Journal of Oral Biology, June 1994, p. 13-21 Vol. 18, No. 1

Alteration of DNA Synthesis and p53 Level by Modulators of Adenylate Cyclase System and Other Second Messenger Systems in Human Oral Keratinocytes

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To study the role of adenylate cyclase system and other mediators of signal transduction pathway in the cell cycle arrest which provides enough time for the cells to repair damaged DNA, we examined the cellular response of primarily cultured human oral keratinocytes (HOKs) to UV irradiation. UV irradiation reduced replicative DNA synthesis of HOKs in a dose-dependent manner without significant changes in viability. Dibutyryl cAMP(5 μM) and forskolin (5 μM) partly inhibited the reduction in replicative DNA synthesis by UV irradiation at 12th hour after irradiation (17% and 31%, respectively) but other agents failed to inhibit the reduction in replicative DNA synthesis at 12th hour after irradiation. At 24th hour after irradiation, none of the agents inhibited the suppression of DNA synthesis. The reduction in DNA synthesis was accompanied by marked p53 induction, which was completely blocked by dibutyryl cAMP (5 μM), forskolin (5 μM), IBMX (5 μM) and dibutyryl cGMP (5 μM). Treatment of TPA (5 nM), staurosporin (10 nM) and dexamethasone (1 μM) reduced DNA synthesis in HOKs and prompted death of the cells. Those drugs, however, did not induce p53 indicating that different mechanism from that for UV irradiation may be involved in the reduction of replicative DNA synthesis Increased reparative DNA synthesis induced by UV irradiation was suppressed by forskolin Our results suggest that cAMP may be involved in DNA damage-induced cell cycle arrest and DNA repair, and cAMP and cGMP may be associated with blockade of p53 induction

Key words cAMP, UV radiation, keratinocytes, p53

Introduction

Carcinogenesis is a multistage process driven by carcinogen-induced genetic and epigenetic damages in susceptible cells that gain a selective growth advantage and undergo clonal expansion as a result of activation of proto-oncogenes and/or inactivation of tumor suppressor genes (Boreck, 1980; Girardi *et al.*, 1965; Milo and DiPaolo, 1978; Namba *et al.*, 1985). The first stage of the carcinogenic process, tumor initiation, involves exposure of normal cells to chemical, physical, or viral carcinogens that cause genetic changes, providing the initiated cells with an altered responsiveness to their microenvironment. Moreover, these changes would exert an advantage of selective clonal expansion compared to the surrounding normal cells (Cerutti, 1985). The initiated cells may have decreased responsiveness to the inter- and intracellular signals that maintain normal tissue architecture and regulate the homeostatic growth and maturation of cells (Parkinson, 1985; Willey *et al.*, 1984; Yuspa and Poirier, 1988).

UV radiation has a range of effects on eukaryotic cells including DNA damage. Exposure to solar simulated radiation stimulates DNA damage and unscheduled DNA synthesis in basal and suprabasal keratinocytes (Freeman *et al.*, 1987). Induction of p53 by UV irradiation in human epidermal kerati-

This work was supported by a grant no. 01-94-267 from the Seoul National University Hospital Research Fund.

nocytes *in vivo* (Hall *et al.*, 1993) and in mouse fibroblast cell line (Fritsche *et al.*, 1993) was also noted previously. Some chemical agents cause DNA damages. Chemicals including actinomycin D, mitomycin C and cisplatin induce p53 (Fritsche *et al.*, 1993) and reduce cell populations entering S phase (Kastan *et al.*, 1991). Actinomycin D is known to induce DNA strand breaks (Trask and Müller, 19 88; Wasserman *et al.*, 1990). Benzo(*a*)pyrene(B(*a*)P), a tobacco carcinogen, is readily metabolized in epithelial cells to yield a highly reactive epoxides which can form DNA adduct (Bartley *et al.*, 1982; Stampfer *et al.*, 1985).

