Induction, not associated with host-cell re-activation of damaged plasmid DNA, of damaged-DNA-recognition proteins by retinoic acid and dibutyryl cyclic AMP in mammalian cells

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Our previous studies [Chao (1992) *Biochem. J.* **282**, 203–207; C. C.-K. Chao, unpublished work] has suggested a correlation between the levels of constitutive UV-damaged-DNA-recognition proteins (UVDRP) and cellular DNA repair in different cell types. In the present study, UVDRP were induced in F9 and NIH3T3 cells by 0.1 μ M retinoic acid (RA) and 1 mM dibutyryl cyclic AMP (dbcAMP), which is sufficient to induce differentiation in murine F9 stem cells. The induction of UVDRP in F9 and NIH3T3 cells was optimized after 6 and 2 days in-

cubation with RA/dbcAMP respectively. Since NIH3T3 cells were not induced to differentiate by RA/dbcAMP, the up-regulation of the UVDRP in mammalian cells would thus seem not to be mediated directly by differentiation. Using a plasmid re-activation assay to estimate DNA repair, we did not find a correlation between DNA repair and UVDRP in RA/dbcAMP-treated cells. The results suggest that UVDRP may have a function other than, or in addition to, its role in DNA repair.

INTRODUCTION

UV irradiation causes the production of cyclobutane-type pyrimidine dimers, (6-4)photoproducts and other DNA base adducts in cells. In response to DNA damage, cells have evolved various types of repair systems. In mammalian cells, most of UV damage is repaired by nucleotide excision [1]. The UV-induced excision-repair is conducted by multiple enzymes and accessory proteins which, for effective repair to occur, presumably must have access to the damaged DNA. Such enzymes and accessory proteins can therefore possibly be identified by damaged DNA-binding activity. We and others have identified a binding activity towards UV-damaged DNA, termed 'UVDRP' (UV-damaged-DNA-recognition proteins) [2] or XPE factor [3], which is overexpressed in cells resistant to DNA-damaging agents. This agrees with the findings that nuclear extracts isolated from cells with DNA-repair deficiency have a lowered ability or even fail to interact with damaged DNA [4–5].

It is known that nucleotide excision-repair also involves the removal of the major DNA adducts induced by cisplatin (an anticancer drug) in bacterial [6–8] and mammalian cells [9–10]. Thus cells probably process cisplatin-induced DNA adducts and UV-induced DNA adducts in a similar way. We have previously also found that HeLa cells resistant to cisplatin express enhanced binding activity for cisplatin-induced DNA adducts [2,11]. In addition, these resistant cells show an increase in cellular capacity of re-activating damaged plasmid DNA, suggesting a possible correlation between the level of cisplatin-damaged-DNA-recognition proteins (DRP) in cells with DNA repair. Additionally, the same cell line also exhibits increased UVDRP and enhanced DNA repair of UV damage [2]. However, failures in detecting an increase of cisplatin-DNA recognition activity in cisplatin-resistant cells have been reported [12], suggesting complexities in

the regulation of damage-recognition activity in mammalian cells. It is therefore important to investigate the role of DRP in DNA repair. It has been demonstrated that UVDRP are induced in primate cells by UV irradiation [13] and in human cells by cisplatin [13a]. Interestingly, we have recently demonstrated that, in mouse F9 stem cells, UVDRP is inducible by retinoic acid and dibutyryl cyclic AMP (RA/dbcAMP), which are effective in causing differentiation of the cells but do not directly elicit DNA damage [14]. In the present study we further investigate DNA repair and UVDRP in RA/dbcAMP-treated cells. The results do not show a correlation between DNA repair and UVDRP.

EXPERIMENTAL

Cell lines and induction conditions

Mouse F9 embryonic carcinoma stem cells, NIH3T3, XP complementation group A (XPA) fibroblasts (XP12RO from Dr. Phil Hanawalt, Department of Biology, Stanford University, Stanford, CA, U.S.A.) and other cell lines (monkey Cos-7, human hepatocellular carcinoma Hep 3B, Burkitt's lymphoma-Namalwa, and WI-38-VA13) were maintained according to the supplier's (American Type Culture Collection) specifications. HeLa S3 and a cisplatin-resistant variant HeLa-R were grown as previously described [15]. F9 cells were cultured on 0.1% gelatin-coated tissue-culture dishes in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD, U.S.A.), containing 10% (v/v) heat-inactivated fetal-calf serum, and incubated at 37 °C in a humidified atmosphere of CO₂/air (1:19). Inhibition of cell growth was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ('MTT') colorimetric method as previously described [16]. For the RA/dbcAMP induction, cells were treated with 0.1 μ M all-*trans*-RA and 1 mM dbcAMP (Sigma Chemical Co., St. Louis, MO,

Abbreviations used: dbcAMP, dibutyryl cAMP; UVDRP, UV-damaged-DNA-recognition proteins; DRP, damaged-DNA-recognition protein; RA, retinoic acid.

