Ultraviolet irradiation can induce evasion of colon cancer cells from stimulation of epidermal growth factor

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Receptor downregulation is the most prominent regulatory system of epidermal growth factor (EGF) receptor (EGFR) signal attenuation and an critical target for therapy against colon cancer that are highly dependent on EGFR. In this study, we investigated the effect of ultraviolet-C (UV-C) on downregulation of EGFR in human colon cancer cells (SW480, HT29 and DLD-1). UV-C caused inhibition of cell survival and proliferation, concurrently inducing the decrease in cell surface EGFR and subsequent its degradation. UV-C as well as EGFR kinase inhibitors decreased the expression level of cyclin D1 and phosphorylated level of retinoblastoma, indicating that EGFR downregulation is correlated to cell cycle arrest. While UV-C caused a marked phosphorylation of EGFR at Ser1046/1047, UV-C also induced activation of p38 mitogen-activated protein kinase (MAPK), a stress-inducible kinase believed to negatively regulate tumorigenesis, and the inhibition of p38 MAPK canceled EGFR phosphorylation at Ser1046/1047 as well as subsequent internalization and degradation, suggesting that p38 MAPK mediates EGFR downregulation by UV-C. In addition, phosphorylation of p38 MAPK induced by UV-C was mediated through transforming growth factor-\(\beta\)-activated kinase-1 (TAK-1). Moreover, pretreatment of the cells with UV-C suppressed EGF-induced phosphorylation of EGFR at tyrosine residues in addition to cell survival signal, Akt. Together, these results suggest that UV-C irradiation induces the removal of EGFR from cell surface that can protect colon cancer cells from oncogenic stimulation of EGF, resulting in cell cycle arrest. Hence, UV-C might be applied for clinical strategy against human colon cancers.

Members of the epidermal growth factor (EGF) receptor (EGFR) family, which are frequently overexpressed in several types of human cancers, including cancers of the lung (1), head and neck (2), prostate (3), breast (4), pancreas (5) and colon (6), have been associated with abnormal growth of these tumors. It is well known that exposure of cells to EGF results in rapid autophosphorylation of EGFR molecules at the cell surface (7-10), which upon activation lead to cell proliferation, motility and enhanced survival (11). There are several mechanisms by which EGFR becomes oncogenic including: 1) increased EGFR expression levels, 2) autocrine and/or paracrine growth factor loops, 3) heterodimerization with other EGFR family members and cross-talk.
with heterologous receptor systems, 4) defective receptor downregulation and 5) activating mutations (12). In clinical trials, increasing evidence shows the efficacy of EGFR-targeted agents, including monoclonal antibodies on the one hand and tyrosine kinase inhibitors on the other (13).

Following activation, the ligand-receptor complexes are internalized and then enter endosomes, where the receptors and their ligands are sorted to various intracellular destinations (14). Thus, some receptors can be recycled back to the cell surface via early endosomes and others are targeted to late endosomes and lysosomes for proteolytic degradation. There is increasing evidence that receptor internalization acts not only to terminate signaling, but that internalized endosome-associated receptors are also able to stimulate specific signal transduction pathways (15-17). Some agents that induce ligand-independent internalization and degradation of EGFR, such as the 225 mouse antibody (18) and gemcitabine (5) could have promising potential for cancer therapies. By contrast, it has previously been reported that the other factors or agents, such as oxidative stress (19) and cisplatin (20) can induce internalization of the EGFR, but not degradation. They differ in their effects on the fate of the receptors, downstream signaling and cell proliferation.

Receptor downregulation is the most prominent regulatory system of EGFR signal attenuation and involves the internalization and subsequent degradation of the activated receptor in the lysosomes. With the current knowledge of the mechanism underlying EGFR downregulation, this molecular event involves several important phosphorylation sites in EGFR. One is the phosphorylation at tyrosine 1045 (Tyr1045), which provides a docking site for the ubiquitin ligase c-Cbl resulting in ubiquitination of the EGFR (10). The others are the phosphorylation at serine or threonine residues which is thought to represent a mechanism for attenuation of the receptor tyrosine kinase activity (21-23). Among the major sites of serine and threonine phosphorylation of the EGFR, it has previously been shown that serine 1046/1047 (Ser1046/7) phosphorylation site is required for EGFR desensitization in EGF-treated cells (23). Moreover, mutant EGFR at Ser1046/7 reportedly causes the inhibition of the EGF-induced endocytosis and downregulation of cell surface receptors (22).