DNA damages caused by physical and chemical carcinogens are known to be repaired before the replicative synthesis of DNA. Arrest in the cell cycle by DNA damage has previously been reported in mouse 3T3 fibroblasts after UV irradiation (Malzman and Czyzyk, 1984), in ML-1 leukemia cells after γ irradiation or actinomycin D treatment (Kastan *et al.*, 1991) and in lymphoblastoid cell line after γ irradiation (Kastan *et al.*, 1992). Arrest in cell cycle may be necessary to provide ample time for DNA repair. The most promising candidate mechanism underlying this arrest is the participation of p53 tumor suppressor protein which have a role in controlling cell proliferation (Malzman and Czyzyk, 1984; Shohat *et al.*, 1987). Primary murine fibroblasts became deficient in the G_1 arrest activated by γ radiation when both wild-type p53 alleles were disrupted (Kastan *et al.*, 1992). Cells from patients with ataxia-telangiectasia, the radio-sensitive, cancer prone disease, were reported to lack γ irradiation-induced increase in p53 protein level (Kastan *et al.*, 1992). According to the previous reports, the increase in p53 after DNA damage seems to be the result of stabilization of p53 transcript rather than the result of increased transcription rate. In ML-1 myeloblastoid leukemia cells the rise in p53 protein levels by γ irradiation or actinomycin D treatment was temporally correlated with the transient G_1 arrest without any apparent changes in p53 mRNA level (Kastan *et al.*, 1991). Since caffeine, a cyclic nucleotide phosphodiesterase inhibitor, abolished the cell cycle arrest and p53 induction after γ irradiation in ML-1 leukemic cells, the involvement of cyclic nucleotides in these responses was also suggested (Kastan *et al.*, 1991). Since the second messengers responsible for the control of cell cycle and DNA synthesis were not documented well, we studied the effects of adenylate cyclase system activation, protein kinase C activation and inhibition, and inhibition of phospholipid metabolism on the cellular response of human oral keratinocytes to UV irradiation. In the present study primarily cultured human oral keratinocyte were used as they are good for the study of oral carcinogenesis and they have quite different characteristics from cells of other region. The results of present investigation suggest that cyclic nucleotides are possible modulators of p53 level and that cyclic AMP is possibly a modulator of cell cycle arrest.

Materials and Methods

Primary Culture of Human Oral Keratinocytes (HOKs)

Primary culture of HOKs was done as described previously (Park *et al.*, 1991). Excised gingival tissue from the human oral cavity was treated with collagenase (type II, 1 mg/ml, Millipore Corp., MA, USA) and dispase (grade II, 2.4 mg/ml, Boehringer Mannheim Biochemicals, IN, USA) for 60 minutes at 37℃ to separate the epithelium from the underlying mesenchyme. Separated epithelial sheets were chopped and then dissociated into single cells by incubation in 2 ml of trypsin with continuous agitation at 37℃ for 8 minutes. The cells were neutralized with 1 ml of fetal bovine serum, washed with 2 ml of keratinocyte growth medium (KGM, Clonetics Corp., CA, USA) and resuspended in appropriate volume of KGM. Cells were plated on 60 mm tissue culture dish at a density of approximately 5×10^4 cells per dish and fed with KGM every second day while maintaining them at 37℃ in 100% humidity and 95% air/5% CO_2. Cells were passed to 24 well plates or 60 mm dishes for the experiment.

UV Irradiation and Drug Treatment of Cells

Actively growing HOKs (passage 1) were subject-

ed to UV irradiation immediately after removal of medium for 5 seconds at 50 cm distance from germicidal UV light (GL-30, National Electric Co., Japan). The cells were refed with either fresh KGM or KGM supplemented with drugs. The drugs were dibutyryl 3',5'-cyclic AMP (dbcAMP, 5 μM), forskolin (5 μM), 3-isobutyl-1-methylxanthine (IBMX, 5 μM), dexamethasone (1 μM), 12-O-tetra-decanoylphorbol 13-acetate (TPA, 5 nM), staurosporin (10 nM) or dibutyryl 3',5'-cyclic GMP (dbcGMP, 5 μM).

Replicative DNA Synthesis

Newly synthesized cellular DNA was labeled with tritium by incubating cells in the presence of [methyl-^{3}H]-thymidine (25Ci/mmol, Amesham Corp., IL, USA) at concentration of 5 μCi/ml during the last 3 hours of incubation. At the end of labeling period, medium was removed and replaced with ice-cold 5% trichloroacetic acid (TCA). Dishes were then left on ice for 15 minutes. After washing 3 times with ice-cold 5% TCA, TCA-insoluble fraction was dissolved in 0.3 ml of 0.5 M NaOH. The incorporated radioactivity was measured with liquid scintillation counter (model LS5000TA, Beckman, CA, USA).

