Role of CD14 Expression in the Differentiation-Apoptosis Switch in Human Monocytic Leukemia Cells Treated with 1α,25-Dihydroxyvitamin D₃ or Dexamethasone in the Presence of Transforming Growth Factor β¹

Yasuhiro Kanatani, Takashi Kasukabe, Junko Okabe-Kado, Yuri Yamamoto-Yamaguchi, Naokazu Nagata, Kazuo Motoyoshi, and Yoshio Honma²

Saitama Cancer Center Research Institute, Ina, Saitama 362-0806 [Y. K., T. K., J. O-K., Y. Y-Y., Y. H.], and Third Department of Internal Medicine, National Defense Medical College, Tokorozawa, Saitama 359-8513 [Y. K., N. N., K. M.]. Japan

Abstract
Transforming growth factor β (TGF-β) enhanced the growth-inhibitory activities of dexamethasone (Dex) and 1α,25-dihydroxyvitamin D₃ (VD3) on human monocytic leukemia U937 cells. TGF-β and VD3 synergistically increased the expression of differentiation-associated markers such as the CD11b and CD14 antigens, whereas TGF-β and Dex did not. On the other hand, TGF-β and Dex synergistically increased the number of Apo2.7-positive cells, which represents the early stage of apoptosis, whereas TGF-β and VD3 did not, suggesting that TGF-β enhanced apoptosis with Dex and enhanced monocytic differentiation with VD3. In the presence of TGF-β, the retinoblastoma susceptibility gene product, pRb, was synergistically dephosphorylated by Dex as well as VD3. TGF similar enhanced the expression of the p21Waf1 gene in U937 cells treated with Dex and VD3. TGF-β dose-dependently increased the expression of Bcl-2 and Bad and decreased the expression of Bcl-XL in U937 cells. Dex enhanced the down-regulation of Bcl-XL expression in TGF-β-treated cells, whereas VD3 blocked this down-regulation of Bcl-XL. However, the down-regulation of Bcl-XL by treatment with the antisense oligomer did not affect the apoptosis or differentiation of U937 cells. The apoptosis of CD14-positive cells was suppressed in the VD3 plus TGF-β-treated cultures. These results suggest that the expression of CD14 is involved in the survival of differentiated cells.

Introduction
Cellular homeostasis is regulated by proliferation, differentiation, and death. The ability of cells to exit the cell cycle, induce apoptosis, and differentiate is mediated by intricate molecular and biochemical mechanisms. Certain myeloid leukemia cells have been used to study the biochemical and molecular mechanisms that govern these processes. Human monoblastic leukemia U937 cells can be induced to differentiate toward monocytes and macrophages by treatment with VD3 in the presence of TGF-β, whereas they are induced to undergo programmed cell death (apoptosis) by treatment with a low concentration of glucocorticoid in the presence of TGF-β. This experimental model is useful for studying the regulatory mechanisms of the signaling pathway of differentiation or apoptosis (1–3). During the early phase of differentiation and apoptosis, cells exit the cell cycle and cease to proliferate (4, 5). For differentiation, cells must possess a mechanism to avoid cell death, thus allowing the development of mature cells to exhibit their functional and morphological phenotypes. The intracellular levels of proteins coded for by apoptosis suppressor and effector genes seem to regulate viability or death (6–10). Glucocorticoid-induced growth arrest is involved in the transcriptional repression of G₁ cyclins and cyclin-dependent kinases (cdks) or in enhancing the transcription of cdk inhibitors by the activated glucocorticoid receptor (11). Dex induces apoptosis in lymphoid leukemia cells by suppressing the expression of Bcl-2 (12), and the monocytic differentiation of myeloid leukemia HL-60 and U937 cells is accompanied by an increased expression of Bcl-6 (13, 14). Moreover, the expression of Bcl-XL is either maintained or increased during the monocytic differentiation of human myeloid leukemia cells (15, 16).

In this study, we examined the events associated with the VD3-induced differentiation of U937 cells to monocytes and macrophages and with Dex-induced apoptosis in the presence of TGF-β. A rapid induction of CD14 expression was observed in cells treated with VD3 plus TGF, but not in those treated with Dex plus TGF-β. Our study suggests that the expression of CD14 is involved in the survival of differentiated cells of the monocyte/macrophage lineage.

