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Chronic Treatment of Human Fibroblasts Cultures with Diacylglycerol Induces Down-Regulation of p53 Functional Activity

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We found that many spontaneous human tumors exhibit increased levels of endocellular diacylglycerol (DAG) which is synthesized *de novo* as a byproduct of glycolysis. It has been shown that DAG mimics phorbol esters as a full tumor promoter in mouse skin carcinogenesis. A short term DAG treatment activates protein kinase C (PKC), while a long term "chronic" treatment down-regulates PKC. We show here that chronic treatment of human fibroblast with DAG induces p53 down-regulation and inhibition of p53 functional activity, and protection from UV-induced apoptosis. As PKC phosphorylation is necessary for p53 functional activity, we propose that chronic DAG treatment mimics the same event occurring *in vivo* for the effect of glycolysis in tumor progression.

The probability that exogenous tumor promoters play any role in spontaneous human cancers is very low. On the contrary, DAG is endogenously produced, either by hydrolysis of membrane phospholipids, or as an intermediate product of glycolysis. The latter is a very important source of DAG (1,2) and the accumulation of DAG in the tumoral cell membrane is a clear cut finding (3,4,5,6). Dominant oncogenes involved in cancer initiation like *src* or *ras* simulate glycolysis and consequently DAG accumulation with a mechanism that has been recently clarified (7): the key glycolysis regulator, fructose 2-6 bisphosphate, is accumulated when transformation occurs and the expression of its regulatory enzymes is under the control of E2F, one of the main cell cycle regulators. A good candidate to connect DAG with the cell cycle regulation is represented by p53, a molecule of basic importance for genome integrity and that is regulated by various kinases, where PKC plays a pivotal role (8). Our working hypothesis is that PKC down-regulation, following DAG accumulation, down-regulates p53 functional activity, leading to tumor progression by increased genome instability, due to lack of repair and loss of regulation of ploidity.

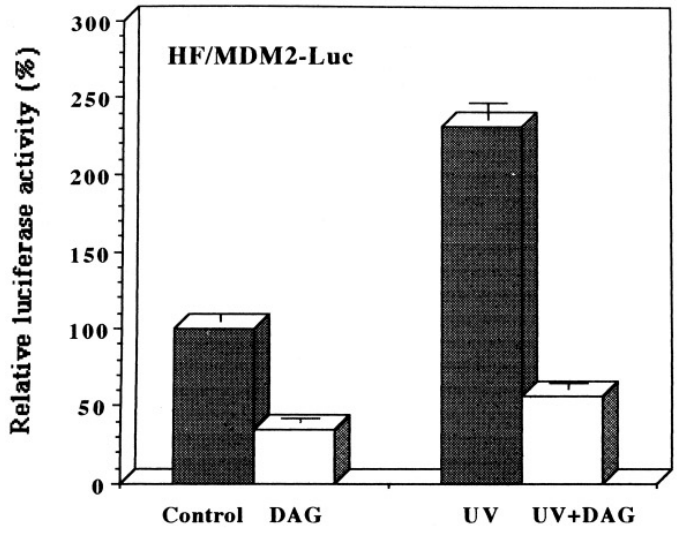

FIG. 1. DAG modulation of p53 activity as a transcription factor: Human fibroblasts were transiently co-transfected with 4μg/plate of pGL2-hmdm2-luc and pEGFP-N constructs by a highly efficient calcium phosphate transfection protocol (12). The plasmid pGL2-hmdm2 was a generous gift of Dr. M. Oren; pEGFP-N was purchased from Clontech (CA, USA). The transfection efficiency was 30–35%. 24 hr following transfection, cells, treated or not with 5μM DAG for 48 hr, were incubated in DMEM supplemented with 1% FCS. Then, cells were irradiated or not with 250J/m^2, using a germicide UV-C lamp (OSRAM, Germany). Twelve hr after irradiation, an aliquot of cells from each sample was assayed for luciferase activity, using a PROMEGA's luciferase assay kit (PROMEGA, U.S.A.). Another aliquot of cells was used to measure the transfection efficiency, as determined by assessing the pEGPDF-N-dependent fluorescence in a Beckton Dickinson flow cytometer, using a Lysis II software (Hewlett Packard, U.S.A.). The results have been normalized for both GFP fluorescence and protein content, using the BCA protein assay reagent (PIERCE, U.S.A.). The error bars indicate the standard error of mean of three independent transfections and treatments.