Unscheduled DNA Synthesis

To measure the unscheduled DNA synthesis which repairs DNA damage, HOKs were synchronized with aphidicolin as previously described (Heintz and Hamlin, 1982) and incubated for 30 minutes in the presence of the drugs and [methyl-^{3}H]-thymidine (5 μCi/ml). Cells were UV irradiated for 8 seconds after removal of medium, refed with KGM supplemented with the drugs, [methyl-^{3}H]-thymidine (5 μCi/ml) and aphidicolin (7 μg/ml), and incubated for an hour. The incorporated radioactivity was determined as described above. Nonspecific labeling was estimated by incubating cells on ice and subtracted from the radioactivity of each group.

Western Blot Analysis of p53 Protein

Cells were lysed with PBSTDS (10 mM Na_2HPO_4, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.2% sodium azide, 0.004% sodium fluoride; pH 7.25) at 5th or 24th hour after UV irradiation.

Aliquots containing 100 μg of protein were analyzed by 10% SDS-PAGE after reduction with 200 mM dithiothreitol. Electroblotting of proteins to PVDF membrane (Immobilon-P, Millipore Corp., MA, USA) at 20 V overnight was followed by washing with PBS for 30 minutes. Detection of transferred p53 was carried out using Western-Light Chemiluminescent Detection System (Tropix Inc., MA, USA). After blocking with PBS containing 0.2% casein and 0.1% Tween-20 for an hour, membrane was subjected to incubation with 1:200 diluted anti-p53 mouse IgG (p53-Ab2, Oncogene Science, NY, USA) and followed by 10 minute washing with PBS containing 0.1% casein and 0.1% Tween-20. Membrane was then subjected to incubation with 1:5,000 diluted alkaline phosphatase-conjugated anti-mouse IgG antibody and followed by 15 minute washing as described above. Chemiluminescent substrate, CSPD™ (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.13,7]decan}-4-yl) phenylphosphate), was applied and drained off briefly after 5 minute incubation at ambient temperature. Wet membrane was covered with plastic. Chemiluminescence light was recorded on Hyperfilm-XP X-ray film (Amersham Corp., IL, USA).

Statistical Analysis

Obtained data were analyzed by Student's *t*-test for their statistical significance.

Results

The DNA synthesis in oral keratinocyte was reduced by UV irradiation in a dose-dependent manner. Approximately half of the DNA synthesis was blocked by 5 seconds irradiation (Fig. 1). The radioactivity in unirradiated cells was 2.28×10^5 dpm/well but it was reduced to 1.29×10^5 dpm/well after 5 seconds irradiation. At 12th hour after UV irradiation, 5 μM forskolin and 5 μM dbcAMP increased DNA synthesis by 31% and 17%, respectively. IBMX and dbcGMP further reduced DNA synthesis over the effect of UV irradiation (Fig. 3).

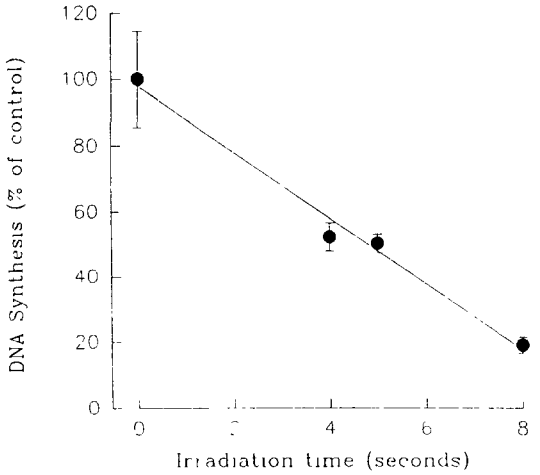

Fig. 1. Effect of UV irradiation on DNA synthesis in human oral keratinocytes.
Cells were UV irradiated for varying time, refed with fresh KGM and incubated for 24 hours. During the last 3 hours of incubation, DNA was labeled with [methyl-^{3}H]-thymidine. Incorporated radioactivity was determined with liquid scintillation counter.
Points are the means± S.E for 4 wells.