¹ The abbreviations used are: VD3, 1α,25-dihydroxyvitamin D₃; TGF, transforming growth factor; Dex, dexamethasone; NBT, nitroblue tetrazolium; cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; GR, glucocorticoid receptor; PBSF, PBS with 2.5% fetal calf serum and 0.01% NaN₃ (w/v); PE, R-phycoerythrin.


**Table 1. Effects of TGF-β on the growth and differentiation of U937 cells in combination with Dex or VD3**

Cells (1 × 10⁵ cells/ml) were cultured with various concentrations of TGF-β and either 3 nm VD3 or 50 nm Dex in the presence or absence of 0.6 ng/ml TGF-β for 72 h. Cell density in control culture was 8.3 ± 0.2 × 10⁵ cells/ml. Values are the mean ± SD for three separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell growth (cells/ml)</th>
<th>NBT reduction (A560/10⁷ cells)</th>
<th>CD11b-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>0.98 ± 0.10</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>TGF-β (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>83.8 ± 0.2</td>
<td>0.93 ± 0.02</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>0.6</td>
<td>78.0 ± 1.5</td>
<td>0.90 ± 0.05</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1.0</td>
<td>73.5 ± 0.9</td>
<td>0.80 ± 0.06</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>5.0</td>
<td>72.3 ± 1.3</td>
<td>0.96 ± 0.04</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>VD3 (3 nm)</td>
<td>68.4 ± 2.6</td>
<td>1.63 ± 0.13</td>
<td>66 ± 1</td>
</tr>
<tr>
<td>+ TGF-β (0.6 ng/ml)</td>
<td>19.6 ± 2.2</td>
<td>5.65 ± 1.06</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>Dex (50 nm)</td>
<td>83.1 ± 1.2</td>
<td>1.06 ± 0.44</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>+ TGF-β (0.6 ng/ml)</td>
<td>26.3 ± 0.1</td>
<td>1.08 ± 0.14</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

**Results**

**Combined Effect of TGF-β with Dex or VD3 on the Growth and Differentiation of U937 Cells.** Dex alone did not inhibit the proliferation of U937 cells, but it did effectively inhibit growth in combination with a low concentration of TGF-β (Fig. 1, left). The combination of TGF-β and VD3 also inhibited the growth of U937 cells (Fig. 1, right). VD3 and TGF-β synergistically induced the monocytic differentiation of several myelomonocytic leukemia cells, including U937 cells (3). However, the combination of Dex and TGF-β did not induce the functional or morphological differentiation of monocytic leukemia cells. TGF-β alone did not induce NBT-reducing activity or the expression of differentiation-associated CD11b antigen by U937 cells (Table 1). VD3 at 3 nm moderately induced NBT reduction and the expression of the CD11b and CD14 antigens (Fig. 2). On the other hand, Dex did not induce monocytic differentiation, and even 500 nm

Dex only slightly inhibited the growth of U937 cells without increasing the NBT-reducing activity or the expression of CD11b antigen. The growth-inhibitory activities of Dex and VD3 were each synergistically enhanced by TGF-β, whereas TGF-β preferentially enhanced differentiation-associated markers in VD3-treated cells, suggesting that the growth-inhibitory and differentiation-inducing effects were regulated differently by TGF-β in U937 cells (Table 1 and Fig. 2).

**Synergistic Effect of TGF-β with Dex or VD3 on the Dephosphorylation of pRb.** TGF-β inhibits the phosphorylation of pRb and blocks progression of the cell cycle from G1 to S phase (5, 17, 18). Therefore, we treated U937 cells with 0–5.0 ng/ml TGF-β for 72 h and examined the phosphorylation status of pRb by Western blotting. TGF-β decreased hyperphosphorylated pRb in a dose-dependent manner, but hypophosphorylated pRb was not recognized at TGF-β concentrations below 0.6 ng/ml (Fig. 3A). Although 50 nm Dex or 3 nm VD3 alone did not substantially suppress the phosphorylation of pRb, Dex or VD3 in the presence of 0.6 ng/ml TGF-β completely suppressed the phosphorylation of pRb at 72 h (Fig. 3A). We further examined time-course changes in the phosphorylation status of pRb in the presence of Dex in combination with TGF-β for up to 96 h and found that the level of the dephosphorylated form of pRb was significant at 48 h (Fig. 3B). A similar result was obtained in cells treated with TGF-β and VD3 (data not shown). A significant decrease in the amount of pRb was observed at 96 h in cells treated with TGF-β and Dex. These results suggest that the dephosphorylation status of pRb is closely correlated with the growth-inhibiting effect of TGF-β with Dex or VD3, but not with the induction of differentiation or apoptosis.