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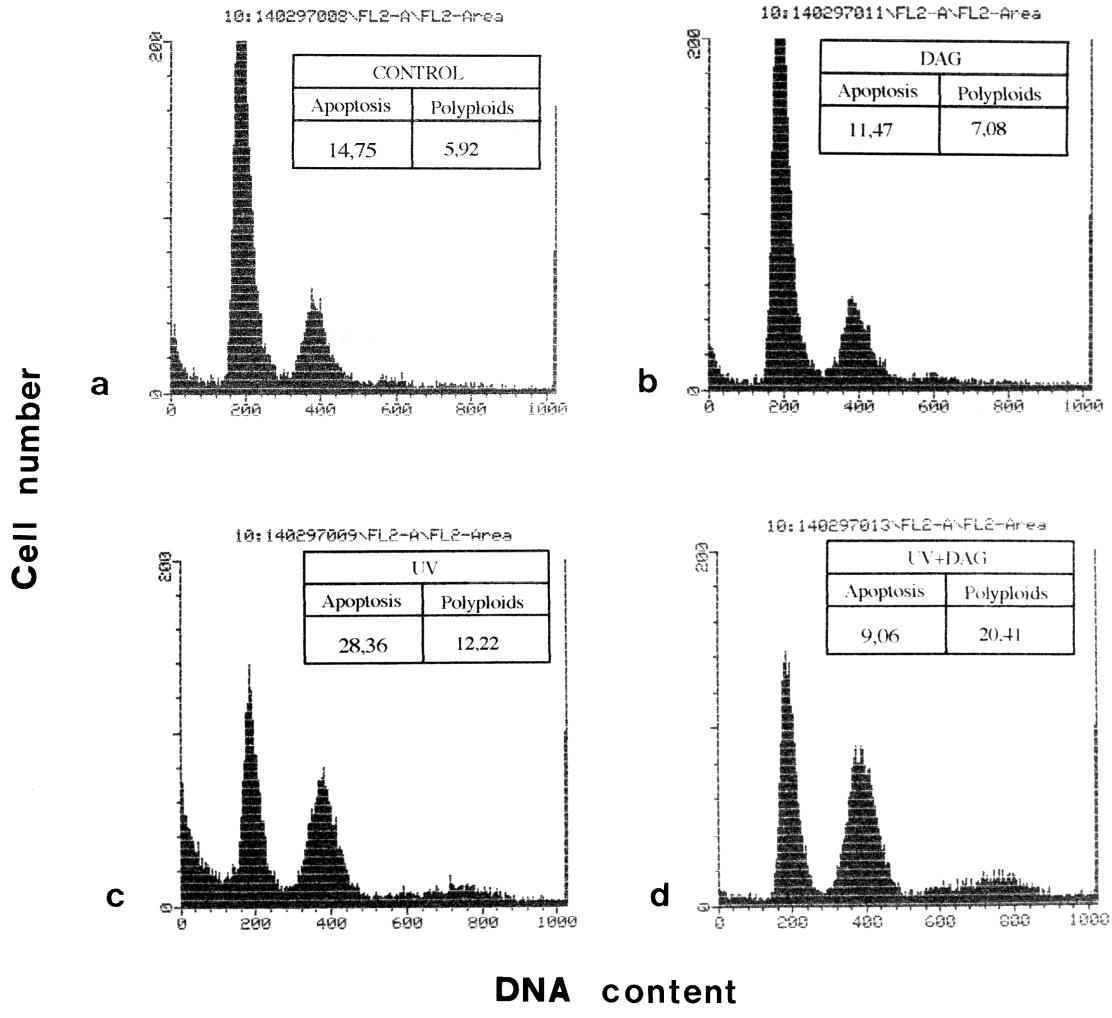

FIG. 2. Protection from apoptosis and induction of polyploidity in DAG chronically-treated UV-irradiated human fibroblasts (HF): Flow cytometric analysis. Primary HF were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS. When subconfluent, cells were washed twice with DMEM, incubated in DMEM 1% FCS, and pretreated for two days with (**b** and **d**) or without (**a** and **c**) 5 μM DAG. All cultures were then washed with a phosphate buffer solution (PBS) and incubated in PBS. After **c** and **d** cultures irradiation with an UV-C lamp (250J/m^2), all cultures were again incubated in the above mentioned conditions for two more days. Finally, cultures were harvested, washed twice with PBS and incubated for 1hr in 0,1% tri-natriumcitrate solution containing 50 μg/ml propidium iodide and 0,1% triton X-100. DNA fluorescence was revealed by flow cytometric analysis with a Becton Dickinson apparatus (Becton Dickinson, France). Data were elaborated by the software program Lysis II (Hewlett Packard, U.S.A.).

