Treatment of Melanoma Cells with the Synthetic Retinoid CD437 Induces Apoptosis via Activation of AP-1 In Vitro, and Causes Growth Inhibition in Xenografts In Vivo

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Abstract. Human malignant melanoma is notoriously resistant to pharmacological modulation. We describe here for the first time that the synthetic retinoid CD437 has a strong dose-dependent antiproliferative effect on human melanoma cells (IC_{50}: 5×10^{-6} M) via the induction of programmed cell death, as judged by analysis of cell morphology, electron microscopical features, and DNA fragmentation. Programmed cell death was preceded by a strong activation of the AP-1 complex in CD437-treated cells as demonstrated by gel retardation and chloramphenicol transferase (CAT) assays. Northern blot analysis showed a time-dependent increase in the expression of *c-fos* and *c-jun* encoding components of AP-1, whereas *bcl-2* and *p53* mRNA levels remained constant. CD437 also exhibited a strong growth inhibitory effect on MeWo melanoma cells in a xenograft model. In tissue sections of CD437-treated MeWo tumors from these animals, apoptotic melanoma cells and *c-fos* overexpressing cells were colocalized by TdT-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (TUNEL) staining and in situ hybridization. Taken together, this report identifies CD437 as a retinoid that activates and upregulates the transcription factor AP-1, leading eventually to programmed cell death of exposed human melanoma cells in vitro and in vivo. Further studies are needed to evaluate whether synthetic retinoids such as CD437 represent a new class of retinoids, which may open up new ways to a more effective therapy of malignant melanoma.

RETINOIDS, structural analogs of vitamin A, are involved in a wide range of biological processes including cell differentiation, morphogenesis, and proliferation (for reviews see Kastner et al., 1995; Mangelsdorf and Evans, 1995). These parameters have been analyzed in various murine and human cancer cell lines including melanoma for more than fifteen years (Lotan, 1980; Meyskens and Fuller, 1980; Hoal et al., 1982). Although their clinical usefulness has so far been limited by various adverse effects, retinoids are of increasing importance for the development of oncological strategies because of their antineoplastic activity (for reviews see Bollag and Holdener, 1992; Smith et al., 1992; Kurie et al., 1994). These may be mediated either directly by toxic membrane damage or by activation of oxidative processes or indirectly by induction of tumor suppressive mediators or by modulation of receptors necessary for growth factor interaction or immune recognition (Evans, 1988; Apfel et al., 1991; Mangelsdorf and Evans, 1995). At the molecular level it is known that retinoids bind to and activate specific nuclear receptors, the retinoic acid receptors (RARs)[1] and retinoid X receptors (RXRs), which in turn, as homo- or heterodimers, recognize specific DNA sequences regulating target gene expression (for reviews see Apfel et al., 1991; Kastner et al., 1995; Mangelsdorf and Evans, 1995). Recently, it has been shown that retinoids interfere with other transcription factors, particularly AP-1, which is formed by members of the Fos and Jun protein family (Schüle et al., 1990, 1991; Yang-Yen et al., 1991; Pfahl, 1993). AP-1 inhibition leads to antiproliferative effects in tumor cells independent of the receptor-transactivating potential of the retinoids (Fanjul et al., 1994; J.-Y. Chen et al., 1995; Li et al., 1996).

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1. *Abbreviations used in this paper*: ATF, activating transcription factor; CAT, chloramphenicol transferase; CREB, cAMP-responsive element-binding protein; RA, all-*trans*-retinoic acid; RAR and RXR, retinoic acid and retinoid X receptors; TUNEL, TdT-mediated deoxyuridine triphosphate-digoxigenin nick end labeling.

In humans, retinoids have so far not been used successfully for the treatment of malignant melanoma (Ho and Sober, 1990; Lotan et al., 1991). Only recently, a RAR-γ–selective synthetic retinoid CD437 has been identified (Bernard et al., 1992) that strongly inhibited in vitro cell proliferation of various human melanoma cell lines in a dose-dependent manner (Schadendorf et al., 1994). Since malignant melanoma cells are notoriously resistant to chemotherapeutic drugs in vitro and in vivo (Ho and Sober, 1990; Schadendorf et al., 1995) and new strategies for the treatment of metastatic melanoma are urgently needed, we used the human melanoma cell line MeWo as a model system for studying the mechanism involved in CD437-induced growth inhibition in greater detail. CD437 is shown to be capable of inducing the transcription factor AP-1 on mRNA and protein levels leading eventually to programmed cell death of MeWo cells in vitro. Furthermore, treatment with CD437 markedly reduced the growth of human melanoma cells in nude mice.

Materials and Methods

Cell Culture and Exposure to Retinoids

Human melanoma cells (MeWo, SK-Mel-23, and MV-3) were grown in RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with charcoal-treated 10% FCS (Schadendorf et al., 1993). For retinoid treatment, the same medium was used. The medium and retinoids were changed every 3 d. The retinoids all-*trans*-retinoic acid (RA), CD437, and CD2398 were used. CD437 is known to activate RAR-γ with high selectivity, whereas CD2398, which is structurally highly homologous to CD437, has no binding affinity to any known retinoid receptor. RA binds to all RAR subtypes with high affinity but not to RXR receptors (Bernard et al., 1992; Schadendorf et al., 1994). All retinoids were kindly provided by Drs. B. Shroot and U. Reichert (CIRD, Galderma, Valbonne, France) and were dissolved in DMSO. The final concentration in all cultures was 0.1% DMSO.