**Effect of Dex or VD3 on the Expression of p21<sup>WAF1</sup> and p27<sup>Kip1</sup> Genes in the Presence of TGF-β.** pRb is phosphorylated by cyclin-CDK complexes through the progression of the cell cycle from G1 to S phase (4, 5, 17). TGF-β inhibits kinase activities of cyclin-CDK complexes mediated by CDK
inhibitors, and the cdk inhibitors may be involved in regulating differentiation or apoptosis (18). Therefore, we examined the expression of the p21Waf1 and p27Kip1 genes by Northern blotting (Fig. 4A). TGF alone induced the expression of the p21Waf1 gene but did not induce the expression of the p27Kip1 gene. Although 50 nM Dex or 3 nM VD3 alone did not affect the expression of the p21Waf1 gene, in combination with 0.6 ng/ml TGF-β, Dex as well as VD3 significantly enhanced the expression of the p21Waf1 gene (Fig. 4B). Western blot analysis revealed that the expression of p21 Waf1 with TGF and Dex was maximally increased at 24 h, and this preceded the appearance of dephosphorylated pRb (Figs. 3 and 5). On the other hand, the p27Kip1 protein level was remarkably increased after the appearance of dephosphorylated pRb (Figs. 3 and 5), suggesting that the induction of p21Waf1 is involved in the dephosphorylation of pRb. Expression of the p27Kip1 gene was greatly enhanced by TGF-β and Dex, but not by VD3 and TGF-β (Fig. 4). Because the accumulation of p27Kip1 is involved in the induction of apoptosis in myeloid leukemia cells (19), the synergistic induction of p27Kip1 by TGF-β and Dex may contribute to the acceleration of apoptosis in treated U937 cells, although the induction of p27Kip1 protein is a later event in apoptosis.

**Down-Regulation of Bcl-X<sub>L</sub> Expression by Dex in Combination with TGF-β.** The expression of Bcl-X<sub>L</sub> has been shown to be either maintained or increased during monocyte/macrophage differentiation (15, 16). Therefore, we examined the expression of Bcl-X<sub>L</sub> and Bcl-2 in VD3- or Dex-treated cells by Western blot (Fig. 6). Untreated U937 cells expressed a high amount of Bcl-X<sub>L</sub> and TGF-β inhibited this expression in a dose-dependent manner (Fig. 6). Although 3 nM VD3 alone barely affected Bcl-X<sub>L</sub> expression, VD3 effectively blocked the down-regulation of Bcl-X<sub>L</sub> expression induced by TGF-β. On the other hand, Dex did not affect the expression of Bcl-X in the cells and accelerated the down-regulation of Bcl-X<sub>L</sub> protein expression in cells treated with TGF-β (Fig. 6). The down-regulation of Bcl-X<sub>L</sub> protein was significant 48 h after exposure to TGF plus Dex. On the other hand, Bcl-2 expression was increased by TGF, but not by Dex or VD3 (data not shown). Bad interacts with antiapoptotic molecules such as Bcl-2 and Bcl-X<sub>L</sub> and promotes apoptosis (20). Bad was dose-dependently up-regulated by TGF-β in U937 cells, but it was not significantly affected by Dex or VD3 (Fig. 6).

To investigate the link between the down-regulation of Bcl-X<sub>L</sub> and apoptosis, we treated U937 cells with antisense oligonucleotides of Bcl-X<sub>L</sub> that successfully induced the apoptosis of vascular lesions (21). Treatment with antisense oligomers decreased Bcl-X<sub>L</sub> protein expression in U937 cells but did not affect the growth or viability of the cells, indicating that the down-regulation of Bcl-X<sub>L</sub> is not sufficient to induce the apoptosis of U937 cells (data not shown). Cells with decreased Bcl-X<sub>L</sub> protein levels showed slightly suppressed responses to the induction of differentiation by VD3 plus TGF-β (a 20–30% decrease compared to U937 cells treated with the reverse control oligomers). These results suggest that Bcl-X<sub>L</sub> is not mainly involved in inducing apoptosis or differentiation in U937 cells.
Induction of Apo2.7 Expression in U937 Cells Treated with TGF-β plus Dex.

