed by insulin (100 nM) for 10 min. Total cell lysates were separated by SDS-PAGE and blotted with Abs against Ser^{473} phospho-Akt (α-phospho-Akt) and total Akt (αAkt). *C*, MIF decreases insulin-mediated 2-deoxyglucose (2-DOG) uptake in cultured adipocytes. Basal and insulin-stimulated uptake of 2-deoxyglucose into adipocytes was analyzed as described in *Materials and Methods*. Results were obtained in triplicate wells. Data shown (mean ± SD) are representative of three independently performed experiments. *, $p < 0.05$ vs control.

IL-6 levels, either at baseline (data not shown) or 2.5 h after LPS (adiponectin: $MIF^{+/+}$, 3.9 ± 0.3 ng/ml, $MIF^{-/-}$, 3.5 ± 0.3 ng/ml, p = NS; TNF-α: $MIF^{+/+}$, 57.0 ± 13.3 ng/ml, $MIF^{-/-}$, 69.6 ± 15.3 ng/ml, p = NS; and IL-6: $MIF^{+/+}$, 310 ± 100 ng/ml, $MIF^{-/-}$, 340 ± 80 ng/ml, p = NS). These genetically based results support an important role for MIF in the dysregulation of carbohydrate metabolism that occurs during endotoxemia.

Certain aspects of the metabolic effects of LPS can be recapitulated by the administration of TNF-α (4). To provide evidence for an effector role for MIF in the action of TNF-α, we next examined circulating glucose levels in $MIF^{-/-}$ and WT mice treated with a single, i.p. dose of TNF-α (160 μg/kg). TNF-α caused a marked reduction of blood glucose level in the WT mice, which is in agreement with prior reports (5, 17), and this effect occurred at least 1 h earlier than in mice administered LPS, which is in accord with the time delay necessary for TNF-α transcription, translation, and secretion. By contrast, $MIF^{-/-}$ mice treated with TNF-α were euglycemic in the acute phase (≤2 h), but then showed a reduction in blood glucose levels after 2 h (Fig. 1*B*). These data are in agreement with a prior report that anti-MIF may prevent TNF-α-induced hypoglycemia (5). The acute phase changes also are consistent with experiments supporting an MIF release response by cells and tissues exposed to TNF-α (5), and the reduction in blood glucose after 2 h may reflect a TNF-α-mediated induction of additional, downstream mediators (1).

Glucose uptake into adipose tissue is increased in endotoxemic, $MIF^{-/-}$ mice

To better assess the impact of MIF deficiency on glucose metabolism during endotoxemia, we measured organ-specific glucose uptake in awake mice at 2.5 h following sublethal treatment with LPS. Hepatic glucose production and glucose uptake into skeletal muscle (gastrocnemius) and adipose tissues were determined using continuous infusion of 3-[^{3}H]glucose and a bolus injection of 2-[^{14}C]DG as a nonmetabolizable glucose analog. As shown in Fig. 2, MIF deficiency was associated with a significant and selective increase in glucose uptake into white adipose tissue. In contrast, glucose uptake in skeletal muscle and brown adipose tissue, and hepatic glucose production were not significantly affected under these experimental conditions.

The inhibitory action of TNF-α on insulin-mediated glucose transport is reversed by MIF immunoneutralization or genetic deficiency

The preceding observations, together with the report that TNF-α-treated adipocytes secrete MIF (9), prompted us to examine the role of MIF in mediating the effect of TNF-α on adipocyte glucose metabolism. We verified that TNF-α (2 ng/ml) induced MIF secretion from cultured adipocytes, and we observed a diminution in MIF release at very high TNF-α concentrations (≥20 ng/ml), which is in agreement with prior observations in TNF-α-treated monocytes/macrophages (6 and data not shown).

TNF-α inhibits the stimulatory action of insulin on glucose uptake into adipocytes (20). We treated adipocytes with TNF-α and measured insulin-stimulated uptake of 2-deoxyglucose in the presence of anti-MIF mAb or an isotypic, IgG1 control. The insulin-stimulated uptake of 2-deoxyglucose was significantly greater upon MIF immunoneutralization (Fig. 3*A*). The inhibitory action of MIF upon 2-deoxyglucose uptake was verified in studies performed in primary adipocytes isolated from $MIF^{-/-}$ mice. As shown in Fig. 3*B*, adipocytes genetically deficient in MIF showed a significant increase in 2-deoxyglucose uptake despite treatment with TNF-α. These data support a model whereby TNF-α inhibits insulin signal transduction via the autocrine/paracrine secretion of MIF. Such a mechanism of TNF-α action also is consistent with studies in differentiated myotubes, where TNF-α was found to induce MIF release, leading to a downstream, autocrine/paracrine response (5).