Electrophoretic Mobility Shift Assay

5×10^6 cells were stimulated with RA, CD437, and CD2398 (10^{-5} M) for the times indicated. Cell extracts were prepared as described previously (Trede et al., 1991; Pahl and Baeuerle, 1995). Total cell extracts were prepared using high-salt detergent buffer (Totex; 20 mM Hepes, pH 7.9, 350 mM NaCl, 20% [wt/vol] glycerol, 1% [wt/vol] NP-40, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% PMSF, and 1% aprotinin). Cells were harvested by centrifugation and washed once in ice-cold PBS and resuspended in 4 vol of Totex buffer. The cell lysate was incubated on ice for 30 min and then centrifuged for 5 min at 13,000 *g* at 4°C. The protein content of the supernatant was determined, and equal amounts of protein (10–20 μg) were added to a reaction mixture containing 20 μg BSA (Sigma Chemical Co., St. Louis, MO), 2 μg poly(dI-dC) (dIdC purchased from Pharmacia LKB Biotechnology, Piscataway, NJ), 2 μl buffer D+ (20 mM Hepes, pH 7.9, 20% glycerine, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF), 4 μl buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCL, 10 mM DTT, 0.1% PMSF), and 100,000 cpm of α-^{32}P–labeled oligonucleotide in a final volume of 20 μl. Samples were incubated at room temperature for 25 min. For the supershift assays, 2.5 μl of antibody were added to the reaction simultaneously with the protein and incubated as described. The following antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA): *4-10G* (mouse IgG_{2a}; broadly reactive with c-Fos, Fos B, Fra-1, and Fra-2), *4* (rabbit polyclonal IgG; reactive with c-Fos), *102* (rabbit polyclonal IgG; reactive with Fos B), *R-20* (rabbit polyclonal IgG; reactive with Fra-1), *Q-20* (rabbit polyclonal IgG; reactive with Fra-2), *N* (rabbit polyclonal IgG; reactive with c-Jun), *N-17* (rabbit polyclonal IgG; reactive with Jun B), *329* (rabbit polyclonal IgG; reactive with Jun D), *C-21* (rabbit polyclonal IgG; reactive with cAMP-responsive element-binding protein 1 [CREB-1]); *C-20* (rabbit polyclonal IgG; reactive with CREB-2 and activating transcription factor 4 [ATF-4]), *F1-1* (rabbit polyclonal IgG; reactive with ATF-1), *C-19* (rabbit polyclonal IgG; reactive with ATF-2), *C-19* (rabbit polyclonal IgG; reactive with ATF-3), and *Z5* (mouse monoclonal IgG_1; reactive with ATF-4). AP-1 oligonucleotide (Promega Corp., Madison, WI) was labeled using [γ-^{32}P]ATP (3,000 Ci/ml; Amersham Corp., Arlington Heights, IL) and T4 polynucleotide kinase (Promega Corp.).

DNA Transfection and CAT Assay

Cells were DNA transfected using cationic liposomes (Lipofectin; Life Technology, Inc., Eggenstein, FRG) under serum-free conditions as described previously (Artuc et al., 1995). For the transfection assay, cells were seeded into 6-well plates at a density of 12,000 cells/cm^2. Cultures were transfected 1 d after they reached 50–60% confluency, and cells were incubated with DNA–liposome complexes (2 μg DNA per well) for 24 h. To exclude differences in transfection efficiency, cotransfection of a cytomegalovirus-driven β-galactosidase construct (Stratagene, Heidelberg, Germany) was performed in each experiment (1 μg per well). 48 h after transfection, cells were washed twice with PBS and harvested. After centrifugation, the pellet was resuspended in 0.125 M Tris HCl buffer, pH 7.8, and lysed by repeated cycles of freezing and thawing at −80°C, and the total protein concentration of the cell lysates was determined. To determine AP-1–dependent CAT activities, a DNA fragment containing five AP-1 DNA-binding sites was generated by PCR (primer: ACTgAATCAAC-TgAATCAACTgAATCAACTgAATCAACTgAATCATT) cloned into the BglII site of the pCAT-promoter vector (Promega Corp.), and the resulting plasmid was transfected into MeWo cells. CAT activity was measured as described previously (Artuc et al., 1995; Nürnberg et al., 1995). Commercially available CAT was used as a standard for the determination of enzyme activity in extracts of transfected cells. Transfection efficiency was estimated by cotransfection of a β-galactosidase plasmid in each experiment.

Northern Blot Analysis and In Situ Hybridization

RNA was isolated using an RNeasy Total RNA Kit (QIAGEN Inc., Chatsworth, CA), electrophoresed through 1% agarose-formaldehyde gels, and then transferred to nylon membranes (Boehringer Mannheim Corp.). Northern blot hybridization to random-primed ^{32}P-labeled DNAs and high stringency washes were performed as described (Sambrook et al., 1989). Signals were visualized by autoradiography. The cDNA probes for *c-fos*, *c-jun* (Nürnberg et al., 1995), and *p53* (Baker et al., 1990) were excised from their plasmid vectors and random-primed with [α-^{32}P]dCTP (3,000 Ci/mmol; Du Pont-New England Nuclear, Boston, MA). For in situ hybridization, digoxigenin-labeled antisense and sense *c-fos* riboprobes were synthesized using T7 and SP6 RNA polymerase (DIG-RNA labeling kit; Boehringer Mannheim Corp.). The hybridization procedure of paraffin-embedded sections of cutaneous tumors followed a standard protocol using a colorimetric detection system (Nürnberg et al., 1995).

Electron Microscopy

The method used has been described in detail elsewhere (Kolde and Knop, 1986). Briefly, the cells were fixed in half-strength Karnovsky solu-

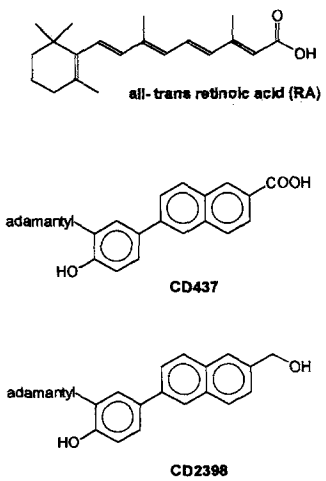

Figure 1. Structural formula. Structures of (RA), 6-[3-adamantyl-4-hydroxyphenyl]-2-naphtalene carboxylic acid (CD437), and 6-[3-adamantyl-4-hydroxyphenyl]-2-naphtalene aldehyde (CD-2398).

tion (0.1 M cacodylate buffer, pH 7.4) for 30 min at 4°C. After washing in PBS, the cells were postfixed in 1.33% osmic acid (0.05 M phosphate buffer, pH 7.4) for 30 min, dehydrated in a graded ethanol series, and embedded in Araldite using beam capsules. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (model 9S; Carl Zeiss, Inc., Thornwood, NY). Necrotic and apoptotic cell figures were counted and expressed by a semiquantitative scoring system as follows: ∅, no cell with altered morphology; +/− single cells (up to 3%); +, few cells (between 3–10%); ++, moderate number of cells (11–50%); +++, numerous cells (>50%).

Melanoma Nude Mouse Model

For the in vivo experiments, male Swiss-nu/nu mice (Bomholtgaard; Ry, Denmark) weighing 20–25 g were used. Mice were kept under sterile conditions at 24–26°C room temperature, 50% relative humidity, and 12 h light–dark rhythm in laminar flow shelves and were supplied with autoclaved food (Sniff; Soest, Germany) and bedding. The drinking water was filtered and acidified (pH 4.0). For treatment of melanoma xenografts, previously established MeWo melanoma tumors of 1–2 mm in diameter were implanted into the right flank of animals. After tumor growth for 10 d,

