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REGULATION OF TNF-α, IL-1 AND IL-6 SYNTHESIS IN DIFFERENTIATING HUMAN MONOBLASTOID LEUKEMIC U937 CELLS

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Abstract—The human monoblastoid tumor cell line U937 was induced to differentiate along the monocyte/macrophage lineage by treatment with 5×10^{-9} M 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Between 2 h and 4 h following TPA-treatment U937 cells started to release significant amounts of TNF-α which remained detectable until 8–10 days. A significant IL-1 β release was measured 24 h–48 h post stimulation and increased levels of IL-1 β persisted until 20–22 days of culture. In contrast no release of either IL-1 α or IL-6 could be detected with 5×10^{-9} M TPA during the whole time course of the experiments. The sequential induction of TNF-α and IL-1 β appeared to be independently regulated since TNF-α release was not required for the onset of IL-1 β production. Northern-blot analysis confirmed the sequential induction and the long term expression of TNF-α and IL-1 β mRNAs. Western-blot analysis predominantly showed a high molecular weight IL-1 β protein of about 35 kD. Further investigations on the regulation of cytokine production and release by TPA-differentiated U937 cells revealed that TNF-α and IL-1 β synthesis was not influenced by exogenously added rhTNF-α or PGE_2, whereas rhγ-IFN specifically enhanced the IL-1 β production. Thus, the regulation and intracellular processing of cytokines generated by differentiating U937 cells shows some differences when compared to mature monocytes/macrophages which may be related to the tumorigenic origin of U937 cells or to an incomplete differentiation.

Key words: TNF, IL-1, IL-6, differentiation, U937, leukemic cell line.

INTRODUCTION

CYTOKINES including TNF-α, IL-1 and IL-6 play a pivotal role in immune reactions and regulate the growth and the activity of many cells in inflammation [1, 2].

TNF, IL-1 and IL-6 are predominantly secreted by activated monocytes/macrophages as part of a general inflammatory response. These cytokines are acting (*in vivo*) as amplification and differentiation factors in a cascade of inflammatory events. They can also be released from *in vitro* induced cells in culture. Many studies have investigated the regulation of TNF-α, IL-1 and IL-6 in macrophage cell lines or macrophages stimulated with various inflammatory agents [3–6].

Abbreviations: TPA-U937, U937 cells treated for 72 h with 5 nM TPA; *TPA/IFN-U937*, TPA-U937 treated for 24 h with 100 U/ml rhγ-IFN; *TPA/Dex-U937*, TPA-U937 treated for 24 h with 10^{-6} M dexamethasone.

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The human monoblastoid U937 tumor cell line is widely used as a model for a differentiation of precursors into macrophage-like cells [7]. U937 cell differentiation can be induced by incubation with different agents, including retinoic acid [8], vitamin D derivatives [9], cytokines [10] and phorbol esters [11]. In the present studies the phorbol ester TPA was used which resulted in a differentiation of each U937 cell since no TPA-resistant subclone could be isolated from the cultures. TPA-treatment of U937 cells is accompanied by several morphological and functional changes such as alterations in cell shape and cessation of proliferation. These include a down-regulation of transferrin-receptors, a reorganization of actin and vimentin filaments and a significant expression of newly synthesized surface antigens [12]. Furthermore, TPA-induced monocytic differentiation of U937 cells is associated with a rapid *de novo* synthesis of cellular lectins and glycoproteins [13] including a markedly increased expression of the leukocyte function antigens LFA-1 (CD11a), CR3/MAC1 (CD11b), gp 150,95 (CD11c) and the β-subunit CD18. Although

these cells acquire many functional parameters in common with monocytes/macrophages during the differentiation process we have previously shown that TPA-treated U937 cells fail to exhibit some important properties associated with mature monocytes including the expression of HLA-class II or CD14 antigens. TPA-treated U937 cells were therefore characterized as immature macrophage intermediate rather than terminally differentiated cells [12].

Here we investigated the regulation of cytokine production during differentiation of human monoblastoid U937 tumor cells. Our observations demonstrate that during differentiation induced with 5 nM TPA the U937 cells sequentially acquire the capacity to produce and release TNF-α and IL-1 β, but not IL-1 α or IL-6. Both, the intracellular IL-1 β as well as the released molecule showed a predominant form of approximately 33–35 kD. In addition, the different regulation of TNF-α and IL-1 β release by exogenous rhTNF-α, rhγ-IFN and PGE_2 in differentiating U937 cells was found to be partially different from that reported for activated monocytes/macrophages. Therefore, differentiating U937 cells provide an excellent model to study cytokine regulation within different steps of development along the monocytic pathway.

MATERIALS AND METHODS

Cell culture

U937 cells were cultured in RPMI 1640 medium and differentiated with 5×10^{-9} M 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Sigma Chemie GmbH, Deisenhofen, F.R.G.) for 3 days as described elsewhere [13]. A long term culture of TPA-differentiated U937 cells (TPA-U937) was performed by replacing the culture medium without TPA every 3 days. These TPA-U937 were cultured for 2–3 weeks without any significant changes in cell morphology and adherence. Cell viability was assessed by trypan-blue exclusion in the non-adherent untreated U937 cells and by acridine orange staining of the adherent TPA-U937. At every time point of the experiments the viability of all cell cultures was greater than 95%.

[^{3}H]TPA was purchased from NEN, Dreieich, F.R.G. TPA-differentiated U937 cells (TPA-U937) were treated with 10^{-6} M dexamethasone (Merck, Darmstadt, F.R.G.) for 24 h (TPA/Dex-U937). In similar experiments TPA-U937 cells were also treated with 100 U/ml rhγ-IFN (kindly provided by Dr Otto, Fraunhofer Institut für Toxikologie, Hannover, F.R.G.) for 24 h (TPA/IFN-U937) or with 2 ng/ml PGE_2 (Sigma) for 24 h.