Immunoneutralization of MIF reverses TNF-α-mediated insulin resistance in cultured adipocytes

A well-characterized action of TNF-α with respect to glucose transport is inhibition of insulin signal transduction leading to insulin resistance (21). We followed a standard protocol for inducing insulin resistance in vitro and treated adipocytes with TNF-α together with a neutralizing anti-MIF mAb or isotype control, followed by stimulation with insulin for 5 min. As expected, insulin induced the rapid tyrosine phosphorylation of the insulin receptor and the IRS-1, and these events were inhibited by TNF-α pretreatment (Fig. 4*A*). The addition of anti-MIF mAb, but not control Ab, significantly reduced the action of TNF-α on the tyrosine phosphorylation of the insulin receptor and IRS-1.

The enzyme PI3K is a downstream mediator of insulin signal transduction, and it plays an important role in the insulin-dependent translocation of GLUT4 (22). PI3K is comprised of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit with Src homology 2 domains that bind to the tyrosine-phosphorylated isoform of IRS-1. The functional significance of the TNF-α-dependent reduction in IRS-1 tyrosine phosphorylation can be monitored by coimmunoprecipitation of the IRS-1 complex containing the p85 regulatory subunit of PI3K (23). We examined the effect of MIF neutralization on the TNF-α-mediated decrease in the association of IRS-1 and PI3K (p85). As shown in Fig. 4*B*, the insulin-induced association of IRS-1 with PI3K (p85 subunit) was reduced by TNF-α treatment, and anti-MIF inhibited this specific action of TNF-α on adipocytes. These data, taken together, are consistent with a role for MIF in mediating the action of TNF-α on the proximal signal transduction events induced by insulin receptor ligation.

Recombinant MIF inhibits insulin signal transduction in adipocytes

To provide a more direct assessment of MIF action on adipocytes, we treated cells with different concentrations of MIF before stimulation by insulin. As shown in Fig. 5*A*, exogenously added MIF decreased the tyrosine phosphorylation of IRS-1 as well as the insulin-induced association of IRS-1 with the p85 regulatory subunit of PI3K. This effect occurred in a dose-dependent fashion, and at concentrations of MIF that are within the range for those reported in patients with sepsis or severe inflammation (7, 8). The serine-threonine kinase Akt is a downstream target of PI3K (24) that is recruited to the membrane by PI3K-generated phospholipids. Akt undergoes phosphorylation and activation, and provides signals for the synthesis of new glucose transporters and enhanced glucose uptake. We examined the effect of exogenously added MIF on insulin-mediated, Ser^{473} phosphorylation of Akt in adipocytes. Cells were treated with MIF followed by insulin (100 nM) for 10 min. As shown in Fig. 5*B*, MIF inhibited the insulin-induced phosphorylation of Akt.

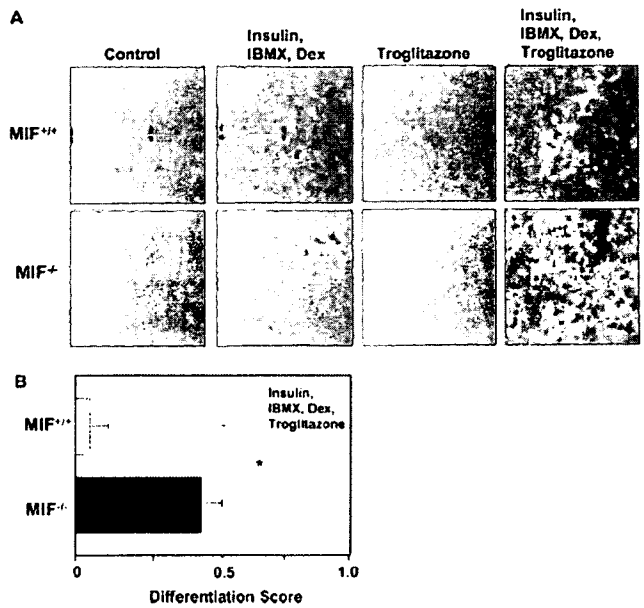

FIGURE 6. MIF deficiency enhances adipocyte differentiation. *A*, MEFs were prepared from $MIF^{+/+}$ or $MIF^{-/-}$ mice, and differentiation into adipocytes induced as shown. Differentiation was assessed visually by staining with Oil red O at a magnification of ×100. *B*, Quantification of color intensity by NIH Image Analyzer software is shown. Data are representative of four experiments each performed with independently derived fibroblast cell lines. *, $p < 0.001$.

Next, we investigated the action of MIF on basal and insulin-mediated glucose uptake. The addition of MIF to adipocytes did not cause an appreciable change in basal 2-deoxyglucose uptake (Fig. 5*C*). A decrease in insulin-stimulated glucose uptake was observed in this cultured cell system at 200 ng/ml MIF. These data differ from those reported in differentiated myotubes, where MIF was found to augment basal glucose uptake and not to influence insulin-mediated glucose uptake (5). This difference in response likely reflects the selective use of insulin-sensitive vs insensitive glucose transporters in these two differentiated cell types (25).