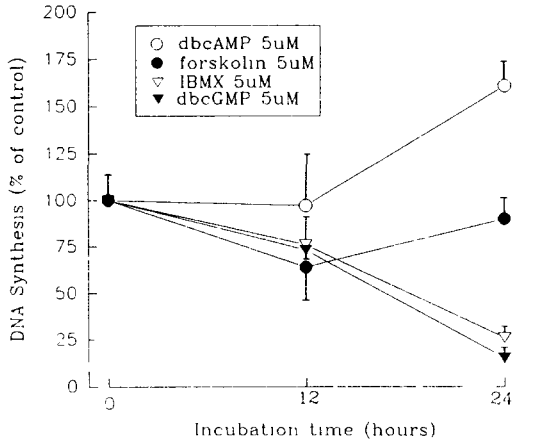

Fig. 2. Efeect of dbcAMP, forskolin, IBMX and dbcGMP on DNA synthesis in human oral keratinocytes.
Cells were treated with each drug for 12 or 24 hours. DNA synthesis was measured as described in Fig. 1.
Points are the means± S.E for 3~6 wells.

dbcAMP is converted to monobutyryl cyclic AMP which acts as a cAMP analog and forskolin activates adenylate cyclase to increase the synthesis of cAMP. IBMX inhibits cyclic nucleotide phosphodiesterase to reduce the metabolic degradation of endogenous cAMP. dbcGMP is converted to monobutyryl cyclic GMP which is a cGMP analog. At 24th hour after irradiation these four agents enhanced the suppression of DNA synthesis in HOKs by UV irradiation (Fig. 3). dbcAMP increased DNA synthesis in unirradiated HOKs at 24th hour of incubation (Fig. 2) showing differential effect of this agent between UV-irradiated cells and unirradiated cells. TPA, staurosporin and dexamethasone prompted cell death which was noted by loss of intact cellular structure. Accordingly reduction of DNA synthesis in HOKs was resulted (Fig. 4). Dexamethasone is a glucocorticoid which is known to inhibit phospholipid metabolism. TPA is a protein kinase C activator and staurosporin is reported to counteract the effect of TPA. These three agents enhanced the reduction in DNA synthesis by UV irradiation (Fig. 5). Western blot analysis of p53 revealed that UV irradiation markedly increased p 53 level and this effect was almost completely abolished by dbcAMP, forskolin, IBMX and dbcGMP (Fig 6A). The elevated level of p53 persisted up to 24 hours after UV irradiation (Fig. 7). Neither

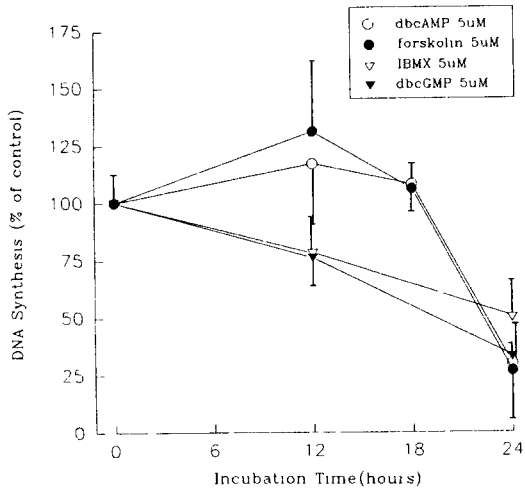

Fig. 3. Effect of dbcAMP, forskolin, IBMX and dbcGMP on DNA synthesis in UV-irradiated human oral keratinocytes.
Cells were UV irradiated for 5 seconds, refed with KGM supplemented with drugs and incubated for 12, 18 or 24 hours. DNA synthesis was measured as described in Fig. 1.
Points are the means± S.E for 3~6 wells.

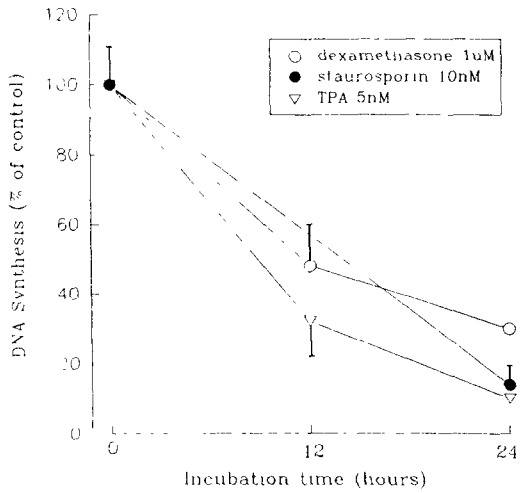

Fig. 4. Effect of TPA, staurosporin and dexamethasone on DNA synthesis in human oral keratinocytes.
Cells were treated with each drug for 12 or 24 hours. DNA synthesis was measured as described in Fig. 1. Points are the means± S.E for 3~6 wells.