Detection of TNF-α and IL-1 β in culture medium

Radioimmunoassays of cell supernatants from both U937 control cells and stimulated U937 cells were performed using specific antibodies without cross-reactivities to other known cytokines as described elsewhere for TNF-α [14], IL-1 α [15] and IL-1 β [16].

Biological activity of TNF-containing samples was assayed by measuring neutral red dye uptake of L929 cells using the method as described by Wallach [17]. IL-1-activity was examined with the mouse thymoma EL4.16 cell line which can be stimulated by IL-1 to produce IL-2. These supernatants were transferred to an IL-2-dependent cytotoxic-T-lymphocyte line (CTLL) and the proliferation of these CTLL cells was measured by [^{3}H]thymidine incorporation. The biological activities (U/ml) were calculated in (pg/ml) by the specific activities: 1 U (TNF-α or IL-1 β) = 50 pg.

As controls rhTNF-α (Boehringer Mannheim GmbH, Mannheim, F.R.G.), rhIL-1 α and rhIL-1 β (both from Biogen Inc., Geneva, Switzerland) were used in the appropriate assay systems.

FPLC-separation of proteins released into the culture medium

TPA/IFN-U937 were incubated for 4 days with FCS-free culture medium. The medium was changed and collected every 24 h. This medium was concentrated 1:1000 in an ultrafiltration chamber (Amicon GmbH, Witten, F.R.G.) using a membrane with molecular weight cut off of approximately 5 kD (YM5 membrane, Amicon). The concentrate was applied on a FPLC superose 12 column (Pharmacia, Freiburg, F.R.G.) pre-equilibrated with PBS. Fractions were collected in 500 µl aliquots. To estimate the molecular weight of the protein containing fractions, the FPLC column was calibrated by using the standard proteins α-amylase (200 kD), BSA (67 kD), ovalbumin (43 kD), carboanhydrase (29 kD), cytochrome C (12 kD) and aprotinin (6.5 kD) (all from Sigma).

Western-blot analysis of IL-1β proteins

For Western-blot analysis the homogenate, cytosol and membrane fraction was prepared from U937, TPA-U937, TPA/Dex-U937 and TPA/IFN-U937. The cells of each treatment group were washed twice with PBS and disrupted in PBS by sonication (3×5 s/50W) with a labsonic cell-disrupter (Braun AG, Melsungen, F.R.G.). The cell fragments were centrifuged ($900 \times g$/10 min) to remove nuclei. The supernatant was termed as homogenate. For further fragmentation, this cell homogenate was centrifuged ($120\,000 \times g$/30 min) in a Beckmann L8-70M ultracentrifuge (Beckmann Instruments GmbH, München, F.R.G.). The resulting supernatant was used as cytosol and the pellet after resuspending in PBS was used as membrane fraction.

Homogenate, cytosol and membrane fraction of all samples were adjusted to the same protein concentration using the microtiter Bradford protein assay [18, 19] with BSA as standard. Aliquots containing 30 µg of protein were run on a 10% polyacrylamide SDS-gel [20] and transblotted to an immobilon-P membrane (Millipore Inc., Bedford, MA) in ice-cold blotting-buffer (15 mM Tris, pH 8.3; 120 mM glycine; 20% (v/v) methanol) at 200 mA for 1 h. After blotting, the protein standards on the reference lane of the blot were stained with amido black. The membrane was then incubated in blocking-buffer (100 mM Tris, pH 7.4; 0.9% (w/v) NaCl; 0.02% (w/v) NaN_3; 5% (w/v) low fat dry milk) for 4 h to block non-specific binding sites. Thereafter, a monoclonal mouse anti-rhIL-1 β (peptide: 165–186) antibody (kindly provided by Dr H. Herzbeck, Forschungsinstitut Borstel, F.R.G.) was used in blocking-buffer for 12 h. Following 3–4 washes of the blot with blocking-buffer for 10 min, an [^{125}I]-labeled rabbit anti-mouse IgG (Dacopatts GmbH, Hamburg, F.R.G.) was applied in the same buffer for a further 12 h. After

removing the iodinated antibody the blots were washed 8–10-times in 100 mM Tris, pH 8.0 to remove non-specifically bound radioactivity. The blots were air dried and exposed to an X-ray film.

Northern-blot analysis of cytokine mRNAs

U937 cells and TPA-U937 cells cultured for the time points indicated (10^7 cells per culture) were solubilized with 1 ml 7.6 M guanidine–HCl in 0.1 M potassium acetate buffer pH 5.0 and DNA was sheared by 5-times aspiration through a 21 gauge needle. Isolation of total RNA was performed according to Khandjian [21]. The RNA was electrophoresed on 1% agarose/formaldehyde gels, transferred to nylon filters and hybridized as described by Chirgwin *et al.* [22].

A TNF-cDNA probe (BASF AG, Ludwigshafen, F.R.G.) (425 bp PstI-fragment of the non-translated 3'-region of human TNF), the IL-1 α and IL-1 β probes (generous gift from Dr U. Gubler, Hoffmann LaRoche, Nutley, N.J.) (460 bp human IL-1 α EcoRI-BamHI cDNA fragment and a 530 bp human IL-1 β BamHI-NdeI cDNA fragment of the coding region, respectively) and the IL-6 probe (kindly provided by Dr M. Revel, Weizmann Institute, Rehovot, Israel) were labeled by the random primer method [23] and exposed to X-ray film at −70°C for 1–3 days.

RESULTS

Characterization of monocytic markers and growth arrest during TPA-induced monocytic differentiation of U937 cells

To determine differentiation of U937 cells after treatment with 5 nM TPA we measured both the expression of some markers associated with the monocytic phenotype and cell growth. The capacity to generate oxygen-derived free radicals was detected by reduction of nitroblue-tetrazolium (NBT). After 72 h of TPA-treatment approximately 14% of the cells showed positive staining with NBT. Furthermore, measurement of the monocyte specific marker α-naphthylacetate esterase revealed a rapid expression by 84% of U937 cells after 24 h of incubation with 5 nM TPA. These levels were elevated to nearly 100% of the TPA-treated cell population after 72 h. In contrast, untreated U937 control cells showed neither positive staining with NBT nor expression of α-naphthylacetate esterase (Table 1). According to the expression of these monocytic markers, differentiating U937 cells ceased to grow as measured by [^{3}H]thymidine incorporation. After 72 h no proliferation could be detected in TPA-treated U937 cells (Table 1).