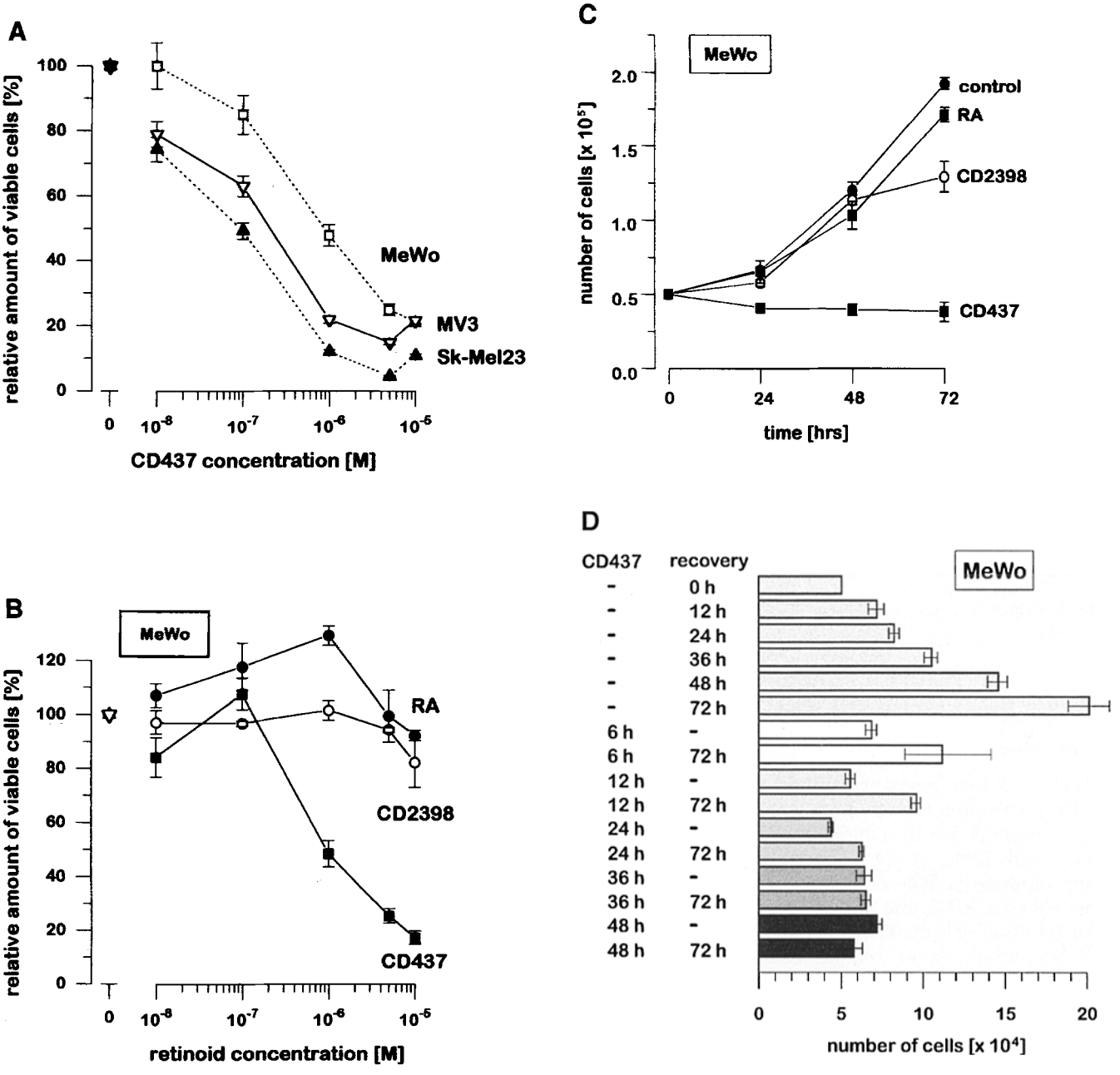

Figure 2. Growth inhibitory effects of retinoids on melanoma cells in vitro. Human melanoma cells (MeWo, MV3, SK-Mel-23) were grown to ~70% confluency, and at time zero, retinoids were added. (*A*) CD437 was added over a concentration range from 10^{-8} to 10^{-5} M, and viable cells were counted after 72 h. (*B*) MeWo cells were cultured in the presence of retinoids (10^{-8} to 10^{-5} M), and viable cells were counted after 48 h. (*C*) MeWo cells were cultured in the presence of retinoids (10^{-5} M each), and viable cells were counted at the times indicated. (*D*) MeWo cells were treated with CD437 (5×10^{-6} M) for the times indicated. After removal of CD437, cells were allowed to recover in retinoid-free medium for 72 h. As vehicle control, DMSO (0.1%) was used in all experiments. Results are expressed as means ± SEM ($n = 5$).

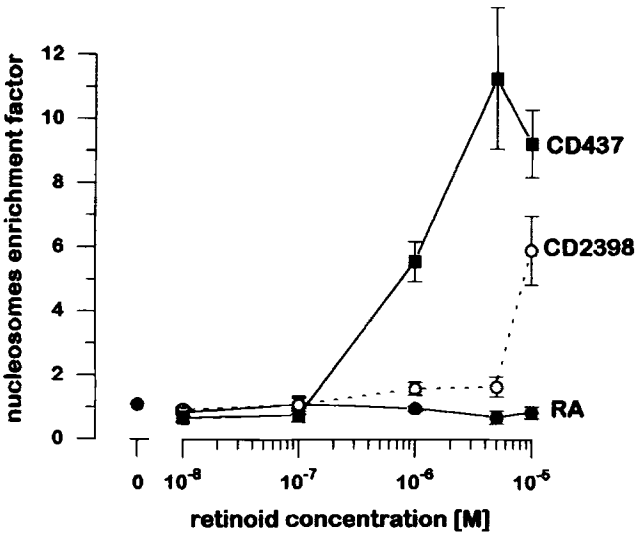

Figure 3. Analysis of DNA fragmentation of human melanoma cells upon treatment with retinoids in vitro. MeWo melanoma cells were treated with CD437, CD2398, and RA over a concentration range from 10^{-8} to 10^{-5} M for 48 h, and control cultures were incubated with DMSO (0.1%) as vehicle control. Histone-associated DNA fragments released from the cytoplasmic fractions of retinoid-treated cells were quantified using a Cell Death Detection ELISA (Boehringer Mannheim Corp.) according to the manufacturer's instructions. Data are given as means ± SEM ($n = 5$).

groups of mice ($n = 8$) were either treated with saline p.o. or were injected intratumorally for 3 wk or were fed with various concentrations of CD437 (10 mg/kg/body weight and 30 mg/kg/body weight). In addition, tumors of a fifth group were injected with CD437 (10 mg/kg/body weight) each day. Mice were visited daily and growing tumors were measured twice weekly with a caliperlike instrument. No signs of major toxicity were observed, as judged by loss of body weight over the treatment period. CD437-treated animals showed, however, some scaling of the skin, which is typical for retinoid treatment.