We have recently reported that phosphorylation of EGFR at serine 1046/7 via activation of p38 mitogen-activated protein kinase (MAPK) plays a pivotal role in downregulation of EGFR induced by (-)-epigallocatechin gallate (EGCG) (24), anisomycin (25) and HSP90 inhibitor (26). Moreover, there is an evidence that cisplatin also induces EGFR internalization, which is mediated by p38 MAPK dependent phosphorylation of the receptor at threonine 669 (20). As well, it has been shown that gemcitabine induces EGFR internalization and subsequent degradation which may be a novel mechanism for gemcitabine-mediated cell death (5), whereas the activation of p38 MAPK is necessary for gemcitabine-induced cytotoxicity (27,28). Taken together, serine phosphorylation of EGFR via p38 MAPK might be considered a new therapeutic target especially to counter cancer cells of the colon, lung, pancreas and breast that highly express EGFR.

Ultraviolet (UV) radiation from sunlight is sorted by wavelength regions: long-wavelength UV-A (320–400 nm), medium-wavelength UV-B (280–320 nm) and short-wavelength
UV-C (200–280 nm). In general, UV-A and UV-B are recognized the major carcinogenic components of sunlight (29). As for UV-C, it is used for studying DNA damage and cellular DNA repair process, although it does not actually exist in earth’s surface since they are filtered out by the atmosphere. While UV-C has been commonly applied for equipment such as water sterilization, recent studies show the possible application of UV-C against human cancer (30). However, its exact mechanism has not been fully clarified.

We have recently reported that the blockade of EGF stimulation significantly suppressed SW480 cell growth, suggesting that EGFR pathway plays an important role in proliferation of colon cancer cells (26). Therefore, we herein investigated the effect of UV-C on the downregulation of EGFR in these cells and found that this induces the internalization and degradation of EGFR that indicates the removal of EGFR from cell membrane, and this action of UV-C can protect colon cancer cells from oncogenic stimulation such as EGF, resulting in cell cycle arrest.

**Experimental Procedures**

**Materials**  
SB203580, (5Z)-7-oxozeaenol, AG1478 and PD153035 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). BIRB0796 was obtained from Dr. Philip Cohen (University of Dundee, UK). Antibodies against total EGFR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-EGFR (Tyr1045, Tyr1068 and Ser1046/7), phospho-p44/p42 MAPK, p44/p42 MAPK, phospho-p38 MAPK, p38 MAPK, phospho-stress-activated protein kinase/c-Jun-N-terminal kinase (SAPK/JNK), SAPK/JNK, phospho-transforming growth factor-β (TGF-β)-activated kinase-1 (TAK-1), phospho-MKK3/6, cyclin D1 and phospho-retinoblastoma (Rb) were purchased from Cell Signaling (Beverly, MA). Antibodies against phospho-γH2A.X at Ser139 (γH2AX) were purchased from Abcam (Cambridge, MA). ECL Western blot detection system was purchased from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources.

**Cell culture**  
SW480 and HT29, human colon cancer cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, San Diego, CA), containing 10% fetal calf serum (FCS) with penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO₂ incubator at 37°C. DLD-1, other human colon cancer cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen). Two days after seeding, they were incubated in serum free medium for 24 h as described previously (31).

**Cell proliferation assay**  
The cells (3 x 10³/well) were seeded onto 96-well plates and 24 h later, the cells were exposed to the indicated doses (0 to 200 J/m² [J]) of UV-C after the aspiration of the growth medium. The cells were then incubated in DMEM medium with 1% FCS in a humidified 5% CO₂ incubator at 37°C for 48 h. The remaining cells were finally counted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit I (Roche Diagnostics Co., Indianapolis, IN) in accordance with instructions of the manufacturer. BrdU incorporation was measured using cell Proliferation ELISA (BrdU; Roche Diagnostics Co.). The cells (7 x 10³/well) were seeded onto 96-well plates and 48 h later, the cells were exposed to the indicated doses (0 to 50 J) of
UV-C, just after the aspiration of the growth medium. The cells were then incubated in DMEM medium with 1% FCS for 24 h. They were then used for measurement according to the manufacturer’s protocol. All assays were done in triplicate.