These data are in concert with extensive studies on monocytic surface receptor expression performed previously under the same experimental conditions [12] and it is therefore suggested that U937 cells treated with 5 nM TPA were differentiated along the monocyte/macrophage lineage.

Release of TNF-α and IL-1 β from TPA-treated U937 cells

U937 cells released increasing levels of both TNF-α and IL-1 β into the culture medium during induction of monocytic differentiation with 5×10^{-9} M TPA. Investigation of the time course of this cytokine release by radioimmunoassay showed that the level of released TNF-α became significant by 4 h following TPA-treatment and reached a maximum after 48 h (Fig. 1a). In long term cultured TPA-U937 in the absence of TPA, maximal TNF-α release was measured until day 6. Between 8 days and 10 days in culture this TNF-α release dropped to detection limit. TPA-treatment of these long term cultured TPA-U937 after 20 days, however, re-induced the TNF-α production (Fig. 1c). In contrast, the amount of TNF-α in the culture medium of untreated U937 control cells was always at the detection limit (49 pg/ml) during the whole time course of the experiment (Fig. 1a).

IL-1-production was not detectable in U937 control cells and during the first 24 h of TPA-treatment. A significant release of IL-1 β, however, was measured between 24 h and 48 h following addition of TPA and reached a maximum after 72 h (Fig. 1b). This maximal level was continuously decreased during long term culture of TPA-U937 and reached detection limit between 20 days and 22 days. At this time point IL-1β production could be induced again by a further stimulation with TPA (Fig. 1d). There was no detectable production of IL-1α in the culture medium of either U937 or phorbol ester-treated U937 cells at any time point of the experiments (data not shown).

In order to test the bioactivity of the released TNF-α and IL-1 β proteins the appropriate biological assay systems were employed. TPA (5×10^{-9} M) significantly interferred with both the TNF and IL-1 biological assay. Therefore, it was removed after 72 h by changing the culture medium and the cytokine release was measured after a further 24 h culture. Control experiments by labeling of U937 cells with [^{3}H]TPA, demonstrated that only a very low concentration of [^{3}H]TPA (about 10^{-11} M) was still present in these 24 h supernatants. This amount of TPA did not interfere with either of the biological assay systems (data not shown). Measurements of the 24 h supernatant from TPA-U937 cells by the indirect IL-1-assay using the EL4.16 and CTLL cell lines showed significant levels of biologically active IL-1 (260 pg/5×10^5 cells). The presence of biologically active IL-1 was also detected in long term cultured TPA-U937 (until day 16). In contrast, no IL-1 activity was measured in the supernatant of undifferentiated U937 control cells. In addition, no

TABLE 1. CHARACTERIZATION OF MONOCYTIC MARKERS AND GROWTH ARREST DURING TPA-INDUCED DIFFERENTIATION OF U937 CELLS

	NBT [%]	α-NAEST [%]	[^{3}H]thymidine [%]
U937	<1	<1	100.0 ± 7.8
24 h TPA	3.3 ± 0.7	84.3 ± 4.7	35.2 ± 8.2
48 h TPA	10.3 ± 0.9	96.9 ± 1.2	1.1 ± 0.2
72 h TPA	13.8 ± 3.5	98.3 ± 0.3	0.3 ± 0.1

U937 cells (5×10^5 cells/ml) were treated with 5 nM TPA for the time points indicated. The differentiation along the monocytic pathway was investigated by evaluating the percentage of the different cell populations to reduce nitroblue tetrazolium (NBT) [53] and to express α-naphthylacetate esterase (α-NAEST) [54]. Data represent the means ± S.D. of three different experiments. The cell growth was measured by [^{3}H]thymidine incorporation [12] and the percentage of proliferating cells was calculated with U937 cells as reference (U937 = 100%). Data represent the means ± S.D. ($n = 8$).

biological TNF-activity was detectable in the supernatant of undifferentiated U937 cells whereas the subsequent 24 h supernatant from TPA-U937 showed cytotoxic activity (150 pg TNF/5×10^5 cells) using the L929 cell assay and the cytotoxic activity was still detectable in long term cultured TPA-U937 (until day 6).

The sequential induction of TNF-α and IL-1 β in U937 cells following TPA-treatment occurred independently as demonstrated in experiments where a parallel incubation of TPA-treated U937 cells with a monoclonal TNF-α antibody reduced the amount of TNF-α in the supernatant by 92.1% and 90.4% at 12 h and 48 h, respectively. Under these conditions, IL-1 β production was not significantly reduced. Accordingly, incubation of U937 cells with rhTNF-α for 24 h at concentrations between 100–3000 U/ml did not result in a significant release of IL-1 β at any time point measured (Table 2).

Regulation of cytokine release in TPA-differentiated U937 cells

TPA-U937 were treated for 24 h with different concentrations of either rhTNF-α (1–1000 U/ml), dexamethasone (60 nM–1 μM), PGE_2 (0.5 ng/ml–3 ng/ml) or rhγ-IFN (1–1000 U/ml). The medium was changed and the subsequent 24 h and 48 h supernatants were collected and assayed for TNF-α and IL-1 β. The data are shown for the optimal concentrations of the exogenous stimuli, including TNF-α (100 U/ml), PGE_2 (2 ng/ml) and rhγ-IFN (100 U/ml).

Treatment of TPA-U937 with rhTNF-α did not significantly alter the level of TNF-α and IL-1 β released from TPA-differentiated U937 cells (Fig. 2). Although a slight reduction of IL-1 β was measured after 48 h this effect by TNF-α was not significant as calculated by the two sided Student *t*-test for dependent measurements.