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U.S.A.). A differentiated phenotype of F9 cells was evident from morphological changes in response to RA/dbcAMP exposure. Biochemical differentiation was verified by analysing cell cultures for expression of plasminogen activator [17].

Flow cytometry

Cell-cycle analysis was performed by a modification of the propidium iodide/citrate method of Vindelov and Christenson [18]. Briefly, cells were rinsed twice with PBS, resuspended twice respectively in 10 ml and 200 μ l of citrate buffer [40 mM sodium citrate (pH 7.6)/250 mM sucrose]. The cells were adjusted to 10^6 per 200 μ l and processed immediately for staining or stored at -70°C until use. For staining, the fixed cells were thawed, gently mixed in 1.8 ml of solution A {0.03 mg/ml trypsin in stock solution [0.1% Nonidet P40/3.4 mM sodium citrate (pH 7.6)/1.5 mM spermidine tetrahydrochloride/0.5 mM Tris]} and incubated at 25°C for 20 min. Routinely, 1.5 ml of solution B (1 mg/ml RNase/0.5 mg/ml trypsin inhibitor in stock solution) was then added and incubated at 25°C for an additional 20 min, followed by the addition of 1.5 ml of solution C (0.5 mg/ml propidium iodide/3 mM spermidine tetrahydrochloride in stock solution) and kept at 4°C until analysis. Cells were analysed on a FACScan flow cytometer (Becton-Dickinson) equipped with a 15 mW air-cooled argon ion laser. On FACScan (488 nm excitation), fluorescence emission was collected after passage through band-pass filters (585 nm). Data were collected and analysed on a Hewlett-Packard model 340 computer interfaced with the FACScan.

DNA probes

^{32}P -labelled f130 DNA probes were prepared as previously described [19]. The DNA fragment f130, which is part of the simian-virus-40 early-region promoter, was originally isolated from plasmid pSVT [20] by restriction with *SphI*--*BglI* (blunted) and inserted into *SphI*--*SmaI*-opened vector pBS(+) (Stratagene). The *HindIII*--*EcoRI* f130 fragment (termed f130) containing a 17 bp dA/dT-rich region is a potential target for UV modification. The f130 fragments were labelled with [α - ^{32}P]dCTP (3×10^4 c.p.m./ng of DNA) at the *HindIII* end using Klenow DNA polymerase and purified in spin columns by standard methods [21]. The f130 DNA at a concentration of 100 $\mu\text{g/ml}$ was irradiated with UV germicidal lamps in semi-darkness to avoid photoreversal of the UV-induced DNA adducts as described in [22]. DNA was irradiated with a VL-100C UV irradiation unit (Vilbert Lourmat, Marne La Vallee, France) at a fluence rate 25 J/s per m^2 , measured with a VLX-254 radiometer (Vilbert Lourmat).

Cell extracts and DNA-mobility-shift assay

Nuclear and cytosolic extracts were prepared as described by Dignam et al. [23]. The protein concentration was measured via the Bradford assay using the Bio-Rad dye reagent [24] and proteins were also revealed by SDS/PAGE [25]. Protein-DNA binding was allowed to take place in 15 μ l of buffer containing 12% (v/v) glycerol, 12 mM Hepes, pH 7.9, 100 mM KCl, 5 mM MgCl_2 , 4 mM Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, 300 $\mu\text{g/ml}$ BSA at 25°C for 30 min as described in [26]. The reaction mixtures were then subjected to 4%-PAGE at 15 mA constant current under low ionic strength [6.7 mM Tris/HCl (pH 7.9)/3.3 mM sodium acetate/1 mM EDTA] at room temperature. The resolved gel was dried and exposed to Kodak XAR-5 X-ray film at -70°C with an intensifying screen. The intensity of the DNA bands was determined on the X-ray film

with an appropriate exposure by scanning densitometry using a Hoefer GS300 instrument. An average of three scans was taken.