**Clonogenic survival assay** The cells were grown in regular media to 70% confluence and exposed to UV-C, AG1478 and PD153035 at the indicated doses. Twenty four h after treatment, the cells were trypsinised and counted as usual. The cells (3 x 10^3) were re-seeded into fresh tissue culture dishes and incubated for 7 days. Fresh media were added at day 4. At day 7, the media were removed and the cells were fixed with 2 ml of clonogenic reagent (50% ethanol, 0.25% 1,9-dimethyl-methylene blue) for 45 min. They were then washed with PBS twice and counted the blue colonies.

**UV-C exposure** UV-C exposure of cells was performed in UVC 500 UV Crosslinker (GE Healthcare) by which we used 10-200 J/m^2 (J) of UV at 254 nm. After aspiration of the growth media, the cells were exposed to the indicated doses of UV-C in 20 seconds, and then incubated for the indicated times.

**Western blot analysis** The cells were lysed in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 2.5 mM sodium pyrophosphate, 50 mM NaF, 50 mM HEPES, 1 mM Na_3VO_4 and 2 mM phenylmethylsulfonyl fluoride (PMSF)] and scraped from the Petri dishes. Protein extracts were examined by Western blot analysis as previously described (32). The protein was fractionated and transferred onto an Immune-Blot PVDF Membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% fat-free dry milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) for 30 min before incubation with the indicated primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG were used as second antibodies. Peroxidase activity on the membrane was visualized on X-ray film by means of the ECL Western blot detection system.

**Immunofluorescence microscopy studies** Immunofluorescence microscopy studies were performed as described previously (25). The cells grown on coverslip-bottom dishes were first treated with either SB203580 or siRNA-p38 MAPK, followed by exposure to anti-EGFR antibodies for 15 min in RPMI containing 1 % bovine serum albumin (BSA). They were then exposed to UV-C (30 J) and incubated in DMEM without FCS for additional 30 min. They were then fixed with 4% paraformaldehyde for 10 min on ice and then exposed to 0.1% Triton X-100 for 10 min to permeabilize the cell membrane. They were followed by exposure to Alexa Fluor 546® conjugated donkey anti-mouse IgG antibodies (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Wako, Tokyo, Japan) for 1 h. The cells were then examined by fluorescence microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer’s protocol.

**siRNA protocol** Predesigned siRNAs targeting p38 MAPK (On-TARGET plus Duplex J-003512-20, Human MAPK14) was purchased from Thermo Fisher Scientific K.K. (Yokohama, Japan). Sequences are as follows; sense: GGAAUUAUGAGUGUAUUU and antisense: AUACACAUCAUUGAAUCCU. Transfection was performed according to the manufacturer's protocol (Bio-Rad, Tokyo, Japan). In brief, 5 μl of siLentFect (Bio-Rad) and finally 100 nM of siRNA were diluted with Opti-MEM, pre-incubated at room temperature for 20 min and then added to the culture medium containing 10% FCS. The cells were
incubated at 37°C for 48 h with siRNA–siLentFect complexes and subsequently harvested for preparation of Western blot analysis.

Quantification of cell surface EGFR by enzyme-linked immunosorbent assay (ELISA)
Quantification of cell surface EGFR was performed as described previously (31). In brief, SW480 cells were pretreated with the indicated compounds and then exposed to the mouse anti-EGFR antibody (Santa Cruz Biotechnology) that recognizes the extracellular domain of the EGFR (1:50 dilution), in DMEM containing 1% bovine serum albumin (BSA), for 15 min at 37°C. The cells were then incubated for the indicated times after exposure to UV-C (30 J) and then fixed with 4% paraformaldehyde for 10 min on ice. After blocking with 1% BSA in PBS for 1 h, the cells were exposed to an anti-mouse IgG, horseradish peroxidase-linked whole antibody (GE healthcare, Piscataway, NJ) for 1 h at room temperature, followed by washing four times with PBS containing 1% BSA. Finally, the cells were exposed to 50 μl of 1-stepTM Ultra TMB-ELISA reagent (Pierce, Rockford, IL) for 5 min at room temperature. Fifty μl of 2 M sulfuric acid was then added to each well to stop the reaction. The absorbance of each sample at 450 nm was then measured.

Densitometric analysis The densitometric analysis was performed using scanner and image analysis software (image J ver. 1.32). The background subtracted signal intensity of each protein signal was normalized by the respective control signal. All data were obtained from at least three independent experiments.