In addition, incubation of TPA-U937 with PGE_2 showed no significant effect on either TNF-α or IL-1 β release within 24 h or 48 h (Fig. 2).

When TPA-U937 were incubated with rhγ-IFN, the release of IL-1 β was specifically enhanced. This superinduction of IL-1 β was increased 8–10-fold when compared to TPA-U937 whereas the level of released TNF-α remained unaltered (Fig. 2).

Dexamethasone inhibited the release of both TNF-α and IL-1 β from TPA-U937 in a dose-dependent manner. The inhibition was highly significant (all $p < 0.01$) and reached 64.2% ± 4.9% ($n = 4$) for TNF-α and 57.0% ± 4.2% ($n = 4$) for IL-1 β compared to the 24 h release from TPA-U937 (Table 3).

FPLC-separation of FCS-free cell supernatant of TPA/IFN-U937 cells

Conditioned medium collected from TPA-U937 treated with γ-IFN was concentrated and then separated by gel filtration FPLC. The elution pattern of proteins in the conditioned medium is shown in Fig. 3. All fractions were tested for TNF-α and IL-1 β using RIA. TNF-α was found in fractions with apparent molecular weight between 50 kD and 100 kD. Investigations of IL-1 β containing samples, identified proteins with molecular weight of 35 kD–45 kD and 10 kD–20 kD. All TNF- and IL-1-containing fractions as evaluated by RIA were also biologically active as measured in the appropriate assay systems. In addition, the IL-1-containing fractions were capable of displacing [^{125}I]IL-1 α in receptor binding studies with the mouse thymoma cell line EL4.16 (data not shown).

Characterization of IL-1 β by Western-blot analysis

Western-blot analysis of IL-1 proteins in U937 cells

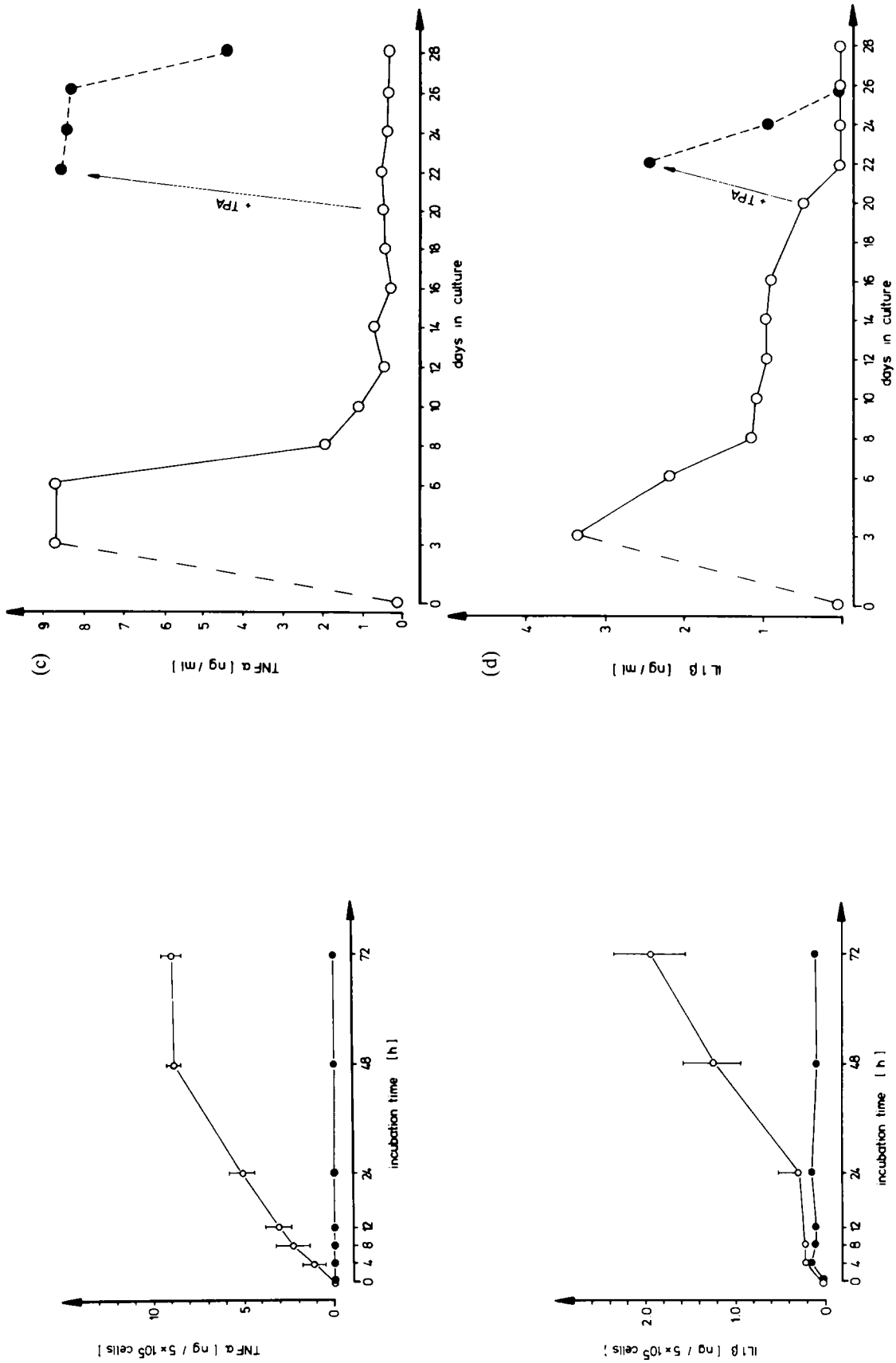

FIG. 1 (a, b). Time course of TNF-α (Fig. 1a) and IL-1 β (Fig. 1b) production by TPA-treated U937 cells. The cytokines were measured by RIA in the supernatants of 5×10^5 cells/ml of either U937 cells (●) and U937 cells treated with 5×10^{-9} M TPA (○) for the time points indicated. Data represent means ± S.D. of 3 different experiments (detection limits: 49 pg/ml TNF-α; 21 pg/ml IL-1 β). (c, d). Time course of TNF-α (Fig. 1c) and IL-1 β (Fig. 1d) production by long term cultured TPA-U937 (○) for the time points indicated. The cytokines were measured by RIA in the supernatants of 5×10^5 cells/ml. The medium including the already produced cytokines was replaced every third day. Therefore, values are given in cytokines/ml. During this period of long term culture the number of cells was not significantly altered as measured previously under the same experimental conditions [24]. After 20 days of these growth arrested cells in culture, cytokine production could be re-induced by a further TPA-stimulus (●). Data are given for 1 representative experiment out of 3 similar ones (detection limits: 46 pg/ml TNF-α; 19 pg/ml IL-1 β).