Results

CD437 Induces Programmed Cell Death In Vitro

CD437 is a new aromatic retinoid that causes selective RAR-γ activation (Bernard et al., 1992) and that has recently been shown to induce apoptosis in human breast cancer cells (Shao et al., 1995). We tested CD437 for its antiproliferative effects on different human melanoma cell lines (MeWo, MV3, and SK-Mel-23) in comparison to the structurally closely related compound CD2398, which does not bind to any known retinoid receptor (Bernard et al., 1992; Schadendorf et al., 1994), and RA (Fig. 1). Treatment for 72 h with CD437 caused a strong dose-dependent growth inhibition in all melanoma cell lines (Fig. 2 *A*). At a concentration of 5×10^{-6} M CD437, only about 5–25% of the cells remained viable after 3 d. The concentrations of CD437 required for 50% growth inhibition (IC_{50}) ranged from 10^{-6} M for MeWo to 10^{-7} M for SK-Mel-23 showing the highest sensitivity. In contrast, proliferation of all cell lines was not affected in the presence of RA (10^{-8}–10^{-5} M) (data not shown). Using MeWo melanoma cells, we studied the effect of retinoids in more detail. In Fig. 2 *B*, the effect of CD437, RA, and CD2398 on MeWo cell growth is shown. CD437 in the range of 10^{-6}–10^{-5} M strongly inhibited cell growth. In contrast, CD2398 had a moderate inhibitory effect only at the highest concentration tested (10^{-5} M), and RA-treated cultures retained normal cell growth. As shown in Fig. 2 *C*, the CD437-induced growth inhibition is time dependent; again, RA did not affect cell proliferation, and treatment with CD2398 (10^{-5} M) resulted in an ~30% reduction in the number of viable cells after prolonged exposure (72 h). At present, the mechanism of the reduced growth inhibitory effect of CD2398 is completely unknown and might be due to interaction of the hydrophobic retinoid with membranes or, since CD2398 does not bind to receptors of the RAR and RXR family (Bernard et al., 1992), activation of a yet unknown receptor. To test whether the effect of CD437 is reversible or irreversible, MeWo cells were treated with CD437 for different time periods (6, 12, 24, 36, and 48 h) followed by a 72-h regeneration period. As outlined in Fig. 2 *D*, the antiproliferative effect could be partially reversed after short-time exposure to CD437 for 6 or 12 h, respectively, with a recovery of around 45–55% of viable cells after the regeneration period compared to controls. In contrast, prolonged CD437 exposure (24–48 h) resulted in an irreversible growth inhibition (Fig. 2 *D*). Furthermore, MeWo cells were sensitive to phorbol ester–induced growth inhibition up to 40% at PMA concentrations ranging from 10–100 ng/ml (data not shown). The same results were obtained with MV3 cells, whereas SK-Mel-23 cells did not respond to PMA treatment under the conditions tested (data not shown).

In comparison to untreated cells, the cell morphology of CD437-treated MeWo cells was changed dramatically (not shown), suggesting the possible induction of programmed cell death. To test this hypothesis, histone-associated DNA fragments (nucleosomes) in the cytoplasmatic fraction of MeWo cells treated with the three compounds over a concentration range from 10^{-8} M to 10^{-5} M were quantified by a cell death detection ELISA. As shown in Fig. 3, RA had no effect and CD2398 caused a 5–7-fold increase of histone-associated DNA fragments only at the highest concentrations tested (10^{-5} M). A dramatic increase in DNA fragmentation (6–12-fold) could be observed in CD437-treated cells over a concentration range from 10^{-6} M to 10^{-5} M. In agreement with these findings, electron microscopy revealed regularly structured cells in untreated (Fig. 4 *A*) or RA-treated preparations, whereas various amounts of apoptotic and necrotic melanoma cells were detectable in CD437-treated groups (Fig. 4, *B* and *C*). After administration of compound CD437, there were numerous apoptotic melanoma cells and a few cells with necrotic alterations, while in the controls, no necrotic and only few if any apoptotic cells were found. Apoptotic MeWo cells were characterized by segregation of compacted chromatin, condensation of cytoplasm and organelles, intact membranes, and fragmentation of the cell (Fig. 4 *B*). In contrast, the rare necrotic cells demonstrated swelling of the cytoplasm and mitochondria, edematous nuclei with clumped chromatin, and dissolution of membranes (Fig. 4 *C*). The relative numbers of apoptotic and necrotic melanoma cells were assessed by a semiquantitative ultrastructural analysis, as summarized in Table I. Whereas necrotic

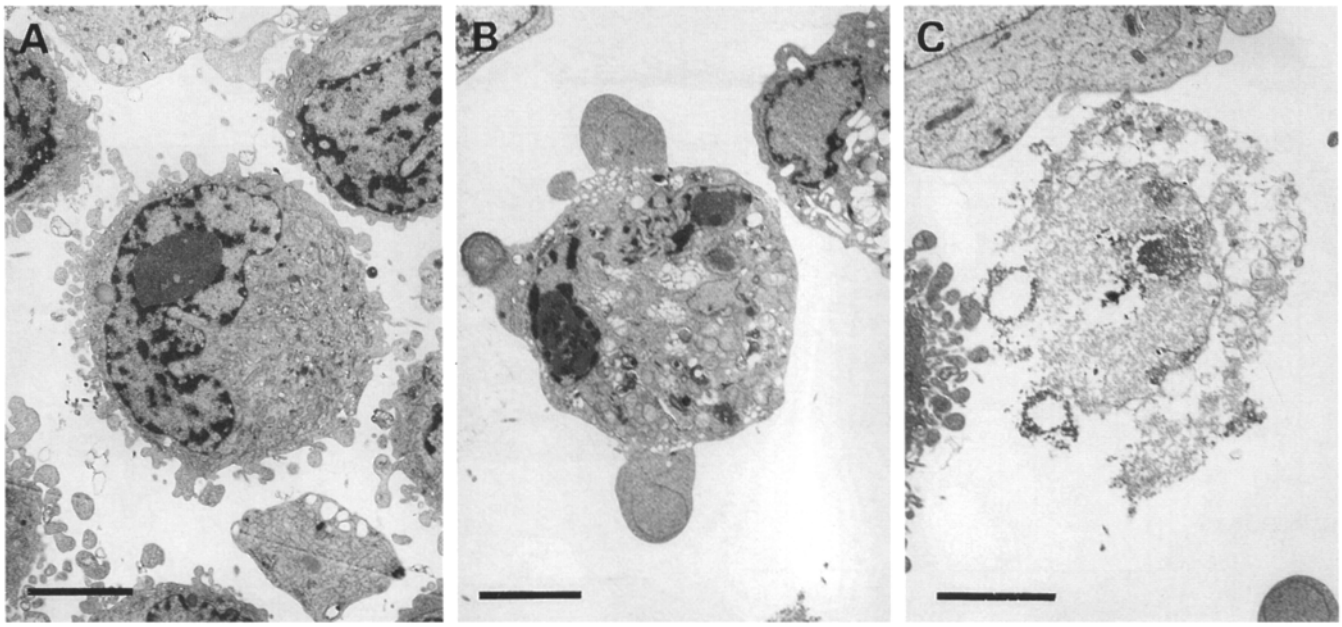

Figure 4. Electron micrographs of untreated and CD437-treated MeWo melanoma cells. (*A*) Regularly structured melanoma cell in DMSO-treated control preparation (4700×). (*B*) Apoptotic melanoma cell with condensed and fragmented nucleus and cytoplasmic blebbing after treatment with CD437 (5×10^{-6} M; 72 h) (6100×). (*C*) Necrotic melanoma cell with toxic alterations such as swelling and dissolution of the nucleus and cytoplasm (5200×). Bars: (*A* and *C*) 3 μm; (*B*) 2 μm.

cells were not detectable in control cells or cells exposed to CD2398 or RA, CD437-treated cells exhibited few cells (up to 10%); however, cells were found to undergo apoptotic death in more than 75% upon exposure. Apoptotic cells were also found in up to 10% of the cells treated with RA or CD2398.