MATERIALS AND METHODS

Detailed explanations are reported in the legends to the figures.

RESULTS AND DISCUSSION

First of all we transfected human fibroblasts with a luciferase reporter gene, driven by a p53 specific binding site, derived from the MDM-2 gene (9). Fig.1 shows that, as compared to control cells, the luciferase response was strongly down-regulated in cells chronically treated with DAG. Furthermore, when cells are chronically treated with DAG, an equally evident repression of UV-C induced high luciferase activity occurs, indicating that p53 transactivation activity is prevented. The higher level of luciferase activity of untreated cells, as compared with the DAG treatment, can be explained with the p53 activation that occurs when DNA enters the cell following transfection. Figure 2 shows the flow cytometric analysis of control [untreated (a), DAG treated (b)] and UV-irradiated [untreated (c), DAG treated (d)] human fibroblast cultures: it is evident that a massive apoptosis (first peak on the left) and a very scarce polyploidity (last peak) occurs, following UV irradiation in the absence of DAG; in its presence, on the

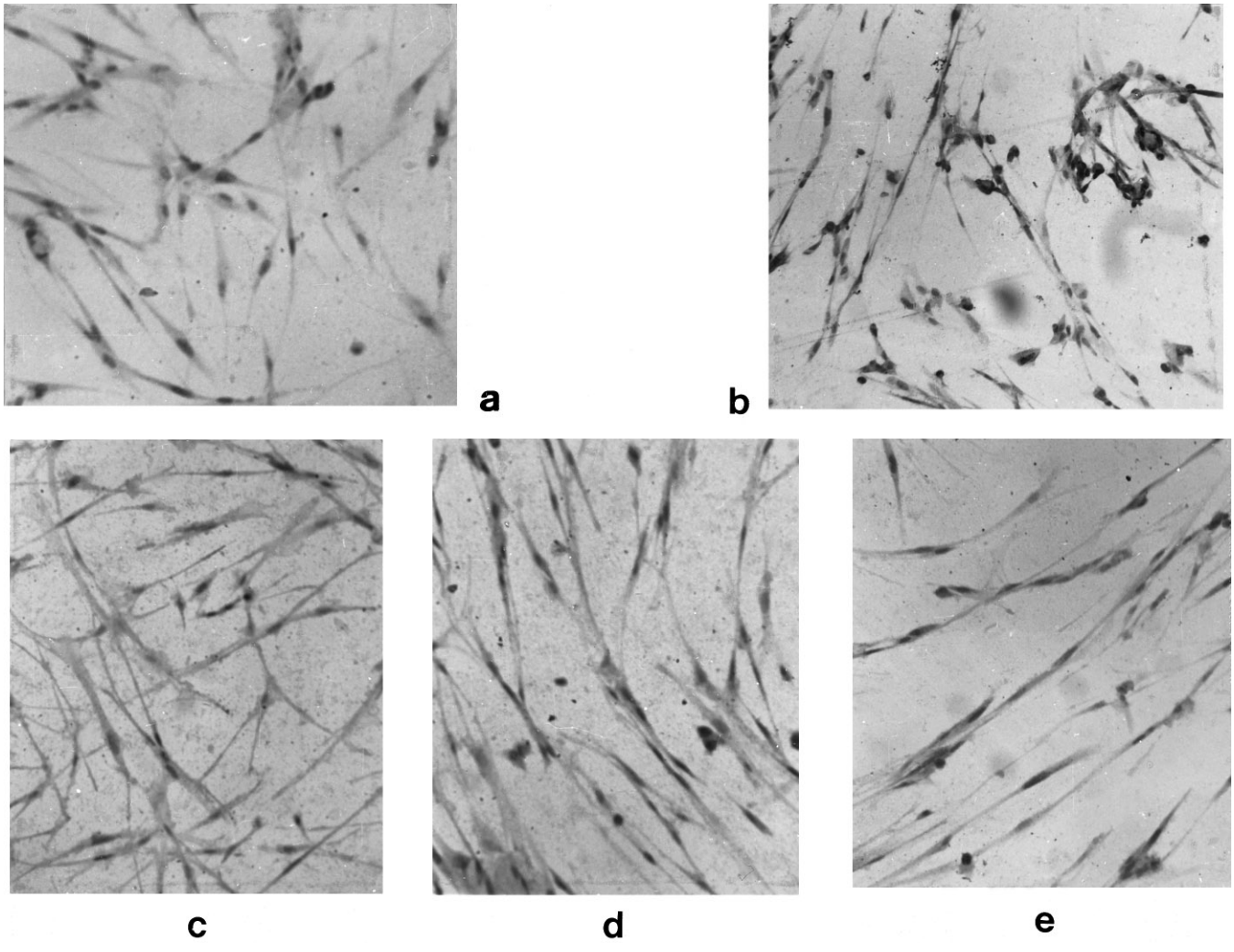

FIG. 3. Morphological features (×16) of protection from apoptosis in DAG chronically-treated/UV-irradiated HF and synergy with the PKC inhibitor RO-31-8220 (Calbiochem, CA, U.S.A.): Cells were cultivated, incubated and irradiated as reported in Figure 2; RO-31-8220-treated cells were incubated in the presence of 1 μM PKC inhibitor, during all the experiment. **a:** Untreated cells; **b:** UV-irradiated cells; **c:** UV-irradiated cells + RO-31-2220; **d:** UV-irradiated cells + DAG chronic treatment; **e:** UV-irradiated cells + DAG chronic treatment + RO-31-8220. Cells were fixed with methanol and colored by May-Grünwald-Giemsa staining.

contrary, the apoptosis rate strongly decreases and polyploidity increases. Thus, an impaired p53 activity induced by chronic DAG is inefficient, in both inducing apoptosis of UV damaged cells and protecting genome integrity at chromosomal level. Fig 3 shows the same phenomenon morphologically, with a clear cut protection of chronic DAG treatment of UV irradiated fibroblasts. Furthermore, the addition of the specific PKC inhibitor RO-31-8220 (c) exhibits a protective effect, which is even synergic with DAG (e). Fig.4 shows an immunoblotting experiment performed with the antibody anti p-53 DO-1. UV-irradiated