DNA transfection and chloramphenicol acetyltransferase (CAT) assay

Portions (20 μg each) of pRSVcat and pSV β (Clontech Laboratories) DNA were co-transfected into cells by using the electroporation technique involving the use of a GenePulser (Bio-Rad) with 1000 μF capacity and at 200 V as previously described [15]. The cells were fed the following day with fresh media and incubated for another 40 h in the presence or absence of RA/dbcAMP. In most cases the transfected cells exhibited an optimal expression of the CAT activity around 40 h. Cells were harvested into 1 ml of PBS, centrifuged, then assayed for CAT and β -galactosidase activity [21] using 200 μg and 400 μg of cellular protein extracts respectively. The CAT assay reaction was incubated at 37°C for 1 h, followed by development on a silica TLC plate (Macherey-Nagel). After autoradiography, density on the X-ray film corresponding to the modified chloramphenicol was quantified through a scanning densitometer. After being normalized to β -galactosidase activity, relative CAT activity was determined. CAT activity was calculated as the percentage of chloramphenicol converted into acetylated derivatives.

RESULTS AND DISCUSSION

Differential inhibition of cell-cycle progression and induction of UVDRP in cells by RA/dbcAMP

The RA/dbcAMP effect on the progression of the cell cycle was investigated from $(1-2) \times 10^4$ events using flow cytometry (Figure 1). Quantification of the cell-cycle distribution from three independent experiments of these cells was determined. Initially, the G1-phase + G0-phase population of F9 cells was accumulated by RA/dbcAMP within 2 days (upper panel, Figure 1), whereas the S-phase population was gradually reduced immediately following the treatment (middle panel, Figure 1). Reduction of S cells within 2 days is probably due to block of cells at G1 + G0. The continuous reduction of F9 cells from the S may explain a rapid increase of G2 + M-phase cells after 2 days treatment (lower panel, Figure 1). The data is consistent with the idea that RA/dbcAMP conditions are sufficient to prevent F9 cells from DNA synthesis, a phenomenon of cell differentiation. In contrast, inhibition of cell-cycle progression in NIH3T3 cells is different by the same treatment. It appeared that a transient decrease of G1 + G0 population is associated with a transient accumulation of NIH3T3 cells in S phase, whereas G2 + M NIH3T3 cells were least affected.

Under the standard DNA-binding conditions, a UVDRP was detected in RA/dbcAMP-induced F9 and NIH3T3 cells (Figure 2, panel A). The probe (6000 J/ m^2 UV-irradiated f130) was bound by a factor or factors in 8 μg of nuclear extracts following 2, 4, 6, or 8 days of induction (indicated above the lanes). The specificity of the UVDRP in F9 and NIH3T3 cells was characterized by a competition experiment [2,6]. A specific binding activity (indicated by an arrowhead) transiently increased with the period of induction. The optimal UVDRP binding in F9 cells was detected at day 6, followed by a rapid decrease at day 8. It should be noted that morphological and biochemical markers indicate that more than 70% of F9 cells after 6 days RA/dbcAMP treatment were in the process of differentiation. Only the free probe (indicated with 'f') was detected in the extract-free reaction (the far-left lane). UVDRP induction was also detected in NIH3T3 cells, with an optimal induction at 2 days. A shorter

Figure 1 Effect of RA/dbcAMP on the cell-cycle distribution of F9 and NIH3T3 cells

At 0, 1, 2, or 4 days after RA/dbcAMP addition, F9 or NIH3T3 cells were harvested, fixed, stained with propidium iodide, and analysed for DNA content by flow cytometry, with the percentage of cells at G1 + G0, S and G2 + M plotted as a function of the period (in days) of RA/dbcAMP treatment. Points represent the means $\pm$ 5–10% S.D. for three experiments.