Activation of AP-1 upon Treatment with CD437 In Vitro

Since antiproliferative effects of synthetic retinoids have recently been attributed to an anti–AP-1 activity (Fanjul et al., 1994; J.-Y. Chen et al., 1995; Li et al., 1996), we studied the effects of CD437 on AP-1 activation. Using a vector containing five repeated AP-1 DNA-binding sites cloned in front of the CAT-reporter gene, we observed a 20-fold CAT induction after treatment with CD437 (5×10^{-6} M, 72 h) (Fig. 5). In contrast, MeWo cells treated with CD2398 or RA showed no increase in AP-1–dependent CAT activity. Cotransfection of a full-length *c-fos* expression plasmid led to a 2.5-fold CAT induction (Fig. 5), similarly to treatment of MeWo cells with PMA (100 ng/ml), a well-known activator of AP-1. The latter is in agreement with the observed moderate effect of PMA on growth of MeWo cells.

Furthermore, electrophoretic mobility shift assays using an oligonucleotide containing an AP-1 DNA-binding motif as a probe confirmed upregulation of the AP-1 complex after exposure to CD437 (Fig. 6 *A*). This protein–DNA interaction was specifically competed for by an excess of cold probe. In contrast, no such dramatic changes were observed in the presence of CD2398 and RA (5×10^{-5} M, each) (Fig. 6 *A*). Nuclear extracts of MeWo cells treated with these compounds showed only weak binding of the AP-1 sites. Since DNA binding of other unrelated transcription factors such as Nil-2 (not expressed) or Oct-1 (highly expressed) was not changed by the retinoid treatment (Fig. 6 *B*), it can be assumed that CD437 specifically activates DNA binding of the AP-1 complex. AP-1 activation became visible as early as 3 h after exposure to CD437 and remained high for up to 48 h (Fig. 6 *C*). Cells treated with RA or CD2398 also showed a slight increase in AP-1 DNA-binding activity (Fig. 6 *C*); however, AP-1 complexes built upon RA or CD2398 treatment were not transcriptionally active as suggested by the activation of the CAT gene (Fig. 5) and the lack of growth inhibition (Fig. 2). The AP-1 complex upon treatment with CD437 is formed by multiple members of the Fos and Jun protein family (Fig. 6 *D*). Dissection of the AP-1 complex by gel retardation assays using nuclear extracts of melanoma cells and monospecific antibodies in a supershift assay revealed that

Table I. Semiquantitative Ultrastructural Evaluation of Apoptotic and Necrotic MeWo Melanoma Cells Treated with either CD437, CD2398, or RA (5×10^{-6} M) for 72 h, Compared to DMSO (0.1%) Treatment and Untreated MeWo Cells (control).

Incubation	Apoptosis	Necrosis
Control	+/−	∅
DMSO	+/−	∅
CD437	+++	+
CD2398	+	∅
RA	+	∅

∅, no cell with altered morphology.
+/−, single cells.
+, few cells.
++, moderate number of cells.
+++, numerous cells.

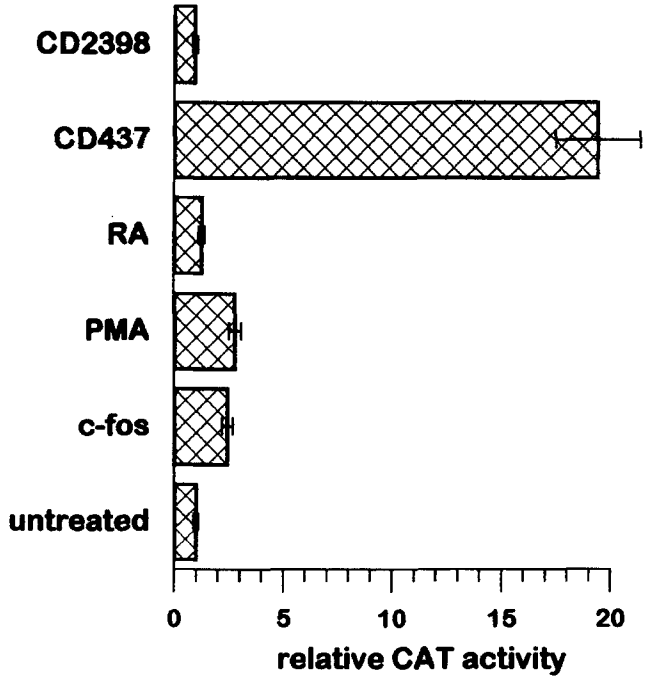

Figure 5. AP-1-dependent CAT expression is increased by treatment with CD437. Retinoid treatment (*RA*, *CD437*, and *CD2398*) at a concentration of 5×10^6 M was started 24 h before transfection. For control, a full-length *c-fos* expression plasmid was cotransfected in a fourth set of experiments. CAT activities (β-galactosidase corrected) shown as bars were calculated as relative activities by arbitrarily setting the activity of untreated but control-transfected cells at 1. Results are expressed as means ± SEM ($n = 4$).

c-Fos and the Jun family members c-Jun and JunD are involved in AP-1 complex formation in CD437-treated cells (Fig. 6 *D*, *upper*). In contrast with cell extracts prepared from RA-treated cells, weak signals were obtained after coincubation with antibodies against Fra-1, c-Jun, and JunD (Fig. 6 *D*, *lower*). Under both conditions, ATF and CREB proteins do not participate in the AP-1 complex.

Northern blot analysis confirmed that mRNA expression of the protooncogenes *c-fos* and *c-jun*, encoding members of the AP-1 complex, was concurrently upregulated after a 48- to 72-h exposure to CD437 (Fig. 7), whereas CD2398 and RA had no such effect. Expression of *p53* (Fig. 7), which encodes a negative growth regulator and expression of the antiapoptotic gene *bcl-2* (not shown), was not altered in MeWo cells upon treatment with any of the compounds (Fig. 7). Taken together, gel shift assays demonstrated a CD437-induced increased AP-1 DNA-binding activity already 3 h after exposure to the retinoid (Fig. 6), suggesting that posttranslational activation of the transcription factor is a primary event during CD437-induced apoptosis. As shown by Northern blot analysis (Fig. 7), prolonged exposure to CD437 also stimulates expression of genes encoding components of the AP-1 complex.