TABLE 2. TNF-INDEPENDENT INDUCTION OF IL-1 DURING TPA-INDUCED DIFFERENTIATION OF U937 CELLS

	2 h		12 h		48 h	
	TNF-α [pg/ml]	IL-1 β [pg/ml]	TNF-α [pg/ml]	IL-1 β [pg/ml]	TNF-α [pg/ml]	IL-1 β [pg/ml]
U937	ND	ND	ND	ND	ND	ND
U937 + TPA	390 ± 18	ND	3349 ± 712	77 ± 51	3532 ± 458	350 ± 77
U937 + TPA + mab(TNF-α)	ND	ND	264 ± 29	67 ± 18	254 ± 45	337 ± 172
U937 + TNF-α (100 U/ml)	2282 ± 561	ND	794 ± 59	ND	745 ± 60	ND
U937 + TNF-α (300 U/ml)	over	ND	over	ND	over	ND
U937 + TNF-α (1000 U/ml)	over	ND	over	ND	over	ND
U937 + TNF-α (3000 U/ml)	over	ND	over	ND	over	ND

U937 control cells were treated with either 5×10^{-9} M TPA alone or with both 5×10^{-9} M TPA and 10 µg/ml of a monoclonal TNF-α antibody (mabTNF-α). Furthermore, control cells were incubated with different concentrations of rhTNF-α for the time points indicated. The concentrations of TNF-α and IL-1 β in the supernatants were measured by RIA (detection limits: 49 pg/ml TNF-α; 21 pg/ml IL-1 β). Data represent means ± S.D. (n = 4); ND = not detectable; over = out of range.

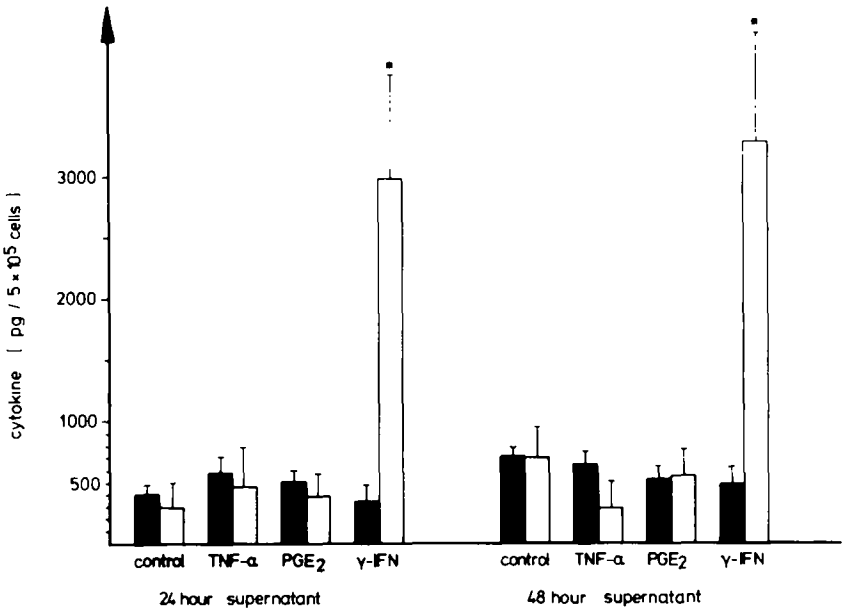

FIG. 2. Effects of rhTNF-α, PGE_2 and rhγ-IFN on TNF-α release (■) and IL-1 β release (□). TPA-differentiated U937 cells (5×10^5 cells/ml) were incubated with either culture medium (control), 100 U/ml rhTNF-α, 2 ng/ml PGE_2 and 100 U/ml rhγ-IFN for further 24 h. After removing the stimuli the cells were cultured further on in culture medium and the cytokine release was measured by RIA in the subsequent 24 h and 48 h supernatants (detection limits: 49 pg/ml TNF-α; 21 pg/ml IL-1 β). Data represent means ± S.D. of 3 different experiments.

were shown in Fig. 4a. In addition to the 18 kD rhIL-1 β standard protein a strongly expressed band with apparent molecular weight of 33–35 kD and a high molecular weight protein of approximately 70 kD was recognized by the IL-1 β antibody in TPA-U937, these proteins being even more strongly expressed in

TABLE 3. DOSE-DEPENDENT INHIBITION OF TNF-α AND IL-1 β RELEASE FROM TPA-DIFFERENTIATED U937 CELLS BY DEXAMETHASONE

	TNF-α [pg/ml]	IL-1β [pg/ml]
TPA-U937	4089 ± 506	1083 ± 191
TPA-U937 + 6×10^{-8} M Dex	2872 ± 395	1277 ± 240
TPA-U937 + 10^{-7} M Dex	2079 ± 142	842 ± 15
TPA-U937 + 3×10^{-7} M Dex	2224 ± 288	749 ± 116
TPA-U937 + 6×10^{-7} M Dex	2042 ± 168	627 ± 78
TPA-U937 + 10^{-6} M Dex	1463 ± 202	463 ± 45

TPA-U937 (5×10^5 cells/ml) were cultured for 24 h with the concentrations of dexamethasone indicated. The amount of released cytokines was detected by RIA (detection limits: 56 pg/ml TNF-α; 81 pg/ml IL-1 β). Data represent means ± S.D. ($n = 4$).

