Inhibition of p70S6 Kinase during Transforming Growth Factor-β1/
Vitamin D₃-induced Monocyte Differentiation of HL-60 Cells Allows
Tumor Necrosis Factor-α to Stimulate Plasminogen Activator
Inhibitor-1 Synthesis*

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We investigated intracellular mechanisms involved in the up-regulation of plasminogen activator inhibitor I (PAI-1) synthesis by human recombinant tumor necrosis factor-α (TNF) during monocyte differentiation of HL-60 cells triggered by the transforming growth factor-β1/vitamin D₃ (TGF/D₃) mixture. TGF/D₃-treated cells expressed surface monocyte markers and produced noticeable amounts of PAI-1 but stopped to proliferate. A reduced p70 S6 kinase (p70S6K) phosphorylation was also observed and, in this situation, TNF dramatically enhanced PAI-1 synthesis. Similarly, TNF significantly up-regulated PAI-1 synthesis when p70S6K phosphorylation was inhibited by rapamycin. This phenomenon was not due to a general decrease in protein synthesis but involved the activation of gene transcription rather than PAI-1 mRNA stabilization. The level of the transcriptional regulator factor E2F1, a repressor of PAI-1 gene expression, was shown to be down-modulated in TGF/D₃- as well as in rapamycin-treated cells. Furthermore, the apoptotic effect of TNF in HL-60 cells appeared to be prevented by the addition of either TGF/D₃ or rapamycin. In conclusion, these results indicate that inhibition of p70S6K phosphorylation during TGF/D₃-induced monocyte differentiation of HL-60 cells is a determinant factor that allows TNF to exert its up-regulating effect on PAI-1 synthesis while protecting cells from apoptosis.

The key role of macrophages derived from blood-borne monocytes in inflammatory diseases and particularly in atherosclerosis development has been well documented (1–3). A property that can be considered essential for monocytes/macrophages to exert their roles is their ability to emigrate from the circulating blood pool into different tissues or structures. This ability is mainly secured by the expression of pericellular proteolytic activities, with the partial involvement of the fibrinolytic system (4–6). Because of its potency to inhibit plasminogen activators, Plasminogen activator inhibitor I (PAI-1) can be considered the cornerstone of fibrinolytic system regulation (7, 8) and thus involved in the regulation of cell surface proteolytic activity (9, 10).

Changes in gene expression during monocyte/macrophage differentiation have been investigated (11–13), and, in these studies, PAI-1 mRNA levels were reported to increase. During differentiation of HL-60 cells, which are usually used as a model to study monocyte/macrophage differentiation, Krichevsky et al. (14) described a cluster of mRNAs, including PAI-1 mRNA, that are more associated to polysomes, which are more susceptible to translation into proteins. Thus, during monocyte differentiation, PAI-1 synthesis was unanimously reported to increase. We investigated pathways leading to PAI-1 production during HL-60 cell differentiation toward the monocyte lineage. We showed that, upon treatment of these cells by the TGF/D₃ mixture, PAI-1 synthesis largely increased and this increase could be up-regulated by the addition of TNF (15). Signaling pathways allowing this cellular event were reported to be different from those responsible for PAI-1 synthesis induced by phorbol esters, which involves activation of the pathway protein kinase Cβ/ extracellular-regulated kinases (ERK1/2) followed by autocrine stimulation of neosynthesized TNF (15).

Cell differentiation and cell proliferation arrest can be considered as two associated features. For instance, differentiation of monocyte precursors occurs when cells stop growing (16, 17). On the contrary, during the atherosclerotic process smooth muscle cell dedifferentiation is accompanied by their proliferation (18).

The enzyme p70S6K was originally described as responsible for the phosphorylation of the ribosomal protein S6 (19, 20). Its specific inhibition by rapamycin (RAP) was shown to reduce the translation of 5’-TOP mRNA (21) mainly coding for proteins involved in the translation process, which results in a decrease in their amount and in turn leads to a general decrease in translation. The activity of the p70S6K closely correlates with cell proliferation (22) and cell cycle regulation (23–25), and it was suggested that p70S6K activity promotes proliferation and negatively regulates differentiation (26, 27).