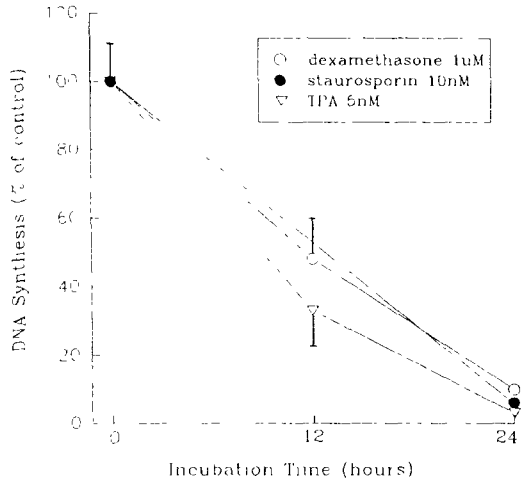

Fig. 5. Effect of TPA, staurosporin and dexamethasone on DNA synthesis in UV-irradiated human oral keratinocytes.
Cells were UV irradiated for 5 seconds, refed with KGM supplemented with drugs and incubated for 12 or 24 hours. DNA synthesis was measured as described in Fig. 1.
Points are the means± S.E for 3~6 wells.

TPA nor dexamethasone induced p53 (Fig. 8). Dexamethasone, staurosporin and TPA did not block induction of p53 protein (Fig. 6B) although they

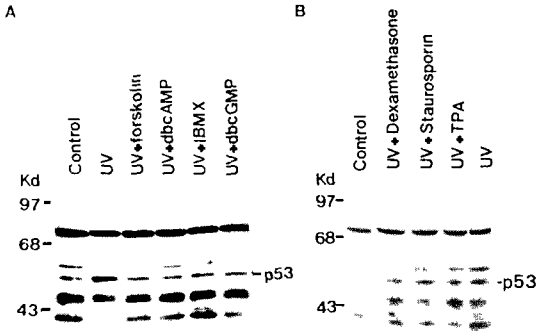

Fig. 6. Western blot analysis of p53 in human oral keratinocytes 5 hours after UV irradiation and drug treatment.
Cells were UV irradiated for 5 seconds, refed with KGM supplemented with drugs (5 μM forskolin, 5 μM dbcAMP, 5 μM IBMX, 5 μM dbcGMP, 1 μM dexamethasone, 10 nM staurosporin and 5 nM TPA) and incubated for 5 hours. At the end of incubation cells were lysed with PBSTDS and subjected to western blot analysis.

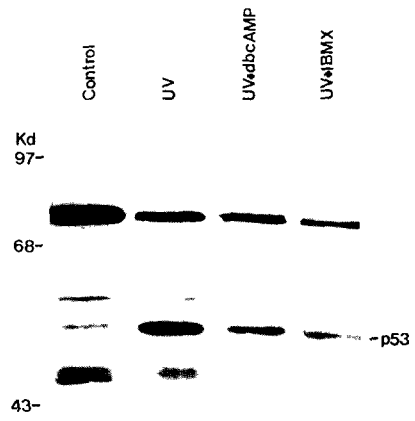

Fig. 7. Western blot analysis of p53 in human oral keratinocytes 24 hours after UV irradiation and drug treatment.
Cells were UV irradiated for 5 seconds, refed with KGM supplemented with drugs (5 μM dbcAMP and 5 μM IBMX) and incubated for 24 hours. At the end of incubation cells were lysed with PBSTDS and subjected to western blot analysis.

blocked DNA synthesis (Fig. 4). UV irradiation induced unscheduled DNA synthesis in HOKs. The induction of unscheduled DNA synthesis was blocked by forskolin treatment (Fig. 9).