Genetic deficiency in MIF increases adipogenesis

We next examined the influence of MIF in adipogenesis, which is known to be inhibited by TNF-α (26). We prepared embryonic fibroblasts from $MIF^{+/+}$ and $MIF^{-/-}$ mice and subjected them to defined adipocyte differentiation protocols (27). We found that although $MIF^{-/-}$ cells responded similarly to $MIF^{+/+}$ cells when exposed to a standard differentiation medium (insulin, isobutylmethylxanthine, and dexamethasone) or to the peroxisome proliferator-activated receptor (PPAR)γ agonist, troglitazone, the

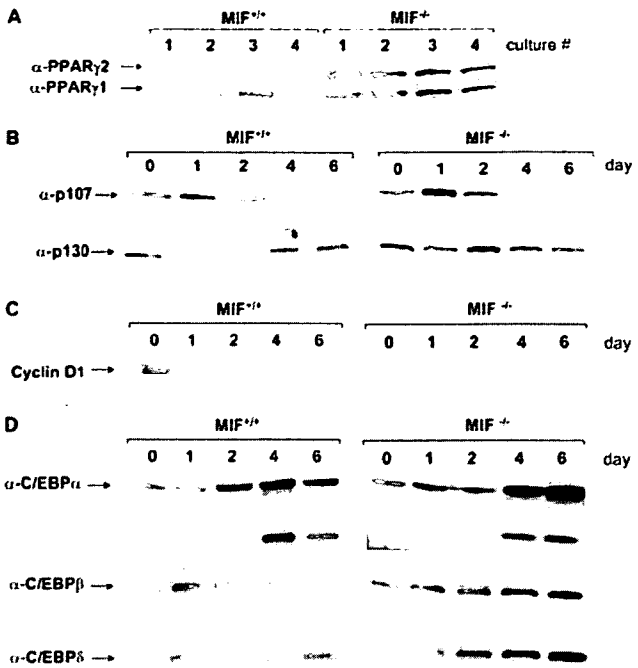

FIGURE 7. Impact of MIF deficiency on signaling pathways associated with adipogenesis. *A*, The expression of PPARγ proteins after induction of adipogenesis. Cells were grown in standard differentiation medium in the presence of troglitazone and harvested at day 9. The Western blot analysis of lysates from cultures of four different MEF cell lines (*lanes 1–4*) is shown. *B*, The expression of the p107 and p130 transcriptional regulatory proteins during adipocyte differentiation. $MIF^{+/+}$ and $MIF^{-/-}$ MEFs were grown in standard differentiation medium in the presence of troglitazone and harvested at the indicated days for Western blot analysis with anti-p107 (α-p107) or anti-p130 (α-p130) Ab. *C*, The expression of cyclin D1 protein during adipocyte differentiation. $MIF^{+/+}$ and $MIF^{-/-}$ MEFs were grown in standard differentiation medium in the presence of troglitazone and analyzed by Western blotting with anti-cyclin D1 Ab. *D*, The expression of C/EBPα, C/EBPβ, and C/EBPδ proteins during adipogenesis. Cells were grown as described, harvested on the indicated days, and Western blot analyzed with the C/EBP-specific Abs shown. The blot for C/EBPα shows both the p42 and the p30 isoforms.

$MIF^{-/-}$ cells were significantly more sensitive to the combination of standard differentiation medium plus troglitazone (Fig. 6). Adipocyte differentiation is known to be linked to the transcriptional activation of the PPARγ, which is a target for the differentiation agent, troglitazone. We observed higher expression of the PPARγ1 and PPARγ2 isoforms during differentiation of the $MIF^{-/-}$ cells vs the $MIF^{+/+}$ cells (Fig. 7*A*). Fibroblasts exposed to adipogenic factors enter a stage of postconfluent, cell division that is associated with a switch in the expression of the p130:p107 transcriptional regulatory proteins (28). We analyzed the expression of p130:p107 during adipocyte differentiation and found a similar pattern of expression in both the $MIF^{-/-}$ and $MIF^{+/+}$ cells (Fig. 7*B*). The expression of cyclin D1 and the subsequent activation of cyclin-dependent kinases has been shown to inhibit adipocyte differentiation (38). We analyzed cyclin D1 levels by western blotting and observed the expected down-regulation of this protein after the addition of differentiation medium to $MIF^{+/+}$ cells. By contrast, the expression of cyclin D1 was significantly reduced at baseline in $MIF^{-/-}$ cells, which appears consonant with the increased sensitivity of these cells to adipocyte differentiation (Fig. 7*C*).

Finally, we examined the expression of C/EBPα, C/EBPβ, and C/EBPδ, which are transcriptionally activated during adipogenesis.