In this work, we investigated possible links between TGF/D₃-induced HL-60 cell differentiation, p70S6K activity, and up-regulation of PAI-1 synthesis by TNF. Our results strongly
suggest that, during TGF/D3-triggered monocyte differentiation of HL-60 cells, the inhibition of p70S6K activity allows TNF to fully stimulate PAI-1 production. This likely occurs through a decrease in the level of the PAI-1 transcriptional inhibitor E2F1.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Monoclonal antibodies (12A4 and 15H12) specific for human PAI-1 enzimelinked immunosorbent assay were generously given by Dr. P. Declerck (Center for Thrombosis and Vascular Research, Leuven, Belgium). Antibodies raised against p70S6K and E2F1 (C-20) were from Santa Cruz Biotechnology Inc. The phospho-p70S6K-specific antibody recognizing threonine 389 when phosphorylated was from New England BioLabs. Recombinant human transforming growth factor-β1 (TGF) and TNF-α were from Amersham Pharmacia Biotech. 1,25-Dihydroxyvitamin D₃ (D₃) was from Biomol. Rapamycin (RAP) was from Calbiochem. Cycloheximide was from Sigma Chemical Co. Molecular biology products (dNTP, random hexaprimers, RNasin, and appropriate buffers) were from Life Technologies Inc.

**Cell Culture**—The human promyelocytic HL-60 leukemia cell line was kindly provided by Pr. E. Huberman (Argonne National Laboratory, Argonne, IL) and cultured as described previously (28). Cells in 72-h culture medium were incubated for 1 h in fresh medium and then treated. Human monocyte-derived macrophages were prepared as described previously (29). Proper controls made with a similar concentration of vehicle alone were without effect on the parameters studied.

**Western Blot Analysis**—Cells were lysed in the buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease and phosphatase inhibitors. Cell lysates were centrifuged, and proteins of the supernatant were separated using an 8% SDS-PAGE according to Laemmli (30), electrotransferred onto a polyvinylidene difluoride membrane, which was hybridized with the antibody. Detection was performed by the use of alkaline phosphatase-coupled secondary antibodies and the colorimetric substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

**Flow Cytometry Analysis of CD11b and CD71 Surface Expression**—A population of 10⁶ HL-60 cells (5.10⁵/ml) was incubated either with the fluorescein isothiocyanate-conjugated monoclonal antibody raised against CD11b (Beckman-Coulter) washed and fixed in 2% paraformaldehyde, or with the unlabeled monoclonal antibody directed against CD71 (Dako), washed and incubated with the appropriate fluorescein isothiocyanate-conjugated secondary antibody (Beckman-Coulter), washed again and fixed as described above. Cells were analyzed on a XL-cytofluorograph (Coulter Electronics Inc.) at 488 and 525 nm, corresponding to excitation and detection wavelengths, respectively.

**Total Protein Synthesis Analysis**—Cells were treated for 12 h with TNF or TNF + RAP, then 1 nCi of [³⁵S]methionine/10⁶ cells was added to the media for 30 min. Cells were harvested, washed with phosphate-buffered saline, and lysed using Triton X-100 0.1%. Proteins were precipitated by two volumes of aceton, and pellets were suspended in the same lysis buffer. The amount of precipitated counts was measured by scintillation counting, and proteins were assayed according to specifications of the biocinchonic acid protein assay kit (Sigma). Protein synthesis is expressed as counts per minute.

**DNA Fragmentation Detection**—DNA was extracted using a QIAamp DNA mini kit from Qiagen according to the manufacturer’s instructions. The same amounts of DNA were loaded and separated by electrophoresis on a 2% agarose gel and visualized under UV irradiation.