time course for UVDRP induction in NIH3T3 cells was also observed, but was not consistent (results not shown). A non-specific binding activity (the fast-migrating band) was also detected in both cells [14]. Cellular localization of the UVDRP in cells was also investigated. Nuclear (NE) or cytosolic (CE) extracts from optimal induction, 6-day-induced F9 cells, or 2-day-induced NIH3T3 cells, were used for the DNA-binding assay. A majority of the UVDRP binding activity appeared in the nuclear fraction. The same nuclear extracts of F9 or NIH3T3 cells showed no change in Sp1 binding as a negative control (results not shown). Relative UVDRP binding (mean $\pm$ S.D.) of the RA/dbcAMP-treated cells, setting that of untreated cells as 1, from three independent experiments, was determined (panel B). These results indicate that UVDRP binding activity is inducible by RA/dbcAMP in mammalian cells. Since NIH3T3 cells do not show differentiation in response to RA/dbcAMP, the induction of UVDRP binding activity is unlikely initiated by, or be solely associated with, differentiation. The estimated abundance of the maximum UVDRP in induced F9 and NIH3T3

Figure 2 Induction of UVDRP binding activity in F9 and NIH3T3 cells

Panel A: portions (8 μ g) of nuclear extracts isolated from cells induced with 0, 2, 4, 6, or 8 days of RA/dbcAMP were incubated with probe f130-uv (panel A). A shorter time course for UVDRP induction in NIH3T3 cells was also shown in the right panel. The far left lane is nuclear extract-free reaction. Symbols: f, free probe; arrowhead, bound probe. Panel B: quantification of UVDRP binding activity in induced F9 and NIH3T3 cells. Relative binding activity was calculated by scanning densitometry, taking the binding activity of untreated cells as the reference point. Values are means $\pm$ S.D. ($n = 3$). Left panel, F9 cells; right panel, NIH3T3 cells.

cells is about 0.04 molecule/Mb, that is, about 50% of HeLa UVDRP binding activity [14]. Compared with 5% of HeLa UVDRP in uninduced F9 and NIH3T3 cells, the UVDRP binding activity in mammalian cells is similar under the induced conditions.

There is a dramatic difference in the rate of UVDRP induction in F9 and NIH3T3 cells: F9 takes 6 days, whereas NIH3T3 takes only 2 days, to optimize the induction of UVDRP binding activity. The induction may be regulated by, or co-regulated with, cell growth conditions. F9 and NIH3T3 cells were effectively blocked at G1 + G0 and S respectively by RA/dbcAMP. The effect of the cell-cycle block and UVDRP induction in NIH3T3 cells is reversible or transient. In contrast, the cell-cycle block following longer treatment with RA/dbcAMP is irreversible in F9 cells, associated with G2 + M-cell accumulation and the appearance of cell death. The pattern of the UVDRP induction in F9 cells is somewhat different from that in NIH3T3 cells. There is a rapid decrease in the UVDRP level following maximal induction with RA/dbcAMP in F9 cells, distinct from a gradual decline in NIH3T3 cells by the same treatment (see Figure 2). The rapid decrease of UVDRP activity in F9 cells may be explained by the cell death induced by RA/dbcAMP. The time-dependent characteristics of cell-cycle inhibition and induction of UVDRP in F9 and NIH3T3 cells suggests that the hindrance of events controlling cell-cycle progression, probably at G2 + M, may signal the induction of UVDRP. Although RA/dbcAMP does not directly cause DNA damage, the induction of UVDRP

Figure 3 Re-activation kinetics of plasmid pRSVcat in F9 and NIH3T3 cells

The F9 and NIH3T3 cells were treated with RA/dbcAMP for the indicated number of days. A 20 μ g portion of unirradiated or 250 J/m²-UV-irradiated pRSVcat DNA was electroporated together with 10 μ g of pSV β into cells and expression was allowed to proceed for 40 h. Portions (200 μ g) of cellular extracts were used for the CAT activity assay. Relative CAT activity, being normalized to the β -galactosidase activity (mean $\pm$ S.D.; $n = 3$), was calculated, taking the CAT activity of untreated cells as the reference point. Upper panel, F9 cells; lower panel, NIH3T3 cells.

in rodent cells probably mimics the proliferative response induced by phorbol ester or growth factors [27]. Additionally, the induction of the immediate target sequences for AP-1 and NF κ B by UV takes place within minutes. In contrast, induction of UVDRP by either UV or RA/dbcAMP takes at least hours or days. Perhaps UVDRP induction is secondary to activation of AP-1 and NF κ B type proteins or genes. The up-regulation of UVDRP is either directly enhanced at UVDRP gene(s) by RA/dbcAMP or by a post-translational mechanism like the phosphorylation pathway.