Apo2.7 reacts with a Mr 38,000 mitochondrial membrane protein that appears to be exposed on cells undergoing apoptosis (22). Expression of Apo2.7 protein has been suggested to be an early event in apoptosis. Although Dex or TGF-β alone did not increase the number of Apo2.7-positive cells, treatment with the combination of TGF-β and Dex synergistically increased the number of Apo2.7-positive cells (Fig. 7). VD3 alone did not increase the number of Apo2.7-positive cells, and treatment with the combination of TGF-β and VD3 also did not affect Apo2.7 expression (Fig. 7), suggesting that TGF-β induced apoptosis in combination with Dex, but not in VD3-treated cells. The expression of Apo2.7 with TGF and Dex was significantly increased at 24 h (data not shown).

CD14 Expression Prevents the Apoptosis of U937 Cells Treated with VD3 and TGF-β. Because CD14 expression may be a negative regulator of apoptosis in monocytes (23), the time course of CD14 expression was examined (Fig. 2). Treatment with 3 nM VD3 and TGF-β induced CD14 expression within 12 h, and this expression was more prominent 24 h after exposure to TGF-β with VD3. On the other hand, treatment with TGF-β and Dex did not have a similar effect.

To induce growth arrest, U937 cells were cultured in serum-free RPMI 1640 in the presence or absence of VD3 plus TGF-β. The detection of phosphatidylserine exposed during the redistribution of the plasma membrane by annexin V has been shown to be a general and early marker of apoptosis in hematopoietic cells and blood cells (24, 25). Annexin V-FITC labeling was combined with a determination of DNA stainability of permeabilized cells by propidium iodide as a well-established flow cytometric procedure for quantifying apoptosis. Untreated cells in serum-free medium for 24 h showed no annexin V staining, whereas cells with annexin V staining increased thereafter. Fig. 8 shows that 45% of the cells cultured in the serum-free medium for 72 h were annexin V positive, whereas almost none of the treated cells were positive. A previous report (23) indicated that CD14 molecules regulate the apoptosis of normal macrophages. To investigate the link between CD14 expression and apoptosis, differentiated U937 cells were further incubated in serum-free culture medium. CD14 expression by VD3 plus TGF-β-treated cells was gradually down-regulated, and the cells progressively became annexin V positive, resulting in 64%
CD14-negative and annexin V-positive cells (Fig. 8E). On the other hand, very few annexin V-positive cells were also CD14 positive, suggesting that CD14 expression prevented apoptosis. To confirm that CD14 prevents apoptosis, we pre-treated U937 cells with VD3 plus TGF-β to induce surface CD14 expression and then treated cells with Dex plus TGF-β (Fig. 9). When U937 cells were treated with Dex plus TGF-β for 30 h, 13.3% of the cells were annexin V positive, whereas only 2.8% of the VD3 + TGF-β-pretreated cells were positive, suggesting that CD14 prevents annexin V binding.

Discussion

Glucocorticoids induce the apoptosis of lymphoid leukemia cells through the activation of the GR, and a dysfunction of the GR is thought to be one of the mechanisms of Dex resistance (26, 27). On the other hand, myeloid leukemia cells are resistant to Dex, despite having a functional GR (28). The transactivation activity of GR has been suggested to be influenced by cell cycle-regulated protein kinases and phosphatases (11). Recently, a protein kinase A activator, 8-Br-cAMP, and a protein phosphatase inhibitor, okadaic acid, have each been shown to potentiate GR-mediated transactivation (29). Although Dex alone barely inhibited the growth of U937 cells, it synergistically inhibited the growth of human monocytic leukemia cells in the presence of a low concentration of TGF-β. TGF-β potently inhibits the growth of many different cell types and inhibits progression of the cell cycle from G1 to S phase by suppressing the phosphorylation of pRb (17). In this study, TGF-β inhibited the growth of U937 cells and suppressed the phosphorylation of pRb in a dose-dependent manner at concentrations above 1.0 ng/ml (Fig. 3A). Dex alone, even at a high concentration, neither inhibited the growth of U937 cells nor suppressed the phosphorylation of pRb. However, Dex enhanced the dephosphorylation of pRb induced by suboptimal concentrations of TGF-β, and hypophosphorylated pRb was recognized at 24 h (Fig. 3B). Because the dephosphorylation of pRb occurred earlier than the growth inhibition after treatment with TGF-β plus Dex, the dephosphorylation of pRb might be associated with synergistic growth inhibition by TGF-β and Dex. A recent finding indicates that dephosphorylated pRb may play a role in inducing the differentiation of leukemia cells (30). Whereas TGF-β and Dex induced the dephosphorylation of pRb, they hardly enhanced the monocytic differentiation of U937 cells, suggesting that the dephosphorylation of pRb is not sufficient for monocytic differentiation of U937 cells. This discrepancy between the previous and present results may be due to clonal variation of this cell line.