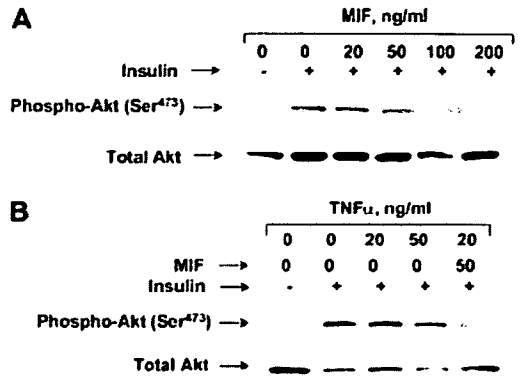

FIGURE 8. The effect of MIF or TNF-α on the insulin-mediated phosphorylation of Akt in $MIF^{-/-}$ adipocytes. *A*, Cultured adipocytes were pretreated with MIF for 6 h followed by insulin (100 nM) for 10 min. Total cell lysates were separated by SDS-PAGE and blotted with Abs against Ser^{473} phospho-Akt and total Akt. *B*, Cultured adipocytes were pretreated with TNF-α, or TNF-α plus MIF, for 6 h followed by insulin (100 nM) for 10 min. Total cell lysates were separated by SDS-PAGE and blotted with Abs against Ser^{473} phospho-Akt and total Akt.

C/EBPβ and C/EBPδ are induced early and activate the expression of PPARγ, and C/EBPα is induced later in time (29). MIF deficiency was associated with an up-regulation of C/EBPα, C/EBPβ, and C/EBPδ during the six-day period of adipocyte differentiation (Fig. 7*D*). MIF-deficient cells thus show an increased ability to differentiate into adipocytes in response to PPARγ ligands, and this effect may result from a constitutive reduction in baseline cyclin D1 levels and an enhancement in cellular C/EBPα, C/EBPβ, and C/EBPδ content.

The effect of MIF or TNF-α on insulin-mediated phosphorylation of Akt in $MIF^{-/-}$ adipocytes

To further evaluate the functional phenotype of $MIF^{-/-}$ adipocytes, we examined the effect of MIF or TNF-α on insulin-mediated signal transduction in these cells. $MIF^{-/-}$ adipocytes were exposed to MIF or TNF-α and the insulin induced phosphorylation of Akt was analyzed by western blotting. As shown in Fig. 8, MIF inhibits the insulin-mediated phosphorylation of Akt, whereas TNF-α showed no significant effect. We also examined the presence of TNFRs during the differentiation of both $MIF^{-/-}$ and $MIF^{+/+}$ adipocytes, because a reduction in the TNFR could offer an explanation for the TNF insensitivity of $MIF^{-/-}$ cells. The expression of TNFR-1 protein was induced in equivalent levels in $MIF^{-/-}$ and $MIF^{+/+}$ cells, and no differences were noted in the expression of the TNFR-2 isoform (Fig. 9 and data not shown).

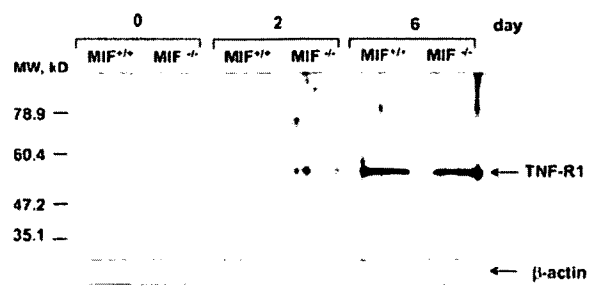

FIGURE 9. The expression of TNFR (TNF-R1) during adipocyte differentiation of $MIF^{+/+}$ and $MIF^{-/-}$ MEFs. Total cell lysates were separated by SDS-PAGE and blotted with Abs against TNFR-1 Ab. The blot was stripped and reprobed with anti-β-actin Ab.

Discussion

Investigations of the metabolic dysregulation that accompanies severe infection or tissue invasion led in the 1980s to the definition of the mediator "cachectin", which was later determined to be structurally identical with TNF-α (30). Among the metabolic changes noted in these studies were insulin resistance and alterations in glucose and lipid homeostasis that affected the rate of substrate production and use by tissues. There has been an accumulation of data supporting the importance of TNF-α in insulin resistance, both in acute disease and in conditions such as diabetes and obesity (3, 20, 21, 31). The importance of metabolic homeostasis to clinical outcome also has been highlighted by observations of decreased mortality in critically ill patients treated with insulin therapy (32).

MIF is an established mediator of sepsis lethality (7), and it is secreted by activated immune cells (6), by the anterior pituitary (10), and by the β-cells of the pancreatic islets, where it is a positive, autocrine regulator of insulin release (19). In a recent study, MIF was described to be released from cultured myotubes stimulated with TNF-α and then to act in an autocrine/paracrine manner to stimulate muscle glucose catabolism (5). In the present study, $MIF^{-/-}$ mice showed a near normalization in glucose metabolism in response to endotoxin, which induces a transient (<2 h) alteration in circulating glucose and lactate levels. Moreover, TNF-α itself did not influence blood glucose in the setting of genetic MIF deficiency. An intrinsic role of MIF in the glycemic response during endotoxemia was verified by in vivo experiments using labeled glucose in awake mice, which showed increased glucose uptake into white adipose tissue in mice genetically deficient in MIF. Anti-MIF mAb also prevented TNF-α inhibition of insulin-mediated glucose transport in adipocytes, which is in accord with a modulating effect of MIF on the action of TNF-α at the level of insulin signal transduction. The precise mechanism for the inhibitory effect of MIF on insulin signal transduction events may involve inhibition of Akt phosphorylation, which together with PI3K is necessary for the serine phosphorylation of the IRS-1 protein (23, 33).