cells show a dramatic increase in p53 level (lane 3), while chronic DAG treatment decreases the DO-1 revealed signal (lane 4). The same effect is true for UV-irradiated cells in the presence of the PKC inhibitor RO-31-8220 (lane 7), while lane 8 exhibits the cooperative effect between DAG and the same inhibitor. All these experimental observations, taken together, indicate a linkage between DAG, PKC and p53. The most important results reported herein are that chronic DAG treatment not only reduces the total p53 amount in cells subjected to UV-irradiation, but also impairs the functional transcriptional activity of p53, evaluated in terms of luciferase activity of the reporter gene. It is of interest that cells expressing aberrant p53 or treated by tumor promoters are characterized by similar genome alterations at chro-

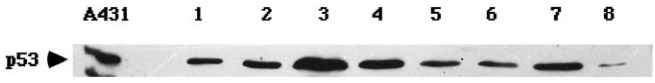

FIG. 4. Down-regulation of p53 following chronic DAG treatment in UV-irradiated HF and synergy with the PKC inhibitor RO-31-8220: Western blotting with DO-1 monoclonal antibody (Santa Cruz Biotechnology, U.S.A.). Cells were cultivated, incubated and irradiated as reported in Figure 3. For each lane 100 μg of total lysate was subjected to SDS-PAGE in a 12% gel. The p53-overexpressing A431 carcinoma cell line (20 μg of total lysate) was used as the p53 marker. Lane **1:** Control, untreated cells; Lane **2:** DAG chronic treatment; Lane **3:** UV-irradiated cells; Lane **4:** UV-irradiated cells + DAG chronic treatment; Lane **5:** RO-31-8220; Lane **6:** RO-31-8220 + DAG chronic treatment; Lane **7:** UV-irradiated cells + RO-31-2220; Lane **8:** UV-irradiated cells + DAG chronic treatment + RO-31-8220.

mosomal level (10) and that DAG perfectly mimics the effect of phorbol esters in mouse skin carcinogenesis, exhibiting all the properties of a full tumor promoter. In conclusion, after having shown that dominant oncogenes induced a sustained neosynthesis of DAG—as demonstrated by the preferential labeling with palmitic, rather than arachidonic acid (11)—and that PKC is down-regulated in the same cells (3), here we show that glycolysis increase in tumors has not only an energetic significance but is rather an indirect mechanism for genome derangement and tumor progression, through neosynthesis and cellular accumulation of DAG and consequent PKC-dependent down-regulation of p53.

ACKNOWLEDGMENTS

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