Growth Inhibition of Human Melanoma Xenograft in Nude Mice with CD437 Treatment

To confirm the significance of the results obtained in vitro

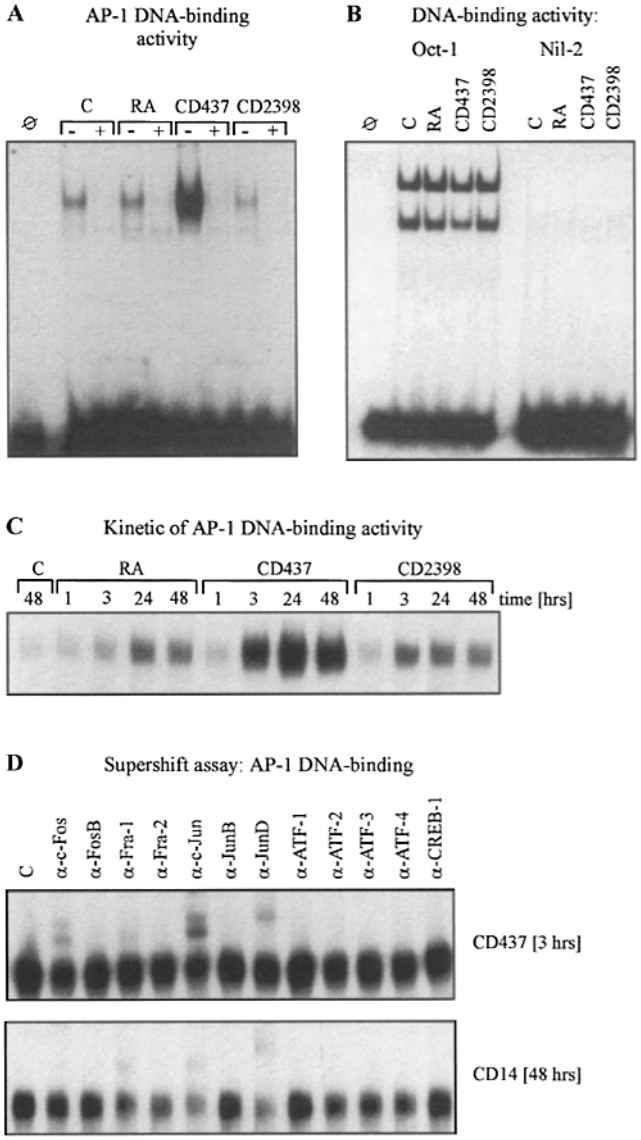

Figure 6. Treatment of MeWo melanoma cells with CD437 upregulates the AP-1 complex. Total cell extracts prepared from unstimulated (control, *C*) or retinoid-stimulated cells (*RA*, *CD437*, and *CD2398*; 10^{-5} M, 24 h) were subjected to electrophoretic mobility shift assays. In *A*, extracts were incubated with a ^{32}P-labeled AP-1 DNA-binding sequence in the absence (−) or presence (+) of unlabeled competitor (100-fold molar excess), and in *B*, oligonucleotides containing either an Oct-1–binding motif (CGA ATG CAA ATC ACT AGA) or a Nil-2 DNA-binding sequence (CAG ACA GGT AAA GAT CAG ACA GGT AAA GA) were used. (*C*) After addition of retinoids (5×10^{-5} M each), cell extracts were prepared at the times indicated, incubated with an oligomer containing an AP-1 DNA-binding site, and analyzed in comparison to the untreated control (*C*). (*D*) Supershift mobility assays of MeWo cells treated with CD437 (3 h, 5×10^{-6} M, *upper*) or RA (48 h, 5×10^{-6} M, *lower*). Cell extracts and antibodies monospecific for various possible components of the AP-1 complex (Fos and Jun proteins, ATF, CREB) were coincubated as described in Materials and Methods. The original data were digitally processed using a Hewlett-Packard Scan Jet IIcx.

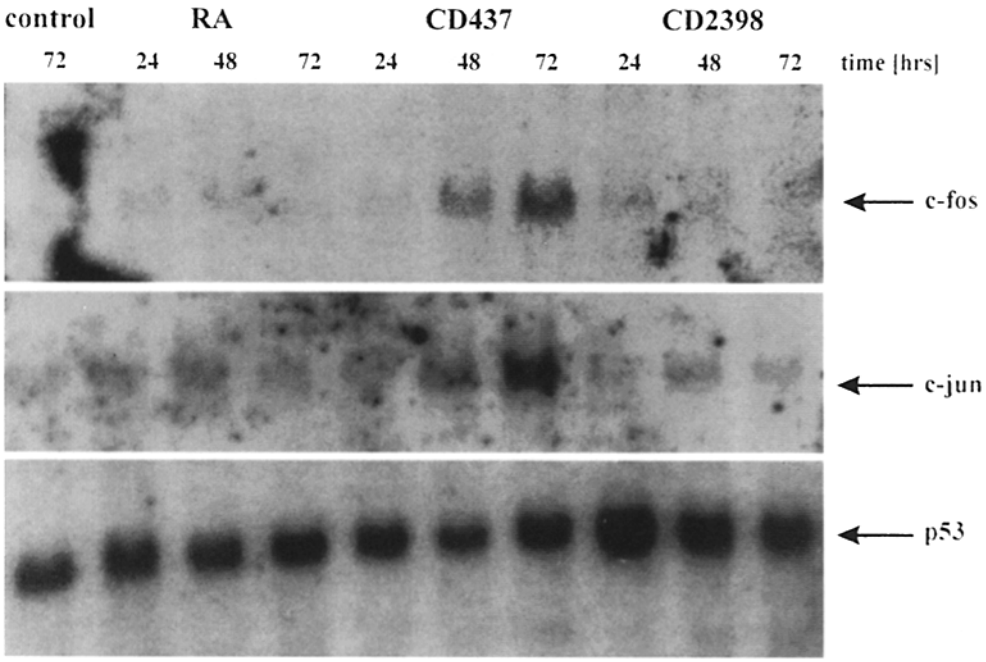

Figure 7. MeWo melanoma cells express *c-fos* and *c-jun* after CD437 treatment. RNA was isolated from cultures at intervals as indicated after exposure to RA, CD437, and CD2398 (10^{-5} M each). 10–12 μg of total RNA was analyzed on 1% agarose-formaldehyde gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled *c-fos*, *c-jun*, and *p53* probes. The original data from Northern blots were digitally processed using a Hewlett-Packard Scan Jet IIcx.