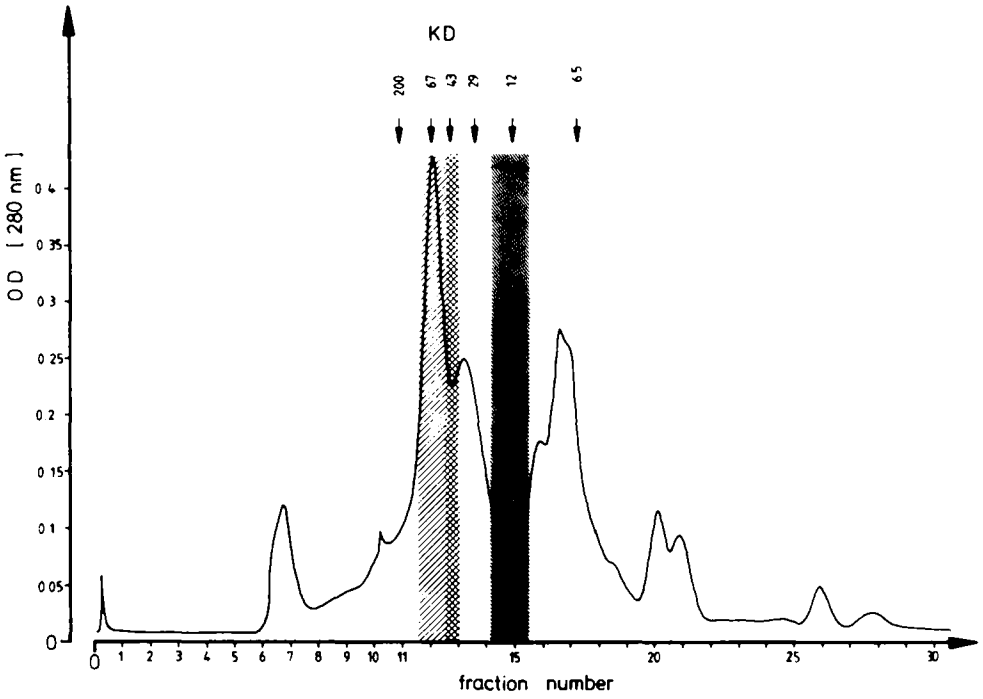

FIG. 3. FPLC-separation of 1:1000 concentrated supernatant from TPA/IFN-U937 cells as described in the Methods section. Fractions from the gel-filtration superose 12 column were collected in 0.5 ml aliquots. TNF-α and IL-1 β in all fractions were investigated by RIA (detection limit: 46 pg/ml TNF-α; 19 pg/ml IL-1 β). The data are shown for 1 representative experiment out of 4.

= TNF-α containing fractions (11.5; 12).

= IL-1 β containing fraction (12.5).

= IL-1 β containing fractions (14; 14.5; 15).

TPA/IFN-U937. Both, the 33–35 kD and the 70 kD proteins were also present in the TPA/Dex-U937 homogenate. In comparison to TPA-U937 and TPA/IFN-U937, however the expression of these proteins was markedly reduced. The concentrated supernatant of TPA/IFN-U937 showed only the 35 kD band. After a long exposition of the blot to the X-ray film (>4 weeks) a very weak 18 kD band could be detected in this sample (data not shown).

In a corresponding experiment a more detailed intracellular localization of IL-1 β in U937 cells was investigated (Fig. 4b). The results demonstrate clearly that IL-1 β is predominantly located in the cytosol of differentiated U937 cells. Furthermore, no IL-1 β was detectable in undifferentiated U937 control cells and TPA/Dex-U937 showed a markedly decreased expression of IL-1 β when compared to TPA-U937 or even TPA/IFN-U937.

Northern-blot analysis of TNF-α and IL-1 β mRNA

The investigation of a time course of mRNA expression for TNF-α (Fig. 5a) and IL-1 β (Fig. 5b) resulted in a sequential induction of both cytokines. A strong expression of TNF-α mRNA was observed in 48 h TPA-treated U937 cells (b) compared to U937 control cells (a). Following an extended culture of TPA-treated U937 cells for 7 days (c), 9 days (d) and 12 days (e) a continuously decreasing expression of TNF-α became obvious reaching again the level of U937 control cells after 16 days following treatment with TPA (f).

In contrast to TNF-α IL-1 β mRNA was not detectable in undifferentiated U937 cells (Fig. 5b, lane (a)). Following 2 days of TPA-treatment a weak band with apparent molecular weight of 1.8 kB became obvious (b), this band being markedly enhanced in TPA-treated U937 cells cultured for 7 days (c). The extended culture of TPA-treated U937 cells for 9 days up to 16 days revealed continuously increasing levels of IL-1 β mRNA. In addition to this long termed IL-1 β signal, an increased expression of a high molecular weight mRNA became detectable.

Equal loading of mRNA was determined by ethidium bromide-stained 18S and 28S RNAs (data not shown).

DISCUSSION

The human monoblastoid tumor cell line U937 is a model in which differentiation of precursor cells into macrophage-like cells can be studied conveniently. Upon treatment with the phorbol ester TPA, all U937 cells undergo significant morphological and functional changes during the differentiation process reaching a transient macrophage intermediate state rather than a terminally differentiated state [13, 24]. This tumor cell line was used to investigate and characterize induction of TNF, IL-1 and IL-6 production and to examine the regulation of these cytokines during monocytic differentiation.

Within 2–4 h following TPA-treatment we measured a rapidly increased TNF-α production, which reached a plateau after 48–72 h and then slowly (within 10 days) decreased to the level of undifferentiated U937 control cells. These data are in accordance with the results obtained by Northern-blot analysis demonstrating a marked increase of TNF-α mRNA after 48 h of TPA-treatment followed by a continuously decreasing TNF-α mRNA signal in a long term culture of TPA-U937. A similar induction of TNF was also observed in TPA-treated resting macrophages, however, the maximal signal of TNF mRNA transcription occurred after 12 h followed by a decreased signal after 24 h of TPA-treatment [25].