The phosphorylation of pRb is suppressed by cdk inhibitors, and VD3 induces the expression of the p21Waf1 and p27Kip1 genes during the monocytic differentiation of U937 cells (31). Although both Dex and VD3 synergistically enhanced the expression of the p21Waf1 gene in the presence of TGF-β, expression of the p27Kip1 gene was preferentially enhanced by TGF-β and Dex rather than by TGF-β and VD3. Western blot analysis indicated that the induction of p21Waf1 by TGF-β and Dex preceded the onset of the dephosphorylation of pRb, whereas p27Kip1 increased at 72 h, when the phosphorylation of pRb was already suppressed. p27Kip1 has been shown to be degraded by the ubiquitin-protea-
some pathway, and quiescent cells exhibited a smaller amount of p27Kip1-ubiquitinating activity, which accounted for a marked increase in the half-life of p27Kip1 (32). Moreover, a recent report also showed that p27Kip1 increases during the apoptosis of myeloid leukemia HL-60 cells (19).

Because the number of apoptotic cells was synergistically increased by TGF-β plus Dex but not by TGF-β plus VD3, the accumulation of p27Kip1 might be associated with the induction of apoptosis in U937 cells.

Liu et al. (31) report that overexpression of p21Waf1 or p27Kip1 can promote differentiation of U937 cells. When p21Waf1 and p27Kip1 were coexpressed after transient transfection, the percentage of transfected cells that stained positively for CD14 was 35%, whereas almost all the VD3-treated cells were positive. The results suggest that p21Waf1 and p27Kip1 directly lead to the differentiation program. In the present study, treatment with Dex plus TGF-β promotes both p21Waf1 and p27Kip1 expression in U937 cells, but the cells do not differentiate. Rigg et al. (33) also indicate that expression of p21Waf1 is observed in sphingosine-treated U937 cells, whereas sphingosine does not induce detectable differentiation. These results suggest that extremely high levels of p21Waf1 and p27Kip1 may be required to promote differentiation of the cells.

Recent findings indicate that the expression of Bcl-XL, but not of Bcl-2, is either maintained or increased during the monocytic differentiation of myeloid leukemia cells (15, 16). Thus, the reduced expression of Bcl-XL caused by TGF-β and Dex might impaire monocytic differentiation, whereas the increased expression of Bcl-XL caused by TGF-β and VD3 might not only protect cells from apoptosis but may also enhance monocytic differentiation (Fig. 6). The expression of Bcl-XL protein increases within intimal cells on vascular lesions, and the down-regulation of intimal cell Bcl-XL expression with the use of antisense oligonucleotides induces apoptosis and the acute regression of vascular lesions (21). However, the down-regulation of Bcl-XL expression with the use of antisense oligomers did not affect the apoptosis or differentiation of U937 cells (data not shown), suggesting that Bcl-XL did not play an important role in the induction of differentiation or apoptosis produced by VD3 or Dex in the presence of TGF-β.

Expression of the CD14 antigen has been shown to rescue monocytes from apoptosis (23). Our previous study indicated that TGF-β plus VD3 synergistically enhanced the expression of CD14 antigen, whereas combined treatment with TGF-β and Dex did not. CD14-positive U937 cells were highly resistant to apoptosis in serum-free medium (Fig. 8, C and D). Glucocorticoids suppress the up-regulation of CD14 in endotoxin-treated monocytes (34). The up-regulation of CD14 is an early event in the differentiation of U937 cells. It has been proposed that the up-regulation of surface CD14 receptor expression is due to the translocation of an intracellular pool of CD14 molecules in normal monocytes (35). These results suggest that this regulation of CD14 expres-

---

**Fig. 9.** Effects of Dex plus TGF-β on annexin V binding in CD14-positive U937 cells. Cells were cultured with (B) or without (A) 5 nM VD3 plus 0.6 ng/ml TGF-β for 72 h in serum-containing medium. The pretreated (D) or untreated (C) cells were washed with fresh medium and cultured with 50 nM Dex plus 0.6 ng/ml TGF-β for 30 h.
sion is important in determining whether growth-arrested cells are induced to undergo differentiation or apoptosis.