Genetic MIF deficiency also promotes adipogenesis in a defined model of adipocyte differentiation requiring PPARγ agonism, and this effect is consistent with an inhibitory action of TNF-α on adipocyte differentiation (34). Among the mechanisms by which MIF may mediate this inhibition it is notable that the E2F family regulates the differentiation of adipocytes. E2F1 induces PPARγ transcription during clonal expansion (35), and Petrenko et al. (36) recently showed that the expression of E2F1 is up-regulated in $MIF^{-/-}$ cells. An additional pathway may relate to the defect in cyclin D1 activity that has been described in fibroblasts deficient in MIF (37). Fu et al. (38) have reported that cyclin D1 inhibits PPARγ-mediated adipogenesis via an action on histone deacetylase. $MIF^{-/-}$ cells showed reduced levels of cyclin D1, which is in agreement with the enhanced adipogenic potential of these cells. MIF also showed an inhibitory action on C/EBPδ. The C/EBPδ transcription factor is induced by the dexamethasone (39), and this action is likely consistent with the glucocorticoid counter-regulating properties of MIF (18, 40).

TNF-α did not affect the insulin-mediated phosphorylation of Akt in $MIF^{-/-}$ adipocytes, further confirming the role for MIF as a downstream effector of TNF-α. This effect was not due to reduced expression of TNFR-1 by these cells, which may be suggested by a prior report (42). It is known, however, that the action of TNF-α on insulin signaling is reduced upon PPARγ activation (41). Although we cannot rule out a role for MIF in affecting PPARγ activation, the PPARγ ligand troglitazone by itself did not show a differential effect on adipocyte development from $MIF^{+/+}$ vs $MIF^{-/-}$ cells. Further studies will be necessary to better define the signaling pathways influenced by the interaction of TNF-α and MIF in this model system.

In conclusion, these studies support the concept that TNF-α mediates insulin resistance in adipocytes by the downstream, autocrine/paracrine action of MIF on key steps in the insulin signal transduction pathway. The present studies nevertheless do not signify that all of the metabolic actions of TNF-α are necessarily attributed to MIF. A broader question regards the potential action of MIF in carbohydrate and lipid homeostasis in physiologic settings outside of severe inflammation. Patients who have type 2 diabetes have increased circulating levels of MIF (43, 44), and the recent discovery of functional alleles in the human *MIF* gene (45), prompt consideration of the role of MIF in the pathogenesis of insulin resistance that occurs more commonly in diabetes, aging, and obesity.

Disclosures

Drs. Leng, Metz, Mitchell, and Bucala are coinventors on patents describing the potential therapeutic value of inhibiting MIF.

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ARTHRITIS & RHEUMATISM
Vol. 56, No. 8, August 2007, pp 2803–2809

CONCISE COMMUNICATIONS

DOI 10.1002/art.22799

A polymorphism in human platelet antigen 6b and risk of thrombocytopenia in patients with systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder characterized by circulating autoantibodies directed against nuclear, cytoplasmic, cell membrane, and other autoantigens. Possible manifestations of SLE include the thrombocyte-associated diseases autoimmune thrombocytopenia and thrombosis.

The platelet surface glycoprotein IIb-IIIa (GPIIb-IIIa) complex plays an important role in platelet aggregation and hemostasis, since it is a receptor for fibrinogen, von Willebrand factor, and fibronectin. This complex constitutes a major target for antiplatelet antibodies responsible for immune-related thrombocytopenia (1).

GPIIIa is highly polymorphic, and 5 human platelet antigen (HPA) systems (HPA-1, HPA-4, HPA-6, HPA-7, and HPA-8) are known to be located in this glycoprotein. Polymorphisms in this region have been associated with neonatal alloimmune thrombocytopenia, posttransfusion purpura, and refractoriness to platelet transfusion in European American populations (2). Thus, these polymorphisms may be important as alloantigen determinants. In addition, evidence has been found to support a significant association between HPA-1 polymorphism and thrombosis (3,4), suggesting that HPA is involved in platelet aggregation.

In the Japanese population, polymorphisms in HPA-1, HPA-7, and HPA-8 are very rare. In a previous study, only 1 of 331 random donors was found to be A/B heterozygous in HPA-1, and no polymorphisms were detected in HPA-7 or HPA-8 in any of the subjects (5). The frequencies of polymorphisms in other HPA systems were 2.2% for A/B in HPA-4 and 5.1% for A/B and 0.3% for B/B in HPA-6 (5).

In the present study, we analyzed HPA-1, HPA-4, and HPA-6 polymorphisms in Japanese patients with SLE to determine the association of these polymorphisms with thrombocytopenia and thrombosis. A total of 134 Japanese patients with SLE (121 women and 13 men) with a mean age of 41.4 years (range 20–72 years), who presented to our Rheumatic and Connective Tissue Disease Unit, were recruited for the study. All of them fulfilled the American College of Rheumatology criteria for SLE (6). Fifteen patients (11.2%) fulfilled the Sapporo criteria for definite antiphospholipid syndrome (7). Sixty-seven healthy Japanese individuals were included as a control group. The study was conducted in accordance with the Declaration of Helsinki and the principles of good clinical practice. Approval was obtained from the local ethics committee, and written informed consent was obtained from each patient before enrollment.