Detection of UV-C-caused DNA damage DNA damage caused by UV-C was examined by detection of phospho-H2A.X at Ser139 (γH2AX). The cells were pretreated with or without 1 μM of BIRB0790 for 1 h and then exposed to UV-C (30 J), followed by incubation for the indicated periods. They were then lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM Na3VO4 and complete protease inhibitor cocktail tablets (Hoffmann-La Roche Inc. Nutley, NJ)]. After sonication for 10 sec, the protein extracts were quantified and examined by Western blot analysis. As for immunofluorescence study, the cells grown on coverslip-bottom dishes were first treated with or without 1 μM of BIRB0790. They were then exposed to UV-C (30 J) and incubated for 3 h, followed by fixation, permeabilization, and exposure to anti-γH2AX for 1 h. They were then exposed to Alexa Fluor 546® conjugated donkey anti-rabbit IgG antibodies and DAPI for 1 h. Finally, the cells were examined by fluorescence microscopy.

Statistical analysis The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between the indicated pairs, and a p<0.05 was considered significant.

RESULTS

UV-C and EGFR kinase inhibitors inhibited colon cancer cell proliferation. We first investigated the effect of UV-C on the proliferation of SW480 cells using MTT. As shown in Fig. 1A, the IC₅₀ value of UV-C in SW480 cells was approximately 80 J. As well, BrdU incorporation assay revealed that SW480 cell proliferation was significantly suppressed when the cells were treated with the increasing dose of UV-C (Fig. 1B, black bar). We also observed similar effects of UV-C in HT29 and DLD-1, other colon cancer cells (Fig. 1B, gray and white bars). Therefore, it is likely that UV-C has suppressive effects on colon cancer cell proliferations. We have recently reported
that anti-EGFR neutralizing antibodies significantly suppressed cell growth in SW480 colon cancer cells (33). In this study, we used two kinds of EGFR kinase inhibitor, AG1478 and PD153035, and examined the inhibitory effect of EGFR kinase activity on colon cancer cell proliferation. MTT assay shows that these inhibitors suppressed cell proliferation (Fig. 1C). In addition, Fig. 1D revealed the suppressive effects of UV-C as well as EGFR kinase inhibitors on colony formations, indicating the reduction of capability of SW480 cells to survive and replicate (34).

The ternary complex of cyclin D/cyclin-dependent kinases (CDK)4 and p27 Kip1 requires extracellular mitogenic stimuli for the release and degradation of p27 concomitant with a rise in cyclin D levels to affect progression through the restriction point and phospho-Rb-dependent entry into S-phase (35), indicating that increasing levels of cyclin D1 and phospho-Rb promote cell cycle, resulting in cell proliferation. Since EGFR kinase inhibitors also suppressed the phosphorylation of Rb as well as the protein level of cyclin D1 (Fig. 1E), it is likely that EGFR signaling plays a critical role in proliferation of colon cancer cells.

**UV-C induced downregulation of EGFR and cell cycle arrest in colon cancer cells.** Overexpressed EGFR plays an oncogenic role in colon cancer cells and its downregulation is the most prominent regulatory system of EGFR signal attenuation (36). Whereas it has previously been reported that UV induces internalization and endosome arrest of EGFR in HeLa epidermal carcinoma cells (30,37), we investigated the effect of UV-C on EGFR signaling pathway in SW480 cells. In Fig. 2A, UV-C caused a marked decrease in the amount of cell surface EGFR within 10-30 min, while they were not affected in unstimulated SW480 cells. Moreover, UV-C markedly decreased the protein level of EGFR in SW480, HT29 and DLD-1 cells (Fig. 2B), thus suggesting that UV-C induces EGFR downregulation in colon cancer cells. Moreover, UV-C (30 J) time-dependently decreased the protein level of cyclin D1 in addition to phospho-Rb (Fig. 2C). As well, increasing dose of UV-C caused the decrease in both of cyclin D1 expression level and phospho-Rb, concurrently with the decrease in EGFR protein level (Fig. 2D). Therefore, it is probable that UV-C-induced EGFR downregulation is correlated to cell cycle arrest in colon cancer cells.