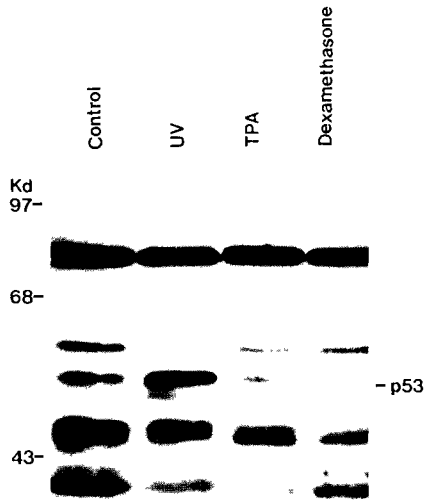

Fig. 8. Western blot analysis of p53 in human oral keratinocytes 5 hours after treatment either with UV radiation or with TPA and dexamethasone.
Cells were either UV irradiated for 5 seconds or added with drugs (5 nM TPA and 1 μM dexamethasone) and continued incubation for 5 hours. At the end of incubation cells were lysed with PBSTDS and subjected to western blot analysis.

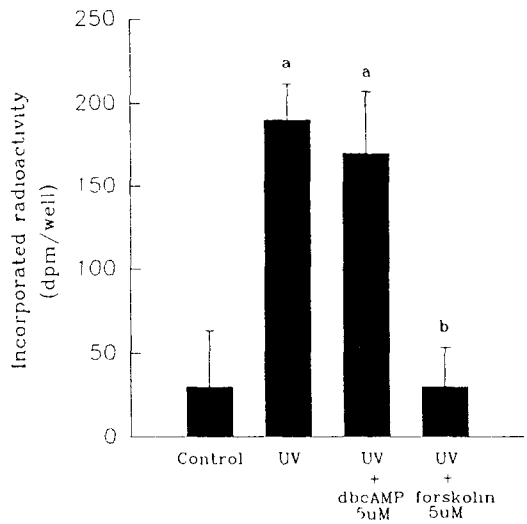

Fig. 9. Effect of dbcAMP and forskolin on unscheduled DNA synthesis in human oral keratinocytes.
Cells were synchronized with aphidicolin and UV irradiated for 8 seconds. DNA was labeled with [methyl-^{3}H]-thymidine for an hour in the presence of aphidicholin (7μg/ml) and each drug. Incorporated radioactivity was measured with liquid scintillation counter. Data are the means± S.E for 6 wells.
a; $p<0.05$, significantly different from control.
b; $p<0.05$, significantly different from UV-irradiated cells.

Discussion

Epithelial cells are frequently challenged by various carcinogens and are preferred site of cancer development. Epithelial cell cultures derived from numerous organs of rodent model systems have been used to study chemically-induced carcinogenesis (Griener *et al.*, 1983; Sima *et al.*, 1982; Stoica *et al.*, 1991) and radiation-induced carcinogenesis. Recently human mammary or foreskin keratinocyte culture systems were developed and utilized for the study of carcinogenesis (Kessis *et al.*, 1993; Milo and DiPaolo, 1978; Stampfer and Bartley, 1985). Moreover several continuous human keratinocyte cell lines were established by transfection of recombinant viral DNA (Münger *et al.*, 1989; Park *et al.*, 1991), or by chemical carcinogen treatment (Stampfer *et al.*, 1981).

Physical and chemical carcinogens may cause DNA damages. DNA damages are known to contribute to the development of cancer if the repair mechanism is defective. Recently it is known that cells exposed to genotoxic stimulation respond by reducing the population of cells entering S phase allowing ample time to repair damaged DNA (Kastan *et al.*, 1991; Malzman and Czyzyk, 1984) or are apoptosized if damage is unrecoverable Clark *et al.*, 1993).

Ineffective DNA repair and subsequent DNA synthesis using defective template expand the defective cell clones facilitating further transformation to occur by additional genetic alterations (Cohen and Ellwein, 1990). Ineffective DNA repair is also associated with the high susceptibility of excision repair-deficient xeroderma pigmentosum cells to radiation transformation (Watson *et al.*, 1987). As keratinocytes are continuously renewed, DNA damages have minimal detrimental effect on the upper layer cells; however, basal cells, which comprise of most primarily cultured HOKs are sensitive to genotoxic stimulation.