Polymorphism status in HPA-1, HPA-4, and HPA-6 was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), as previously described (5), with minor modifications. Briefly, 50 ng of genomic DNA was amplified in a total volume of 10 μl containing dNTPs (200 μM each) in a standard buffer with 1.5 mM $MgCl_2$, 0.5 units of DNA *Taq* polymerase, and 10 pmoles of each primer. The gene-specific primer sequences were as follows: for HPA-1, forward 5′-CTTA GCTATTGGGAAGTGGTAGGGCCTGC-3′ and reverse 5′-ACCTCAGATCTTCTGACTCAAGTCCTAACGTC TCTTATT-3′; for HPA-4, forward 5′-CCTGTGG-ACATCTACTACTTGATGGACC-3′ and reverse 5′-GCCAATCCGCAGGTTACTCGTGAGCATT-3′; and for HPA-6, forward 5′-CTGGCTGGCTGGGATCCCAGTG-3′ and reverse 5′-CCCTGCAGTTCTCCTCACCTGAG-3′. PCRs were performed as follows: for HPA-1, 27 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; for HPA-4, 31 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; and for HPA-6, 27 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. After all cycles were completed, a final extension step of 72°C for 7 minutes was performed in all PCRs.

The amplified products (486 bp, 126 bp, and 240 bp for HPA-1, HPA-4, and HPA-6, respectively) were digested by *Msp* I (New England Biolabs, Beverly, MA) at 37°C for 16 hours for HPA-1, *Bsm* I (New England Biolabs) at 65°C for 1 hour for HPA-4, and *Mva* I (Takara Shuzo, Shiga, Japan) at 37°C for 16 hours for HPA-6. RFLP products were resolved in 9% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light. For HPA-1, two bands of 274 bp and 206 bp were visualized for the A/A homozygous genotype, and 4 bands of 274 bp, 206 bp, 173 bp, and 101 bp were identified for the heterozygous genotype. For HPA-4, a 104-bp band corresponded to the A/A genotype, and a 126-bp band to the B/B genotype. For HPA-6, A/A single band of 240 bp was visualized for the A/A homozygous genotype. Two bands of 165 bp and 75 bp and 3 bands of 240 bp, 165 bp, and 75 bp were identified for the B/B homozygous genotype and the heterozygous genotype, respectively.

All patients were tested for antiphospholipid antibodies (aPL), including anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC). The aCL (IgG, IgM, and IgA) were assayed as previously described (8), and for the detection of LAC the guidelines of the Subcommitte for Standardization of the International Society of Thrombosis and Haemostasis were followed (9).

The records of the patients were reviewed retrospectively, and autoimmune thrombocytopenia, defined as platelet count $<100 \times 10^9$/liter, was detected in 35 patients (26.1%). Transient thrombocytopenia associated with disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, hemophagocytotic syndrome, drug-induced thrombocytopenia, and myelodysplastic syndrome were not considered to be autoimmune thrombocytopenia. Twenty-one patients (15.7%) had a history of thrombotic events. LACs were detected in 43 patients (32.1%) and aCL in 26 (19.4%).

HPA-1 polymorphism was not present in the study population. The A/A genotype was detected in HPA-1 in 100% of SLE patients and healthy individuals, in accordance with the results of a previous study (5).

The HPA-4 A/A genotype was found in 98.3% of SLE patients and in 97% of healthy individuals. The heterozygous

Table 1. Distribution of the HPA-6 genotypes*

Group (n)	A/A genotype	A/B genotype	B/B genotype
Healthy controls (67)	66 (98.5)	1 (1.5)	0 (0)
All SLE patients (134)†	124 (92.5)	10 (7.5)	0 (0)
Patients with thrombocytopenia (35)‡	28 (80)	7 (20)	0 (0)
Patients without thrombocytopenia (99)	96 (97)	3 (3)	0 (0)
Patients with thrombosis (21)	21 (100)	0 (0)	0 (0)
Patients without thrombosis (113)	103 (91.2)	10 (8.8)	0 (0)
Patients with aPL (52)	50 (96.2)	2 (3.8)	0 (0)
Patients without aPL (82)	74 (90.2)	8 (9.8)	0 (0)

* Values are the number (%) of subjects. HPA-6 = human platelet antigen 6; SLE = systemic lupus erythematosus; aPL = antiphospholipid antibody.
† Odds ratio (OR) 5.32 (95% confidence interval [95% CI] 0.66–42.48).
‡ OR 8 (95% CI 1.9–32.9), $P < 0.0028$.

genotype was observed in 2 patients and in 2 controls (1.5% and 3%, respectively), a slightly higher frequency than that previously found by Tanaka et al in 331 random donors (0.3%) (5).