In terms of IL-1 production our results show an enhanced level of IL-1 β mRNA and a continuously increasing amount of IL-1 β release from U937 cells between 24 and 48 h after incubation with TPA. This IL-1 β production was still detectable after 20 days which is also in accordance with the IL-1 β Northern-blots, demonstrating a strong signal after 13 days. Interestingly, Nishida *et al.* have shown that increasing levels of IL-1 β mRNA are expressed already 4 h after TPA-treatment of U937 cells [26] and our data demonstrate furthermore, that transcription of IL-1 β messages continued to increase during long term culture even when the release of cytokine was significantly reduced. These data indicate that the transcription, translation and release of IL-1 β is regulated by different mechanisms which has also been observed in other systems [27]. In contrast to TNF-α and IL-1 β, no increase in either IL-1 α mRNA expression or IL-1 α release could be measured in TPA-treated U937 cells (data not shown) which is in accordance with the findings of Nishida *et al.* [26].

The sequential induction of TNF-α (between 2–4 h) and IL-1 β (between 24–48 h) in TPA-U937 apparently occurs independently, since neutralization of TPA-induced TNF-α release by a monoclonal TNF-α antibody had neither an effect on the TPA-induced IL-1 β release nor any influence on the ongoing differentiation process. Furthermore, treatment of undifferentiated U937 control cells with rhTNF-α did not result in increasing IL-1 β or IL-1 α release, although receptors for TNF were well characterized on these cells [28] and alterations in gene expression following TNF-treatment have been

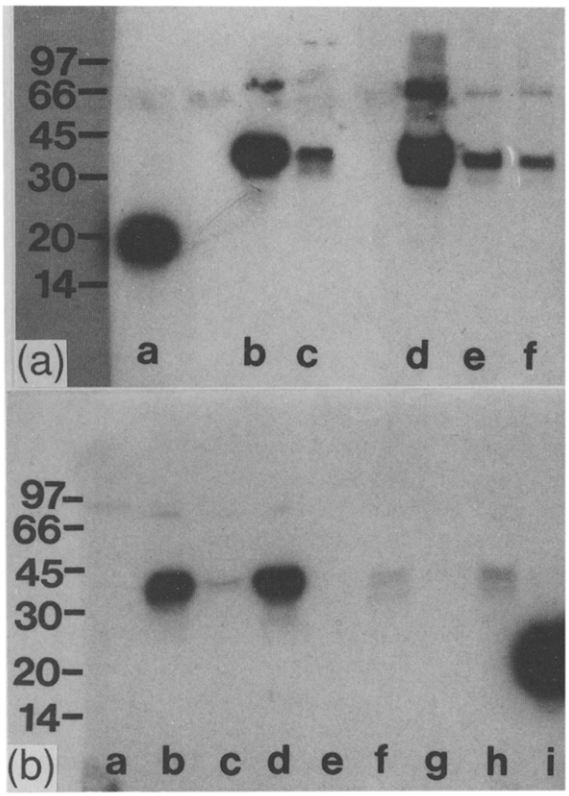

FIG. 4(a). IL-1 β Western-blot analysis of the homogenate from stimulated U937 cells: 50 ng rhIL-1 β as standard (lane a); TPA-U937 (lane b); 1:1000 concentrated supernatant of TPA/IFN-U937 cells (lane c); TPA/IFN-U937 (lane d); TPA/Dex-U937 (lane, e, f). Each lane except the standard (lane a) contained 30 μg protein. (b) IL-1 β Western-blot analysis of the cytosol (lanes a–d) and the membranes (lanes e–h) of U937 control and stimulated cells: U937 cells (lanes a, e); TPA/U937 (lanes b, f); TPA/Dex-U937 (lanes c, g); TPA/IFN-U937 (lanes d, h) and 50 ng rhIL-1 β as standard (lane i). Each lane except the standard contained 30 μg protein.

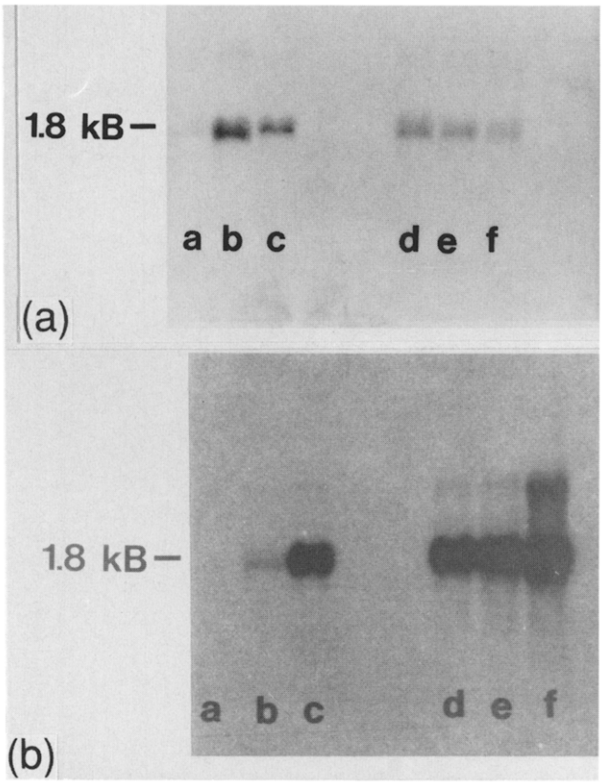

FIG. 5. Northern-blot analysis of TNF-α mRNA (a) and IL-1 β mRNA (b): U937 cells (lane a); U937 cells treated for 48 h with 5×10^{-9} M TPA (lane b); TPA-U937 cultured for further 4 days (lane c); 6 days (lane d); 9 days (lane e) and 13 days (lane f).

described [29]. These findings suggest an independent induction of TNF-α and IL-1 β during differentiation of U937 cells.