**UV-C induced phosphorylation of EGFR at Ser1046/7, not tyrosine residues in colon cancer cells.** In order to elucidate how UV-C causes EGFR downregulation in colon cancer cells, we examined the effects of UV-C on the phosphorylation of EGFR at several residues. While UV-C had slight, but no appreciable effect on phosphorylation of EGFR at Tyr1045 and Tyr1068, both of which are known as autophosphorylation sites (8-10), UV-C markedly induced phosphorylation of EGFR at Ser1046/7 in SW480 cells (Fig. 3). This phosphorylation at Ser 1046/7 appeared at 10 min after exposure to UV-C (30 J) and reached maximum at 60 min and decreased thereafter (Fig. 3A). In addition, this appeared when the cells were exposed to UV-C at a dose over 20 J (Fig. 3B).

**p38 MAPK was involved in the phosphorylation of EGFR at Ser 1046/7 and subsequent its internalization induced by UV-C in colon cancer cells.** In order to clarify how UV-C induces EGFR phosphorylation at serine residues, we next examined the effect of UV-C on the activations of MAPK cascades. UV-C induced phosphorylation of p44/p42 MAPK, SAPK/JNK and p38 MAPK within 10-20 min.

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in SW480 cells (Fig. 4A). These results led us to further investigate which protein kinase plays a critical role in the phosphorylation of EGFR at serine residues induced by UV-C in colon cancer cells.

While we showed that UV-C caused a rapid decrease in the amount of cell surface EGFR (Fig. 2A), we next examined whether UV-C also induces changes in the cellular localization of the EGFR in SW480 cells using fluorescence microscopy. As shown in Fig. 4B, EGFR was mainly localized on the cell surface (Fig. 4B, panel 1). As expected, UV-C caused the internalization of EGFR (Fig. 4B, panel 2) and moreover, when the cells were pretreated with SB203580, a p38 MAPK selective inhibitor (38), the internalization of EGFR was virtually inhibited (Fig. 4B, panel 4). We also obtained similar results using siRNA-p38 MAPK (Fig. 4C). These results strongly suggest that activation of p38 MAPK truly mediates the UV-C-effect on the cellular localization of EGFR in colon cancer cells.

As depicted in Fig. 4D, BIRB0790, another p38 MAPK-selective inhibitors (39) in addition to SB203580 significantly suppressed the phosphorylation of EGFR at Ser1046/7 induced by UV-C (Fig. 4D), although that was not suppressed when the cells were pretreated with PD98059 (40), SP600125 (41), which can inhibit the activation of MEK1/2 and SAPK/JNK, respectively (data not shown). To verify our results shown in Fig. 4D, we further performed siRNA experiment and observed that UV-C-caused phosphorylation of EGFR at Ser1046/7 was clearly suppressed by siRNA-p38 MAPK, whereas negative control (NC)-siRNA did not influence (Fig. 4E). We also verified that these findings were applicable in HT29 and DLD-1 cells (Fig. 4F). Together, we strongly suggest that phosphorylation of EGFR at Ser1046/7 induced by UV-C was also mediated through the p38 MAPK pathway in colon cancer cells.

**Phosphorylation of p38 MAPK induced by UV-C was mediated through TAK-1 in colon cancer cells.** TAK-1 is activated by TGFβ as well as cytokines, including interleukin-1 (42,43). TAK-1 is also known to be a kinase of MAPK kinase (MKK) 3/6 (44-46). Moreover, MKK3 can phosphorylate p38 MAPK (47). Therefore, we examined the effect of UV-C on TAK-1 in order to examine the upstream signaling for activation of p38 MAPK. Of interest, UV-C induced phosphorylation of TAK-1 as well as MKK3/6 (Fig. 5A). Moreover, the inhibition of TAK-1 using (5Z)-7-oxozeaenol restored UV-C-induced phosphorylation of EGFR (Ser1046/7), MKK3/6 and p38 MAPK (Fig. 5B). Therefore, it is most likely that UV-C induces activation of p38 MAPK via TAK-1.

**Degradation of EGFR and cyclin D1 induced by UV-C was blocked by the inhibition of p38 MAPK in colon cancer cells.** We next investigated the involvement of p38 MAPK in UV-C-induced degradation of EGFR and cyclin D1. As depicted in Fig. 6A, the protein level of both EGFR and cyclin D1 were decreased when the cells were treated with UV-C (Fig. 6A, lane 2 compared to lane 1), consistently with the above results shown in Fig. 2B, 2C and 2D. As expected, pretreatment of the cells with SB203580 significantly inhibited UV-C-induced degradation of cyclin D1 as well as EGFR (Fig. 6A, lane 4, compared to lane 3). Similarly, the protein level of both EGFR and cyclin D1 were not decreased by UV-C in the cells that were transfected with p38 MAPK-siRNA (Fig. 6B, lane 4 compared to lane 3), in comparison with the cells transfected with negative control (Fig. 6B, lane 2 compared to lane 1). These results strongly suggest that activation of p38 MAPK plays a critical role in
the downregulation of EGFR induced by UV-C in colon cancer cells. 