Materials and Methods

Materials. Dex and NBT were purchased from Sigma Chemical Co. (St. Louis, MO). VD3 was obtained from Wakо Pure Chemical Industry (Osaka, Japan). Highly purified TGF-β was purchased from R & D Systems (Minneapolis, MN). Monoclonal antibodies against CD11b and CD14 were obtained from Nichirei Co. (Tokyo, Japan). Anti-r-β2, p21Watt, p27Kip1, Bcl-2, Bax, and Bad antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-X₀ antibody was from Transduction Laboratories (Lexington, KY).

Assay of Cell Growth and Properties of Differentiated Cells. Human monocytic leukemia U937 cells were maintained at 37°C under 5% CO₂ in RPMI 1640 (Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (3). The cell number was counted in a model ZM Coulter Counter (Coulter Electronics, Luton, United Kingdom). To assay differentiation, leukemia cells (1 × 10⁶ cells/ml) were cultured with various concentrations of Dex or VDS with or without TGF for the indicated periods. Superoxide-generating oxidase was determined by the ability of the cells to reduce NBT upon exposure to 12-O-tetradecanoyl phorbol-13-acetate. NBT reduction was assayed by incubating 1 × 10⁶ cells in 1 ml of RPMI 1640 containing NBT (1 mg/ml) and 12-O-tetradecanoyl phorbol-13-acetate (100 ng/ml) at 37°C for 60 min. The reaction was stopped by adding 5 ml HCl (1 M, final concentration). The suspension was kept at room temperature for 1 h and then centrifuged. Formazan deposits were solubilized in DMSO, and the absorption of the formazan solution at 560 nm/10⁻⁶ cells was measured in a spectrophotometer. Morphological differentiation was examined in cell smears stained with May-Grnwald-Giemsa. The expression of CD11b and CD14 antigens was analyzed by indirect immunofluorescence staining and flow cytometry as described elsewhere (36). Briefly, leukemia cells (2 × 10⁶ cells) were washed with PBS and incubated in 50 μl of mouse anti-CD11b (Mac-1) or anti-CD14 in PBS containing 0.1% bovine serum albumin at 4°C for 30 min. The cells were washed with PBS and incubated in 50 μl of FITC-conjugated antiumouse IgG (Tago Inc., Burlingame, CA) in PBS containing 0.1% bovine serum albumin for 30 min, washed with PBS, and then analyzed in an Epics XL flow cytometer (Coulter Electronics).

Western Immunoblot. Cells were harvested and lysed in Laemmli buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.003% bromophenol blue]. The protein lysate was electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA). The filters were blocked with 5% non-fat dried milk in 1× TBS buffer [50 mM Tris-HCl (pH 7.4) and 150 mM NaCl] and then incubated overnight with 0.1 μg/ml primary antibodies. Alkaline phosphatase-conjugated IgG (Bio-Rad Laboratories, Hercules, CA) was used as a secondary antibody (1:1000), and the bands were visualized on Kodak X-omat films. An oligonucleotide probe for p21Watt (5'-AGGTGTAAAGAATCTGACTGGTGCTCCGC-CGTTTGGG-3') derived from the human p21Watt gene (37). An oligonucleotide probe for p27Kip1 was synthesized on the antisense sequence (5'-TTATTTGTAGTAAAGAATCTGACTGGTGCTCCGC-CGTTTGGG-3') (38).

Quantitation of Apo2.7-positive Cells. We detected the expression of Apo2.7 (Immunotech, Mardelle Cedex, France) by flow cytometry. After discarding the supernatant, cells were suspended in 100 μl of cold PBS buffer containing 100 μg/ml digitonin and incubated for 20 min on ice. Cells were washed once with cold PBSF and centrifuged at 2000 rpm for 6 min at room temperature. One hundred μl of PE-conjugated Apo2.7 (1:10) was added, and the mixture was incubated for 15 min at room temperature in the dark. The cells were then washed with PBSF buffer and analyzed on an Epics XL flow cytometer (Coulter Electronics).

Simultaneous Detection of CD14 Expression and Annexin V Binding. Cells were double-labeled with PE-conjugated anti-CD14 antibody (TUK4; IgG2a type; DAKO Japan, Kyoto, Japan) and FITC-labeled annexin V (Gendynme, Cambridge, MA) for 30 min on ice, as described previously (23). PE- and FITC-conjugated murine IgG monoclonal antibodies of unrelated specificities were always used as controls. After staining, cells were washed and analyzed by flow cytometry.

References