Dinarello *et al.* reported an induction of IL-1 release following stimulation of human mononuclear cells with exogenous TNF-α [30] and an autocrine TNF-induction has been described by several groups [31, 32]. In TPA-U937, however, no effects on TNF-α and IL-1 β release were detectable when these cells were stimulated with exogenously added TNF-α. The lack of TPA-U937 to respond to exogenous TNF-α similar to macrophages may be explained by the findings of Holtmann and Wallach who reported a rapid down-regulation of TNF-receptors in U937 cells treated with low concentrations of TPA ($<5 \times 10^{-9}$ M) [33]. In this respect it is interesting to note that Brach *et al.* discussed IL-6 mRNA accumulation upon TNF-treatment of U937 cells [34], furthermore suggesting that after TPA-treatment U937 cells are unable to respond to released TNF by an autocrine mechanism since our results revealed only background levels of IL-6 mRNA during the whole time course of the experiment (data not shown). Although IL-6 production by two U937 subclones has been recently reported, this discrepancy may be explained by the 16-fold higher amount of TPA used in the studies of Navarro *et al.* [35], suggesting that induction of the IL-6 gene may depend on the concentration of TPA used but may not necessarily be associated with the differentiation process.

Exogenously added PGE_2 like TNF-α showed no effect on either TNF-α or IL-1 β release from TPA-U937, although an inhibition of either TNF-release [36] or IL-1 production [4, 37] by PGE_2 has been described in stimulated macrophages. Taken together, these data point to a different cytokine regulation in TPA-U937 compared to activated monocytes/macrophages.

Dexamethasone treatment of TPA-U937 caused a dose-dependent inhibition of both, TNF-α and IL-1 β release. Our data are in accordance with the findings of other groups who reported a transcriptional and post-transcriptional reduction of IL-1 expression by a selectively inhibited transcription of the IL-1 β gene and a decreased stability of IL-1 β mRNA upon treatment with dexamethasone [38, 39]. A glucocorticoid inhibited release of TNF and IL-1 in monocytes from several species has also been described [40–42].

Treatment of TPA-U937 with γ-IFN resulted in a strongly enhanced production and an 8–10-fold specifically increased release of IL-1 β, whereas the amount of TNF-α remained unaltered. Similarly, Arenzana-Seisdedos *et al.* reported a potentiation of IL-1 release from human blood monocytes by γ-IFN rather than α-IFN and β-IFN [43]. With respect to the level of released TNF-α, however, Nedwin *et al.* have shown in human blood monocytes that γ-IFN was only capable of inducing TNF production in co-stimulation with IL-2 or mitogens [44], whereas, Beutler *et al.* also discussed a TNF induction by γ-IFN alone in mononuclear phagocytes [45].

A characterization of TNF-α and IL-1 β release from TPA/IFN-U937 by FPLC separation of concentrated FCS-free conditioned medium resulted in TNF with apparent molecular weight of 50–100 kD and 2 fractions of IL-1 β with molecular weight of 35–45 kD and 10–20 kD, respectively. Data obtained by Western-blot analysis under reducing conditions showed an IL-1 β standard protein at 18 kD. In the samples, beside a very weak band at approximately 18 kD, a 33–35 kD IL-1 prevailed and also a cell-associated high molecular weight IL-1 with 70 kD not found in the supernatant was detected. The production of a 33 kD IL-1 β precursor protein by stimulated U937 cells has recently been reported [46]. Extensive studies by Knudsen *et al.* have shown a 14 kD IL-1 protein in the supernatant of *staphylococcus* exotoxin activated U937 cells with pI 5.5 [47] which may correspond to another IL-1 form than those recognized by the antibody in our studies. A high molecular weight IL-1 with approximately 75 kD has been discussed by Togawa *et al.* in human mononuclear cells [48] and has also been found in normal human urine [49].

The data clearly indicate a preferred localization of IL-1 β in the cytosol of differentiated U937 cells which is consistent with the findings of Rubartelli *et al.* [46] and of Sisson and Dinarello who found most of the cell-associated TNF-α, IL-1 α and IL-1 β in the cytosol by sequential and differential centrifugation of rGM-CSF stimulated human monocytes [50]. In this respect it is interesting to note that Merluzzi *et al.* found membrane-associated IL-1-activity in TPA-treated U937 cells [51], suggesting that cytosolic inactive IL-1 precursors may be processed into biologically active IL-1 molecules as discussed by Martin and Resch [52].

The consistency of all IL-1 data, including the release, the FPLC-separation, the Western-blots and the Northern-blots suggests that TPA-U937 acquire the capacity to produce a 33–35 kD precursor IL-1 β in large quantities and release a small amount of IL-1 β with detectable biological activity, whereas about 98% of IL-1 β remains located in the cytosol. It is still unclear whether the intracellular 70 kD IL-1 β, which is reduced by dexamethasone and enhanced expressed by γ-IFN similar to the IL-1 β release data, is a precursor molecule or a tightly associated dimer of the 33–35 kD IL-1 β.

We therefore conclude that the sequential induction of TNF-α and IL-1 β in U937 cells treated with TPA is independently regulated. Although TPA-U937 acquire a glucocorticoid sensitivity similar to macrophages, the newly described specifically super-induced IL-1 β production of TPA/IFN-U937 and the unaltered levels of TNF-α and IL-1 β release by rhTNF-α and PGE_2 indicate differences in the regulation of both cytokines compared to activated monocytes/macrophages. The failure of TPA-U937 to generate detectable amounts of IL-1 α and IL-6 and the production of significant levels of high molecular weight IL-1 β proteins (70 kD, 33–35 kD) may be related to either the tumorgenicity of U937 cells or to an intermediate state of differentiation upon TPA-treatment which then suggests that cytokine processing as in mature monocytes/macrophages is acquired during later or terminal steps of maturation.

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