In the present study, the replicative DNA synthe-

sis of HOKs was reduced by UV irradiation without significant reduction in viability in trypan blue dye exclusion method, and this indicated the occurrence of cell cycle arrest. Reduction in replicative DNA synthesis was accompanied by concomitant induction of p53 tumor suppressor protein as shown by western blot analysis suggesting the association between p53 activity and inhibition of DNA synthesis in HOKs. The induced p53 persisted up to 24 hours after irradiation. This result is in accordance with the effect of γ irradiation on various cell types (Kastan *et al.*, 1991; Lowe *et al.*, 1993). TPA, a protein kinase C activator, and staurosporin, an inhibitor of protein kinase C, both induced loss of intact cell structure and eventually detachment of primarily cultured HOKs after prolonged treatment.

The replicative DNA synthesis was almost completely blocked accordingly. These agents, however, did not induce p53 unlike UV irradiation. This sort of response is previously reported to be a mechanism of immune cell editing. According to this report, in thymocytes, both γ irradiation and phorbol 12-myristate 13-acetate(PMA) in combination with calcium ionophore induced apoptosis (Clark *et al.*, 1993). However, PMA-induced apoptosis was not accompanied by p53 induction unlike γ radiation-induced apoptosis, which is quite in accordance with our result in HOKs. Dexamethasone has been used to intentionally induce apoptosis of various cell types to study physiological process of apoptosis (Clark *et al.*, 1993; Lowe *et al.*, 1993).

In the present study dexamethasone suppressed DNA synthesis inducing loss of intact cell structure as did TPA and staurosporin without any apparent induction of p53 in oral keratinocytes. Previously, caffeine was reported to inhibit the γ ray-induced cell cycle arrest and p53 induction in hematopoietic progenitor cells (Kastan *et al.*, 1991). To find out the second mediators which are responsible in controlling the inhibition of DNA synthesis in HOKs and the upstream mediators responsible for p53 induction, the effects of adenylate cyclase system activation, inhibition and activation of protein kinases C, and inhibition of phospholipid metabolism were studied. Our results show that forskolin and dbcAMP partly inhibited the UV irradiation-induced suppression of replicative DNA synthesis at 12th hour after irradiation though it is still far from complete but IBMX and dbcGMP did not inhibit the suppression. At 24th hour after irradiation, all of these four agents failed to inhibit the UV irradiation-induced reduction in DNA synthesis and even enhanced the effect of UV irradiation. They did not reduce cell viability in 24 hours. G_2 phase arrest which occurs after S phase might explain this result and this is to be proved by other measures.

According to our result it seems that cAMP may work bimodaly, i.e., cAMP may promote cell cycle progression in relatively healthy HOKs and this may also be true in a fraction of UV-irradiated cells. On the other hand, it seems that cAMP enhances cell cycle arrest probably at other phase than G_1 especially after relatively longer exposure to UV radiation. The discrepancy between the capability of the agents to block p53 induction and the capability to increase DNA synthesis still remains to be explained. The difference in the mode of action should be considered as a factor which might contribute to the difference in response of HOKs. Both IBMX and caffeine are phosphodiesterase inhibitor and these agents prolong the action of endogenous cAMP by reducing the enzymatic degradation. On the other hand, forskolin stimulates adenylate cyclase and induces rapid cAMP formation independent of receptor stimulation. Monobutyryl cAMP, the hydrolysis product of dbcAMP, activates cAMP-dependent protein kinases directly. Not only the replicative DNA synthesis was changed but also DNA repair was inhibited by forskolin showing the involvement of cAMP in controlling DNA repair. And as suggested in other types of cells (Clark *et al.*, 1993) it seems that different pathway may exist for the apoptosis of HOKs by TPA, staurosporin and dexamethasone from that associated with p53. We have shown here that UV irradiation causes reduction in DNA synthesis presumably by arresting cells at G1 phase. The arrest of cell cycle in HOKs appears in relatively brief period accompanying induction of p53 tumor suppressor protein. But the role of p53 in HOKs seems not to be the only determinant in DNA damage-induced cell cycle arrest. Increase in DNA synthesis by dbcAMP in nor-

mal HOKs and reversal of UV-induced cell cycle arrest by forskolin and dbcAMP supports, though in part, the role of cAMP-dependent protein kinases in the control of cell cycle arrest and further studies should be focused on the search for the downstream mediator of cAMP in the control of cell cycle and other gene products which may cooperate with p53 or work without the cooperation of p53 to control cell cycle progression.

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