for the in vivo situation, the human melanoma cell line MeWo was established to grow in nude mice. Preexisting tumors of 0.5 cm in diameter were treated for 3 wk with CD437 (10 and 30 mg/kg) in comparison to a vehicle-treated group of control animals. Tumors in CD437-treated mice stopped growing, an effect that became already statistically significant ($P < 0.01$) at day 13, 3 d after first administration of CD437, and was maintained for more than 3 wk after discontinuation of treatment. No difference was observed depending on the route of administration (intratumorally or orally) (Fig. 8). Intratumoral retinoid injections were chosen as one treatment arm, since no pharmacologic information regarding gastrointestinal resorption of CD437 upon oral application were available. Further histologic analysis demonstrated marked *c-fos* mRNA levels at the tumor–stroma edge in CD437-treated tumors, as shown by in situ hybridization (Fig. 9 *E*). TdT-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (TUNEL) staining, a procedure that labels free DNA terminal regions and is considered to be an indicator of cells undergoing apoptosis in situ (Fig. 9 *D*), showed a staining pattern that colocalized to the area of high *c-fos* expression in the CD437-treated tumors (Fig. 9 *E*). Analysis of saline-treated control xenografts demonstrated no comparable *c-fos* expression or TUNEL staining (not shown).

Discussion

In the present study, we have identified the synthetic retinoid CD437 as being able to rapidly induce increased AP-1 DNA-binding resulting in an upregulation of the transcriptional activity of AP-1 and the induction of apoptotic cell death in human melanoma cells. The observed antiproliferative effect of CD437 is in agreement with the findings of others that RA and several synthetic retinoids may function as inductors of apoptosis in various cell lines (Alles and Sulik, 1990; Piacentini et al., 1991; Horie and Broxmeyer, 1995; Shao et al., 1995). Antiproliferative effects of retinoids are presently believed to be mediated by an inhibition of AP-1 activity as a result of the competition for DNA-binding sites and of protein–protein interactions (Schüle et al., 1990, 1991; Yang-Yen et al., 1991; Auwerx and Sassone-Corsi, 1992; Pfahl, 1993; Fanjul et al., 1994; J.-Y. Chen et al., 1995; Li et al., 1996). The present report describes for the first time a retinoid-induced programmed cell death that is accompanied by an activation and upregulation of AP-1. It remains to be shown whether other syn-

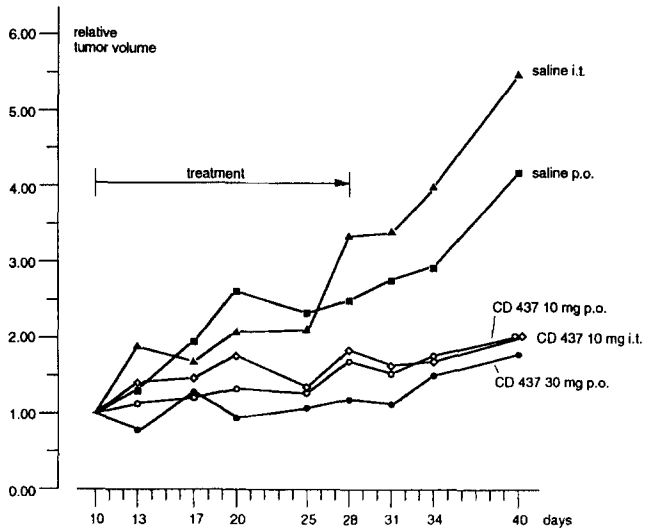

Figure 8. Growth kinetics of MeWo melanoma cells xenografted onto nude mice treated with CD437 or saline. Mice with preexisting MeWo tumors (groups of eight mice each) were fed for 3 wk with various concentrations of CD437 (10 and 30 mg/kg) or saline. In addition, one group was injected with CD437 (10 mg/kg) or saline directly into the tumor (i.t.) each day. Tumor volume differences were statistically significant ($P < 0.01$) starting at day 13.