**Semiquantitative RT-PCR and Real Time Semiquantitative RT-PCR Analysis**—Total RNA extraction, cDNA synthesis, PAI-1, and eEF1a PCR were performed as described previously (28). The mRNA of PAI-1 was measured by real time semiquantitative RT-PCR using a PE Applied Biosystems Prism, model 7700 sequence detection instrument. Sequences of primers and probe for the Taqman PCR were designed using Primer Express software from PE Applied Biosystems. Threshold cycle, C, which inversely correlates with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level.

**Statistics**—Each experiment was performed in triplicate. Results are expressed as mean ± S.D. Comparisons were analyzed by analysis of variance, and significance was calculated at p < 0.01 using the Scheffe F-test.

**RESULTS**

**PAI-1 Synthesis Is Up-regulated by TNF during TGF/D3-induced Monocyte Differentiation of HL-60 Cells**—We reported that HL-60 cells treated with a mixture of TGF/D3 increased PAI-1 synthesis, which was then largely potentiated by the addition of TNF (28), whereas others showed that this mixture triggers HL-60 cell differentiation toward the monocytic lineage (31–33). To clarify if PAI-1 production correlated with monocyte differentiation, we analyzed the effect of TGF/D3, associated or not with TNF on some parameters characteristic of monocyte differentiation. Arrest in cell proliferation is considered as a prerequisite to cell differentiation (34, 35). In our culture conditions, the untreated HL-60 cell population had a doubling time of roughly 24 h and all the treatments used in this study inhibited proliferation (Table I). Treatments with either TGF/D3 or TGF/D3/TNF were the most potent to reduce proliferation, because after 24 h of culture, cells grew by less than 10%. Surface expression of CD11b and CD71 were described to positively and negatively correlate with the process of HL-60 cell differentiation (33, 36), respectively. The increase in CD11b surface expression was optimal upon treatment with the mixture of TGF/D3/TNF (+4.9-fold compared with control cells). This mixture also triggered an optimal, although weak, decrease in CD71 surface expression (+1.5-fold compared with control cells). Compared with control cells, TNF alone exerted intermediate effects on proliferation and surface marker expression. These results indicate that treatment with the mixture of TGF/D3/TNF can be considered as the most potent stimulation used in this study to trigger monocyte differentiation of HL-60 cells. As previously reported (28) and shown herein, PAI-1 accumulation was enhanced upon treatment with the mixture of TGF/D3 (18.3-fold compared with control cells) and greatly increased with the simultaneous addition of TNF (+100-fold compared with control cells). Taken together, these data are in accordance with the existence of a link between HL-60 cell differentiation toward monocytic lineage and PAI-1 production, which suggests that induction of cell differentiation is a prerequisite for PAI-1 synthesis stimulated or not by TNF. We thus investigated which intracellular pathway triggered PAI-1 production and was involved in the early steps of the differentiation process.

**p70S6K Phosphorylation Is Reduced during TGF/D3-induced Monocyte Differentiation of HL-60 Cells**—As mentioned above,
cell growth cessation can be considered as a prerequisite for cell differentiation. Table I shows that reduction in cell proliferation is accompanied by an increase in PAI-1 synthesis. Because an increasing line of evidence suggests that p70<sup>S6K</sup> is a potential regulator of cell growth (22, 37), we investigated possible relationships between p70<sup>S6K</sup> phosphorylation and PAI-1 synthesis. Phosphorylation of p70<sup>S6K</sup> was analyzed by its migration shift on SDS-PAGE and detection by immunoblot and also by immunoblot using a phospho-p70<sup>S6K</sup> antibody able to detect p70<sup>S6K</sup> only when activated by phosphorylation of the threonine at position 389. The phosphorylation status deduced from these two means of investigation was shown by others to closely correlate with p70<sup>S6K</sup> activity (38). The TGF/D3 mixture inhibited p70<sup>S6K</sup> phosphorylation (Fig. 1, A and B), whereas each compound had no effect when used alone (not shown). TNF did not alter the basal or the TGF/D3-modified p70<sup>S6K</sup> phosphorylation (Fig. 1, A and B). All the data above showed that 1) p70<sup>S6K</sup> activity was reduced in the presence of TGF/D3, a situation in which PAI-1 production is increased, and 2) that TNF addition has a minor effect on PAI-1 synthesis, but greatly increased it when added to the mixture of TGF/D3. This prompted us to further investigate relationships between p70<sup>S6K</sup> activity and TNF on PAI-1 synthesis.