**Pretreatment with UV-C can protect colon cancer cells from EGF stimulation.** It is well known that exposure of cells to EGF stimulation results in rapid autophosphorylation of EGFR molecules at the cell surface (7-10), which upon activation lead to cell proliferation, motility and enhanced survival (11). Moreover, through EGF-binding to cell surface EGFR, it activates an extensive network of signal transduction pathways including Akt pathways, which regulates multiple biological processes including survival, proliferation, and cell growth (48,49). We next investigated the effect of pretreatment with UV-C on EGF-induced phosphorylation of EGFR and Akt in SW480 cells. When the cells were not exposed to UV-C or EGF, EGFR at any residues and Akt were not phosphorylated (Fig. 7A, lane 1). Indeed, UV-C caused phosphorylation of EGFR at Ser1046/7 (Fig. 7A, second panel), consistently with the above results shown in Fig. 3. Interestingly, while stimulation with EGF caused the phosphorylation of EGFR at tyrosine residues and Akt (Fig. 7A, first and third panels, lane 2, respectively), these phosphorylations were significantly suppressed when the cells were exposed to the increasing doses of UV-C before EGF stimulation (Fig. 7A: first and third panels and 7B). In cases of HT29 and DLD-1 cells, UV-C also had the suppressive effects on EGF-induced activation of EGFR (Fig. 7B and 7C). These results strongly suggest that UV-C caused the evasion of colon cancer cells from EGF-stimulation because UV-C can remove EGFR from cell membrane. 

**DNA damage is not involved in UV-C-induced EGFR downregulation in colon cancer cells.** DNA damage, provoked by ultraviolet (UV) radiation, evokes a cellular damage response composed of activation of stress signaling and DNA checkpoint functions (29). In addition, DNA double-strand breaks introduced into mammalian cells results in the specific phosphorylation of histone H2A.X at Ser139, named γH2AX (50). We examined whether DNA damage correlates to UV-C-induced EGFR downregulation via p38 MAPK. While BIRB0790 clearly inhibited phosphorylation of p38 MAPK (Fig. 8B), immunofluorescence study and Western blotting showed that it had little effect on UV-C-induced γH2AX (Fig. 8A and 8B). Therefore, it seems unlikely that DNA damage is involved in UV-C-induced EGFR downregulation via p38 MAPK.

**DISCUSSION**

Whereas UV irradiation reportedly has many effects on skin, including inflammation, immunosuppression, and alterations in the extracellular matrix, in addition to accelerated skin aging (51), in the present study we demonstrated that UV-C has a potent anti-cancer effect by decreasing EGFR protein level in colon cancer cells. First, we showed that UV-C caused anti-survival and proliferative effects on SW480, HT29 and DLD-1 cells (Fig. 1). Since EGFR activation has been shown to be oncogenic (9) and we here showed that colon cancer cell proliferation depended on EGFR signaling (Fig. 1C, 1D and 1E), we examined the effect of UV-C on the EGFR signaling and found that UV-C induced internalization and subsequent downregulation of the EGFR as well as the decreased the protein level of cyclin D1 and phospho-Rb (Fig. 2), suggesting that the anti-cancer effect of UV-C could be due to cell cycle arrest in colon cancer cells.