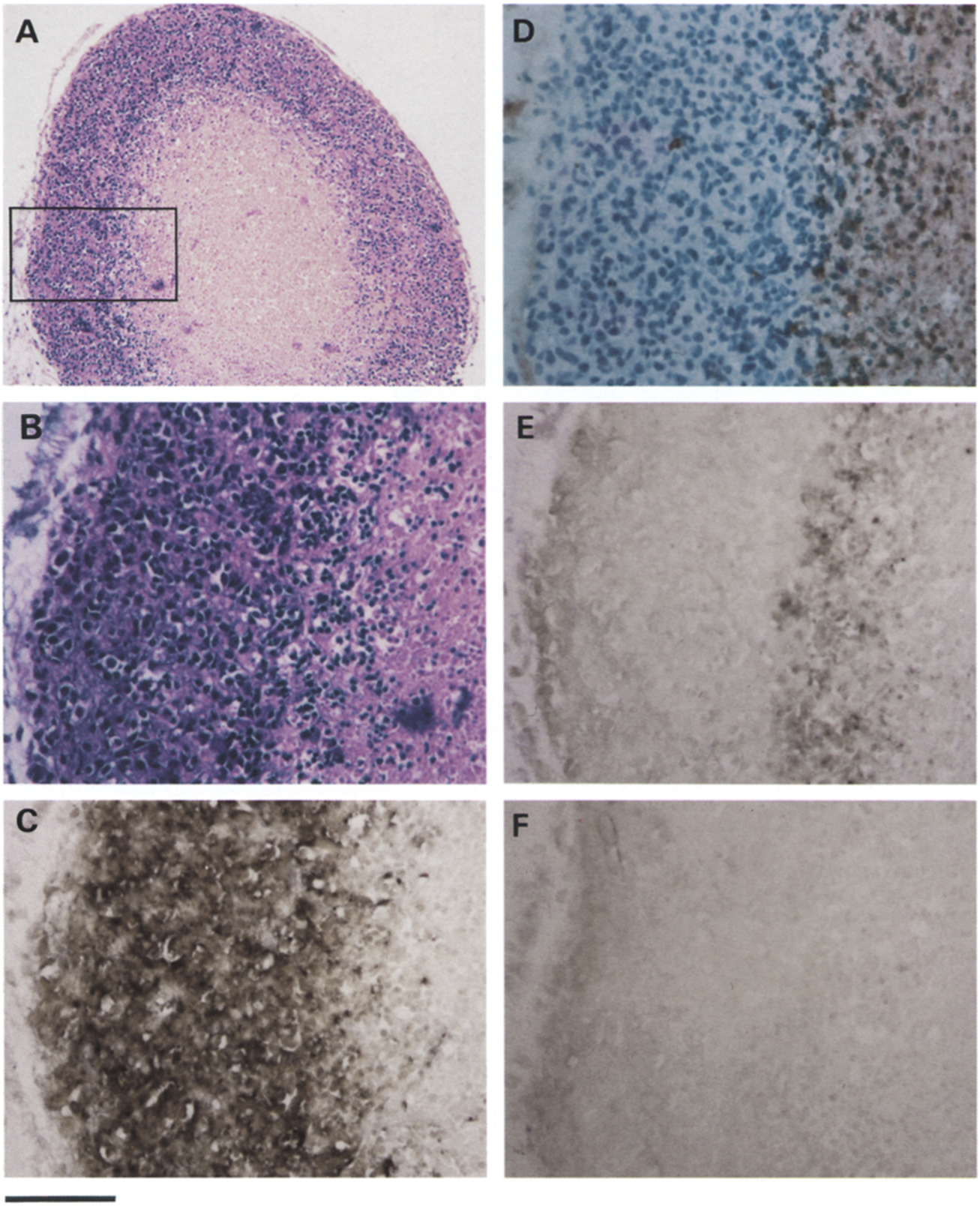

Figure 9. Evaluation of melanoma xenograft treated with CD437 (10 mg/kg body weight) at day 28. (*A*) H&E stain of CD437-treated cutaneous tumor (125×). (*B–F*) Higher magnification (500×). (*B*) H&E stain of the rectangular area marked in *A*. (*C*) In situ hybridization of β-actin to control RNA integrity. (*D*) TUNEL staining of CD437-treated melanoma using the ApopTag in situ apoptosis detection kit (Oncor Inc., Gaithersburg, MD). (*E*) In situ hybridization of *c-fos* in antisense. (*F*) In situ hybridization of *c-fos* in sense orientation. Bar: (*A*) 0.3 mm; (*B–F*) 10 μm.

thetic retinoids with comparable activities exist, suggesting thus a new class of retinoids.

Evidence for the occurrence of apoptotic cell death in human melanoma cells is provided by the demonstration of DNA fragmentation and by electron microscopical analysis of cell morphology. Only very recently, Shao and coworkers have shown that CD437 also induces apoptotic cell death in human breast cancer cells (Shao et al., 1995). The present study provides additional data regarding the molecular mechanisms involved in this process. Thus, melanoma cell death upon treatment with CD437 in vitro was preceded by a substantial upregulation of the AP-1 complex (3 h after exposure) as demonstrated by electrophoretic mobility shift assays (Fig. 6). Neither unrelated transcription factors such as Oct-1 or Nil-2 nor changes in the expression of *p53* or *bcl-2*, which encode proteins known for their critical involvement in regulating apoptotic processes in various cell types, were modulated by CD437, suggesting that cell death was independent of these factors and that AP-1 activation upon CD437 treatment is a specific event. The data presented show that AP-1 activity is regulated at both a posttranslational and transcriptional level. Although it is not known which of the posttranslational mechanisms described (for review see Piechaczyk and Blanchard, 1994) are involved in upregulating AP-1 activity, modification of Fos and/or Jun protein activity seems to be an early event during CD437-induced programmed cell death. In contrast, transcriptional activation of the corresponding genes could be detected by Northern blot analysis only after prolonged treatment with CD437 (24–48 h).

An increase of *c-fos* and *c-jun* expression preceding programmed cell death has been described in diverse systems such as growth factor deprivation of lymphoid cells (Colotta et al., 1992), hormonal deprivation of rat prostate cells (Buttyan et al., 1988), involution of the lactating mammary gland (Marti et al., 1994), or the massive upregulation of the AP-1 complex in UV-damaged cells undergoing apoptosis (Devary et al., 1991; Engelberg et al., 1994; Schreiber et al., 1995). Furthermore, neuronal apoptosis, which has recently been examined in great detail (Smeyne et al., 1993; Estus et al., 1994; S.C. Chen et al., 1995), is characterized by a well-defined change in protooncogene expression with an increase of *c-fos* and *c-jun* expression, whereas expression of other genes encoding AP-1 components such as *fra-1* are downregulated (Estus et al., 1994). At present, it seems safe to conclude from studies in transgenic mice that neuronal death as well as programmed cell death in other mesenchymal tissues such as chondrocytes and bone is preceded by a continuous *c-fos* expression (Smeyne et al., 1993; Estus et al., 1994; S.C. Chen et al., 1995). Since melanoma cells are also derived from neuroectoderm, it is reasonable to suggest that the cascade of gene expression leading to apoptotic cell death might be similar. Presently, it is not entirely clear whether the whole cascade known to occur during neuronal cell death also takes place during CD437-induced melanoma cell death.

In CD437-treated cells studied here, c-Fos, c-Jun, and JunD are involved in AP-1 complex formation as shown by band shift analysis (Fig. 6 *D*). In contrast, in cells exposed to RA, c-Jun, JunD, and Fra-1 were found to be part of AP-1, whereas c-Fos is missing. It is well known that by heterodimerization of Fos (c-Fos, FosB, and Fra-1) and Jun (c-Jun, JunB, and JunD) proteins and homodimerization of Jun proteins, >20 different AP-1 complexes can be formed. Studies with neuronal cells revealed that these complexes represent at least four groups with transactivational abilities ranging from high to very low (for review see Hughes and Dragunow, 1995). c-Fos/c-Jun heterodimers belong to the group exhibiting the highest DNA-binding and transactivational potential, whereas AP-1 complexes built up by Fra-1 and different Jun proteins were much less active (Hughes and Dragunow, 1995). In agreement with these earlier studies, we found that the highest AP-1–dependent CAT activity in cells treated with CD437 (Fig. 5) was most likely due to the formation of c-Fos/c-Jun heterodimers. In contrast, AP-1 constituents detected in RA-treated cells (Fig. 6 *D*) only allow formation of AP-1 complexes with medium to very low activity (Hughes and Dragunow, 1995). The finding that treatment of MeWo cells with different retinoids induces distinct AP-1 complexes is supported by our results of Northern blot analysis (Fig. 7). Induction of *c-fos* mRNA could only be detected in CD437-treated cells.

While the components of the cellular signal transduction cascade responsible for the induction of apoptosis are presently unknown, it is very likely that alterations in gene expression resulting from changes in the composition of AP-1 are instrumental. Novel biochemical evidence indicates that the composition of AP-1 complexes and additional interacting proteins may predict subsequent gene expression and facilitate the switching between proliferation, terminal differentiation, and apoptosis (S.C. Chen et al., 1995; Hagmeyer et al., 1995; Schreiber et al., 1995; Kamei et al., 1996).

Taken together, our data suggest that apoptosis in human melanoma cells may be similar to neuronal cell death and programmed cell death in other mesenchymal tissues, with an upregulation of *c-fos* and *c-jun* expression preceding cell death. Furthermore, we describe CD437 as a member of a possible new class of synthetic retinoids that is capable of initiating the cell death program in human melanoma cells in vitro and that inhibits growth of xenografts in nude mice. Pharmacological induction of apoptosis in neoplastic cells is an aim of cancer therapy (Martin and Green, 1994). Consequently, CD437 might be the first synthetic retinoid of a new generation of therapeutic agents inducing apoptosis via AP-1 activation and might therefore open up new opportunities for the treatment of metastatic melanoma.

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