Lack of enhancement in repair-mediated plasmid re-activation in cells over-expressing inducible UVDRP binding

The question of whether the level of DRPs parallels the cellular ability in DNA excision-repair remains unsolved. In an attempt to answer this question, the repair-mediated plasmid re-activation was measured in F9 and NIH3T3 cells on the assumption that expression of the reporter CAT gene borne on the plasmid depends on repaired plasmid DNA. It is known that transcriptional activity of the Rous-sarcoma-virus promoter is responsive to RA at concentration used in the present study [14,28]. The same RA/dbcAMP conditions used for UVDRP studies was applied to the present study. A typical example is

Figure 4 Re-activation of plasmid pRSVcat in F9 and NIH3T3 cells

F9 and NIH3T3 cells were mock-treated or RA/dbcAMP-treated for 6 days (F9) or 2 days (NIH3T3). pRSVcat DNA (20 μ g) irradiated with 0, 250, 500 or 1000 J/m² of UV was transfected together with 10 μ g of pSV β into F9 or NIH3T3 cells and expression allowed to proceed for 40 h. Cellular extracts (200 μ g portions) were used for the CAT activity assay. The symbols are as for Figure 3. Relative CAT activity (mean $\pm$ S.D.; $n = 3$) was calculated by taking the CAT activity of the unirradiated pRSVcat DNA as the reference point. Upper panel, F9 cells; lower panel, NIH3T3 cells.

shown (Figure 3). A 20 μ g portion of pRSVCAT, either unirradiated or 250 J/m²-UV-irradiated, was transfected into F9 (upper panel, Figure 3) or NIH3T3 cells (lower panel, Figure 3). It should be noted that morphological and biochemical markers indicate that more than 70% of F9 cells following 6 days RA/dbcAMP treatment were in the process of differentiation. However, these changes were not found in NIH3T3 cells. The data indicate a significant increase of the CAT activity (indicated with closed arrowheads) at 6 days in F9 cells, and it increased further at 8 days. For NIH3T3 cells, there is no induction of CAT activity by RA/dbcAMP treatment. In fact, a gradual decline of the CAT activity was detected. The relative CAT activity (mean $\pm$ S.D.) from three independent experiments was quantified. Compared with mock-treated cells, RA/dbcAMP treatment of F9 and NIH3T3 cells, for 8 days for example, did not result in the induction of CAT activity on the UV-damaged plasmid compared with that of the undamaged plasmid. In fact, the relative CAT activity from UV-damaged DNA is slightly lower than that of control plasmid in F9 cells. Thus repair-mediated plasmid re-activation in both cell lines is not quantitatively associated with the level of UVDRP.

CAT activity of the same plasmid treated with different UV doses was also analysed in RA/dbcAMP-induced cells (Figure 4). A typical example of CAT activity from F9 (upper panel, Figure 4) and NIH3T3 cells (lower panel, Figure 4) is shown.

pRSVcat was irradiated with 0, 125, 250, 500 or 1000 J/m² of UV prior to introduction into cells without (□) or with optimal RA/dbcAMP induction (■). There is no difference in CAT activity at lower UV doses between the control and RA/dbcAMP-induced cells. For example, CAT activity was inhibited by $\approx 30\%$ (F9) and $\approx 10\%$ (NIH3T3) in both control and RA/dbcAMP-treated cells following 100 J/m² of UV irradiation. The dose-response curves at the optimal CAT activity in F9 (day 6) and NIH3T3 (day 2) cells was calculated. In both cases the patterns of the relative CAT activity between RA/dbcAMP treated and untreated are similar, if not identical. On the basis of these data, the relationship between the level of inducible UVDRP (fold) and the induction of plasmid re-activation (fold) was determined. A regression line with $R^2 = 0.316$ was estimated, where R is the correlation coefficient. Our previous studies ([19]; C. C.-K. Chao, unpublished work) suggested a correlation between constitutive UVDRP levels and cellular DNA repair ability in different cell types. Our current findings do not show a correlation in RA/dbcAMP-treated cells, raising the possibility that UVDRP may have a function other than, or in addition to, its role in DNA repair.

This research was supported by grants from Chang Gung Medical College (CMRP388, NMRP166) and the National Science Council, Republic of China (NSC83-0412-B182-007 to C.C.-K.C., and NSC80-0203-B001-14 to S.L.-C.).

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