**The Reduced p70<sup>S6K</sup> Activity during TGF/D3-induced Monocyte Differentiation of HL-60 Cells Allows TNF to Up-regulate PAI-1 Synthesis**—To further investigate if a reduced p70<sup>S6K</sup> activity was involved in TNF-stimulated PAI-1 synthesis, we inhibited the kinase activity by RAP (Fig. 1, A and B), a highly specific inhibitor of p70<sup>S6K</sup> activity (39, 40), and measured PAI-1 production. PAI-1 synthesis from D3-treated cells (data not shown) and TGF/D3 (±TNF)-treated cells was not significantly increased by RAP (Fig. 2). The result obtained in this last case could be explained by the fact that, as shown in Fig. 1, TGF/D3 mixture was as effective as RAP in inhibiting p70<sup>S6K</sup> activity, not allowing p70<sup>S6K</sup> activity to be additionally inhibited by RAP. However, the original finding is that inhibition of p70<sup>S6K</sup> activity by RAP was able to enhance the stimulatory effect of TNF on PAI-1 production by a factor of 2.7 (Fig. 2). This suggests that a decrease in p70<sup>S6K</sup> activity allows TNF to exert its full activating potential on PAI-1 synthesis. The activating effect of RAP on PAI-1 production was also significant on cells stimulated by TGF/TNF (factor 2) and by TGF (factor 1.9). The fact that the slight, but non-significant increase in CD11b expression triggered by TNF (Table I) was not further enhanced by RAP treatment (data not shown) ruled out a general up-regulating effect of RAP on TNF-induced gene expression. The permissive effect of RAP on TNF-induced PAI-1 synthesis was also observed on *in vitro*-matured human monocytes/macrophages. The synthesis of PAI-1 was not significantly altered by TNF or RAP (28 ± 3 and 29.4 ± 1 ng/ml, respectively) compared with untreated cells (26.3 ± 2 ng/ml), whereas the association of TNF and RAP increased the level of PAI-1 by 42% (37.4 ± 2 ng/ml).

Because RAP appeared to closely mimic the effect of TGF/D3 on TNF-induced up-regulation of PAI-1, we further investigated how RAP was involved in this process.

**Transcriptional Activation of PAI-1 Gene by RAP Is Not Related to a General Decrease in Protein Synthesis but Associated with a Decreased Amount of E2F1**—The literature documents that total protein synthesis decreases during HL-60 cell differentiation (14), although the synthesis of some proteins, like PAI-1, is specifically increased. We previously reported that PAI-1 synthesis can be down-regulated at the level of translation (41), suggesting that conditions exist during which PAI-1 mRNA are weakly translated. A prediction of the model of Lodish (42) is that a partial block of elongation would impede the initiation of the efficiently translated mRNAs, thereby making translation factors more available for weakly translated mRNAs. In our experimental conditions, RAP was able to completely stop the proliferation of HL-60 cells (not shown) and to decrease total protein synthesis by half when compared with TNF-treated cells, after 24 h of incubation (Fig. 3). We therefore investigated if a general decrease in protein synthesis, rather than a specific decrease in p70<sup>S6K</sup> activity, was involved in the permissive effect of RAP on TNF-stimulated PAI-1 production. We incubated TNF-stimulated HL-60 cells with a low dose of cycloheximide (0.1 μg/ml). In this case, PAI-1 production was neither increased by TNF nor by TNF + RAP treatments (not shown), suggesting that a reduction in protein synthesis itself is not responsible for the increased PAI-1 production triggered by RAP on TNF-treated HL-60 cells.