The HPA-6 A/B genotype was found in 7.5% of patients with SLE, compared with 1.5% of healthy controls. There was no significant deviation from Hardy-Weinberg equilibrium in either group. The HPA-6 A/B genotype was more frequently observed in patients with thrombocytopenia than in those without (odds ratio 8, [95% confidence interval 1.94–32.98], $P = 0.0028$). There was no difference in genotype distribution based on the presence or absence of thrombotic events or aPL (Table 1).

The first recognized platelet alloantigen was seen in 1959 in a patient with clinical features of posttransfusion purpura (10). Since then, >20 alloantigens have been described and attributed to different antigen systems. The HPA-6 polymorphism is due to an Arg–Gln variation at position 489. We found the HPA-6 A/B genotype in 5.5% of subjects, a frequency similar to that previously found in Japanese donors (4.8%) (11), and significantly higher than that observed in a Finnish population (0.7%) (12).

The initial aim of our study was to identify a risk factor for thrombosis, apart from the presence of aPL, in patients with SLE. The strong correlation between HPA-1 polymorphism and coronary events (4) drove us to investigate a link between HPA polymorphisms and thrombosis in SLE. In the present study there was no statistically significant difference between the frequency of the HPA-6 A/B genotype in patients and that in healthy controls. None of the HPA polymorphisms investigated in this study were identified as a risk factor for thrombosis in the study population. Interestingly, the HPA-6 A/B genotype occurred significantly more frequently in SLE patients with autoimmune thrombocytopenia than in those without.

Autoimmune thrombocytopenia, one of the most common features of SLE, occurs in 7–30% of patients during the course of the disease, but its pathogenesis is still controversial. One postulated mechanism of platelet destruction in SLE involves the presence of antiplatelet antibodies, mainly directed against the GPIIb-IIIa complex, which bind to circulating platelets and facilitate the clearance of platelets by the reticuloendothelial system (1,13). Alternatively, aPL may interact with platelet phospholipids, leading to platelet destruction by the reticuloendothelial system (14,15)

The significance of antigen polymorphisms in the development of autoimmune thrombocytopenia is far from clear. Polymorphisms in GPIIb-IIIa may potentially promote the production of autoantibodies. Platelet GPIIb-IIIa is the main antigenic target recognized by antiplatelet antibodies in autoimmune thrombocytopenia, and anti-GPIIb-IIIa antibodies have been measured in SLE-associated thrombocytopenia using an indirect monoclonal-specific immobilization of platelet antigens assay (1) or an enzyme-linked immunosorbent assay, which detects peripheral blood B cells secreting IgG anti–GPIIb-IIIa (16). It would be of interest to analyze the association between anti–GPIIb-IIIa and HPA-6 polymorphism. Unfortunately, our study design did not include the detection of antiplatelet antibodies, and neither of these detection methods was performed.

In our thrombocytopenia patients with the HPA-6 A/B genotype, platelet count ranged from 3.2×10^9 to 9.8×10^9/liter, implying that the association between HPA-6 A/B genotype and thrombocytopenia has an immunogenetic impact rather than clinical significance as a predictor of bleeding tendency.

In conclusion, our preliminary findings show that the HPA-6 A/B genotype is a risk factor for autoimmune thrombocytopenia in SLE. Further investigations are needed to clarify the relationship between this polymorphism and thrombocytopenia in SLE.

Supported by grants from the Japanese Ministry of Health, Labor, and Welfare and the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

AUTHOR CONTRIBUTIONS

Dr. Atsumi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data. Amengual, Atsumi, Komano, Kataoka, Horita, Yasuda, Koike.

Analysis and interpretation of data. Amengual, Atsumi, Komano, Kataoka, Horita, Yasuda, Koike.

Manuscript preparation. Amengual, Atsumi, Komano, Kataoka, Horita, Yasuda, Koike.
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ARTHRITIS & RHEUMATISM
Vol. 56, No. 2, February 2007, pp 393–394
DOI 10.1002/art.22366

EDITORIAL

"Resurrection of Thrombin" in the Pathophysiology of the Antiphospholipid Syndrome

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Antiphospholipid syndrome (APS) is characterized by recurrent thrombosis associated with the persistent presence of antiphospholipid antibodies (aPL) (1). Many of the autoantibodies associated with APS are directed against phospholipid-binding plasma proteins such as β_2-glycoprotein I (β_2GPI) and prothrombin expressed on, or bound to, the surface of vascular endothelial cells, platelets, or other cells.

Beta$_2$-glycoprotein I was first described in 1961 as a component of the β-globulin fraction of human serum (2). However, it was not until the early 1990s that clear evidence emerged of the major role of β_2GPI in the binding of anticardiolipin antibodies to phospholipids in patients with APS. Over the following years, detailed studies have been performed to define its structure and properties. Beta$_2$-glycoprotein I, a 50-kd protein, is a single polypeptide chain composed of 326 amino acid residues with 5 oligosaccharide attachment sites (3). This protein is composed of 5 homologous motifs designated short consensus repeats (SCRs) or sushi domains. Human β_2GPI has been crystallized, and its tertiary structure has revealed a highly glycosylated protein with an elongated fishhook-like arrangement of the globular SCR domains (4,5). Beta$_2$-glycoprotein I has a major phospholipid binding site located in the fifth domain, C^{281}KNKEKKC^{288}, close to the hydrophobic loop.