In the aim to understand how RAP was able to increase PAI-1 synthesis in TNF-treated cells, we investigated at which step RAP action took place. Surprisingly, RAP significantly increased the level of PAI-1 mRNA (Fig. 4). Blockage in protein synthesis with cycloheximide did not impair the increase in PAI-1 mRNA level induced by TNF and TNF + RAP (Fig. 4), suggesting that *de novo* protein synthesis is not necessary in these situations to increase PAI-1 mRNA level. Results obtained from experiments using actinomycin D ruled out a possible stabilization effect of RAP on PAI-1 mRNA to explain the observed increase in PAI-1 mRNA level. Conversely, fine analysis using real time semiquantitative RT-PCR showed that RAP slightly destabilized PAI-1 mRNA (Fig. 5). These results suggest that RAP increased the PAI-1 transcription rate in TNF-treated HL-60 cells.
triggered by RAP and associated with the decrease in p70S6K activity was able to reduce the level of a PAI-1 transcriptional inhibitor. E2F1 was recently demonstrated to bind the PAI-1 promoter and to repress PAI-1 transcription (43). Its level was described to be quickly reduced upon cell growth arrest due to a destabilization of its mRNA (44, 45). Interestingly, as shown in Fig. 6, the protein level of E2F1 was reduced by treatments allowing HL-60 cell differentiation (TGF + D3 and TGF + D3 + TNF). TNF had no effect on E2F1 level, but RAP alone or associated with TNF reduced E2F1 protein level. This result indicates that RAP can mimic the down-modulating effect of TGF + D3 on E2F1 level, which may account for the relief of inhibition in PAI-1 gene transcription and allowing TNF to activate it.

**TNF-induced Apoptosis Is Prevented by TGF/D3 and RAP**—TNF was reported to induce apoptosis in HL-60 cells (46). We investigated this phenomenon in our experimental conditions...
(Fig. 7). After 24 h of treatment, TNF alone induced a detectable DNA fragmentation reflecting the apoptotic process. Incubation with TGF/D3 or RAP clearly protected cells from the TNF-induced apoptotic effect as the DNA pattern closely resembled to that of control HL-60 cells. This protective effect was also induced by D3 alone.

**DISCUSSION**

The literature concerning quantitative and qualitative remodeling of gene expression during monocyte differentiation has been extensively documented by the use of new technologies such as microarray (11–13). However, the identification of signaling components involved in the alteration of the expression of the vast majority of these genes is still missing. We contributed to answer this question by studying PAI-1 expression during HL-60 cell differentiation triggered by the mixture of TGF/D3. We previously reported that stimulation of PAI-1 production by TGF/D3 was further increased following TNF addition, which did not involve the ERK1/2 pathway (28). We therefore investigated the intracellular mechanisms involved in this up-regulation. We first hypothesized that the arrest in cell proliferation, considered as a prerequisite for differentiation, was involved in the stimulation of PAI-1 production. The fact that PAI-1 production is concomitant with proliferation arrest and differentiation supported this hypothesis (Table I). Several papers mentioned the important role of p70S6K activity in the proliferation process (22, 26, 37). Our results (Fig. 1 and Table I) showed that a decrease in p70S6K phosphorylation was closely associated with both TGF/D3-induced monocyte differentiation and PAI-1 production. RAP, which allows the binding of FKBP12 to FRAP (the enzyme responsible for P70S6K phosphorylation), provoked a similar level of inhibition of P70S6K phosphorylation as TGF/D3, although the level of PAI-1 synthesis it induced was lower than that of TGF/D3. The elucidation of the mechanisms responsible for these differences was beyond the scope of this work, because we focused our interest on intracellular mechanisms that allow TNF to up-regulate PAI-1 synthesis during TGF/D3-induced differentiation. When used alone, TNF induced a low level of PAI-1 synthesis. However, when associated with TGF/D3 or RAP, TNF was now able to dramatically up-regulate PAI-1 synthesis (Fig. 2). This supports the contention that a complete decrease in p70S6K activity brought by a differentiative stimulus allowed TNF to exert its full potential of stimulation on PAI-1 synthesis.

Levels of PAI-1 mRNA, undetectable in native HL-60 cells, were induced upon TGF/D3 treatment (15) and we show herein that RAP was also able to increase the level of PAI-1 mRNA when associated to TNF (Fig. 4). Induction of gene expression upon RAP treatment was reported in yeast (47). In mammals, RAP was shown to potentiate glucocorticoid receptor-mediated gene induction, probably by increasing the nuclear translocation of the glucocorticoid receptor (48). However, in this case, FK506 (which allows the binding of FKBP12 to calcineurin) triggered the same effect, ruling out a role of p70S6K activity inhibition in this process. Our findings that RAP, but not FK506,2 potentiates a TNF-mediated increase in PAI-1 synthesis suggests for the first time, to our knowledge, that under particular circumstances, inhibition of p70S6K activity can result in an increased gene expression. This increase was neither due to a prolonged PAI-1 mRNAs half-life, because RAP slightly destabilized PAI-1 mRNA (Fig. 5), nor to de novo protein synthesis (Fig. 4), which was expected because RAP decreased global protein synthesis (Fig. 3). Furthermore, experiments using cycloheximide showed that a global decrease in protein synthesis is not responsible and not favorable for PAI-1 synthesis during TNF stimulation.

The transcriptional regulator E2F1 was recently demonstrated to bind the PAI-1 promoter and to repress PAI-1 gene transcription (43). Its level was reduced during HL-60 cell differentiation and also treatment with RAP (Fig. 6), which makes the relief of E2F1-induced inhibition that follows inactivation of p70S6K a reasonable explanation for the permissive effect of RAP on TNF-induced PAI-1 synthesis. We also show herein that apoptosis triggered by TNF could be circumvented by D3 and RAP (Fig. 7). RAP was shown to protect HL-60 cells from apoptosis triggered by didemnin B (49) through an effect that did not seem to involve p70S6K activity. Inversely, RAP was reported to increase the ability of the alkylating agent, cisplatin, to induce apoptosis in HL-60 (50). Whether RAP inhibited TNF-induced apoptosis through the blockage of p70S6K activity is outside of the confines of this work. Nevertheless, we can hypothesize that, when apoptosis, triggered by TNF, is prevented, the stimulatory effect of TNF on PAI-1 production is exerted on more viable cells for a longer period of time allowing PAI-1 synthesis to be increased. Therefore, apoptosis can be considered as a negative factor for PAI-1 synthesis during HL-60 differentiation. Thus, RAP mimics at least four associated events triggered during TGF/D3-induced HL-60 cell differentiation that allow TNF to up-regulate PAI-1 synthesis: (i) decrease in cell proliferation, (ii) decrease in p70S6K phosphorylation, (iii) decrease in E2F1 protein level, and (iv) decrease in TNF-induced HL-60 cell apoptosis.

Potential pharmacological modulation of vascular remodeling by rapamycin has been highlighted by different results in the literature. Rapamycin was shown to inhibit proliferation and migration of vascular smooth muscle cells in vitro and ex vivo (51, 52). Administration of rapamycin in pig reduced the arterial cell proliferation response after balloon angioplasty by targeting regulators of cell cycle-like E2F (53). The regulatory effect of the fibrinolytic system on smooth muscle cell migration/proliferation (54) and neointimal leukocyte infiltration (55) after arterial injury has been clearly demonstrated. Whether rapamycin may modulate these effects through PAI-1 synthesis could be envisioned, because we observed that RAP up-regulated PAI-1 synthesis in TNF-stimulated human peripheral monocyte-derived macrophages.

In conclusion, our results support the notion that the reduced p70S6K activity that occurs when HL-60 cells differentiate toward a monocyte lineage, driven by the TGF/D3 mixture, is a key event that allows TNF to have its optimal stimulatory effect on PAI-1 synthesis.

**Acknowledgment**—We are indebted to N. Geoffroy for performing cytometry measurements.

**REFERENCES**


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doi: 10.1074/jbc.M103357200 originally published online June 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103357200

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