Recently, great interest has arisen concerning the binding of aPL to endothelial cells or other procoagulant cells and how this binding mediates cell dysfunctions that potentially induce the clinical manifestations of APS. A few years ago, the signal transduction mechanism implicated in the induction of procoagulant substances by aPL was examined. There is now clear evidence that the p38 MAPK pathway of cell activation plays an important role in aPL-mediated cell activation (6–8). Moreover, the role of annexin A2 as a receptor for β_2GPI (9) and the interaction of β_2GPI with different members of the low-density lipoprotein receptor family (10) have been recently reported.

Before this era of cell biology, clinical features of APS were considered as "coagulopathy," and the mechanisms involved in the coagulation cascade abnormality mediated by aPL had been intensively investigated since the early 1980s. Currently, the coagulopathy concept appears to have minor standing within a cluster of reports described in the previous paragraph. However, it is still noted that a significant reduction of in vitro thrombin generation was found in plasma from β_2GPI-null mice, indicating that β_2GPI may play an important role in thrombin generation (11). The in vitro properties of β_2GPI as a natural regulator of coagulation have been proposed in a large number of reports. Beta$_2$-glycoprotein I may act as an anticoagulant by binding to the phospholipids on platelet surfaces and inhibiting the contact pathway. In addition, β_2GPI binds directly to factor XI and inhibits activation of factor XI by thrombin or activated factor XII; this inhibition attenuates thrombin generation (12).

Thrombin, a member of the serine protease family, is a key enzyme in hemostasis. Thrombin is generated from its inactive precursor prothrombin by activated factor X as part of the prothrombinase complex on the surface of activated cells. Thrombin may function as a procoagulant not only by cleaving fibrinogen to fibrin, but also by interacting with protease-activated receptors (PARs) on many types of procoagulant cells and with GPIb-IX-V complex on the surface of platelets, leading to platelet aggregation and activation. On the other hand, thrombin may behave as an anticoagulant upon binding to thrombomodulin to favor activation of protein C. Furthermore, thrombin takes part in the regulation of numerous physiologic and pathophys-

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Submitted for publication September 4, 2006; accepted in revised form October 23, 2006.

iologic functions, such as promotion of inflammation, carcinogenesis, angiogenesis, atherosclerosis, and the tissue reparative process (13).

In this issue of *Arthritis & Rheumatism*, Rahgozar et al (14) elegantly demonstrate that β_2GPI is able to bind exosites I and II on thrombin, and that domain V of β_2GPI is essential for this interaction. The interaction between β_2GPI and thrombin may interfere with the coagulation system as well as with many of the biologic functions in which thrombin participates. The significance of β_2GPI binding to thrombin needs further investigation, but direct interaction of β_2GPI with thrombin, one of the most potent enzymes present in the body, opens a new insight into the pathophysiology of the manifestations of APS. It is recognized worldwide that the mechanisms of thrombosis in APS are multifactorial. The direct involvement of thrombin may be a clue to this pluripathologic process.

A second major antigenic target in APS is prothrombin. Most APS patients have anti-β_2GPI antibodies and/or antiprothrombin antibodies. These 2 populations of antibodies are believed to share pathogenic roles in thrombosis in APS. Prothrombin is a precursor of thrombin; thus, the 2 major antigenic players, β_2GPI and prothrombin, encounter each other around the clot formation process.

Rahgozar et al (14) also investigated the effect of monoclonal anti-β_2GPI antibodies in the inhibition of factor XI activation by β_2GPI and thrombin complex, and they show that anti-β_2GPI antibodies enhance this inhibition, leading to a decreased generation of thrombin. It is apparently paradoxical that anti-β_2GPI antibodies enhance one function of the antigen, but this is similar to the effect seen in the β_2GPI, anti-β_2GPI antibodies, and protein C scenario (15). Anti-β_2GPI antibodies enhanced the inhibitory effect of β_2GPI on activated protein C, which proteolyzes activated factor V/activated factor VIII, and reduced thrombin generation. The direct effect of anti-β_2GPI antibodies on thrombin-dependent factor XI activation may, in part, explain why anti-β_2GPI antibodies have lupus anticoagulant activity, but it cannot explain the increased thrombin generation found in APS patients.

In conclusion, Rahgozar et al provide evidence that β_2GPI and thrombin interact directly with each other and that anti-β_2GPI antibodies may modify the "ultimate" generation of thrombin, reminding us of the importance of thrombin, a main player in the era of "coagulopathy," for explaining the formation of clots. The phenomena provide a step forward in the understanding of the etiologic chart of APS. These data have brought thrombin into the center of the chart again, although many details are still far from being elucidated.

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