In addition, we showed in Fig. 3 that UV-C caused a marked phosphorylation of EGFR at Ser1046/7, however, UV-C failed to induce phosphorylation at Tyr1045, the major c-Cbl...
binding site, as well as Tyr1068, Grb2 adaptor protein binding site (8), in contrast to the prominent phosphorylation induced by EGF as we have previously shown (25). Moreover, while UV-C caused activation of either p44/p42 MAPK, p38 MAPK or SAPK/JNK (Fig. 4A), we observed that the inhibition of p38 MAPK suppressed EGFR internalization (Fig. 4B and 4C). In addition, p38 MAPK was involved in the phosphorylation at Ser1046/7 (Fig. 4D, 4E and 4F) and subsequent degradation (Fig. 6) of the EGFR induced by UV-C. Moreover, UV-C-induced activation of p38 MAPK was mediated through TAK-1 (Fig. 5). We also examined the effect of UV-C on apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase, because ASK1 is activated in response to a variety of stress-related stimuli and activates MKK3, which in turn activate p38 MAPK (52). However, UV-C had no appreciable effect on phosphorylation of ASK1 at Ser967 and Thr845 (data not shown). Furthermore, we found in colon cancer cells that pretreatment with UV-C before EGF stimulation significantly suppressed the phosphorylation of EGFR at tyrosine residues and Akt (Fig. 7), indicating that UV-C can evade cells from oncogenic stimulation of EGF. In addition, as shown in Fig. 8, it seems unlikely that DNA damage is involved in UV-C-induced EGFR downregulation via p38 MAPK. However, our present findings do not evaluate and cannot completely eliminate the possibility that DNA damage plays a role in UV-C-induced cell cycle arrest.

Whereas we have recently reported that the blockade of EGF stimulation significantly suppressed cell growth (33), we herein demonstrated that proliferation of colon cancer cells depended on the EGFR kinase activity, thus suggesting that the desensitization of EGFR signaling is a promising target against human colon cancer. In addition, an early work showed that exposure to UV light induced clustering and internalization of cell surface EGFR, and inhibition of clustering or receptor down-regulation attenuates UV responses (53), which is consistent with our present findings that internalization and subsequent degradation of EGFR induced by UV-C leads to cell cycle arrest of colon cancer cells by causing its phosphorylation at serine residues via p38 MAPK. Since it is generally understood that tyrosine phosphorylation results in cancer cell proliferation (9), these results also suggest the potential availability of UV-C for human colon cancer therapy because UV-C can cause EGFR downregulation without oncogenic activation (Fig. 3). Moreover, while Zwang Y et al. previously reported that abrogating EGFR internalization reduces the efficacy of chemotheraphy-induced cell death and EGFR internalization enhances the cytotoxic effect of cisplatin by preventing EGFR mediated survival signaling, which may underlie interactions between chemotherapy and EGFR-targeting drugs (30). Therefore, our findings also provide a possibility of new combination of conventional chemotherapy and UV-C for human colon cancer.

Our present study combined with previous findings is summarized in Fig. 9 as follows; when the cells are exposed to EGF stimulation, EGFR undergoes dimerization and tyrosine phosphorylation that directs the cells into cell proliferation (9). Subsequently, c-Cbl, a ubiquitin ligase, can bind to EGFR, and cause ubiquitination and degradation of the EGFR (10,54). By contrast, UV-C has little effect on EGFR phosphorylation at tyrosine residues, indicating that adequate dose of UV-C fails to exert the cell growth signals. However, UV-C
induced serial phosphorylation of TAK1, MKK3/6 and p38 MAPK and subsequent phosphorylation of EGFR at Ser1046/7. With time EGFR molecules are internalized and eventually degraded. Therefore, pretreatment of the cells with UV-C can protect colon cancer cells from oncogenic stimulation such as EGF.

We have previously reported that EGCG as well as HSP90 inhibitors cause downregulation of the EGFR via phosphorylation at Ser1046/7 through p38 MAPK in human cancer cells (24,26). Additionally, accumulating evidence shows that activation of p38 MAPK has an inhibitory effect on tumorgenesis (55,56) and that a variety of agents, such as gemcitabine (5) and cisplatin (20) can also induce activation of p38 MAPK and internalization of EGFR into endosomal vesicles. Moreover, it has previously been reported that the Ser1046/7 phosphorylation sites act to suppress the oncogenic signal transduction by the wild-type EGFR (21,23). Hence, our present findings might provide a new therapeutic strategy for human colon cancer, although further investigations are necessary to elucidate the mechanism underlying EGFR downregulation via its phosphorylation at serine residues.

Regarding our concern how these findings would be translated into the clinic, current limitation of the present study is the lack of practical tool to deliver UV-C irradiation onto the colon cancer tissue in the human body. Our hypothetical approaches would include a combination of extracorporeal generator and fiberscopic transmission of UV-C through colon endoscope, or possible expansion of light emitting diode (LED) technique to exert shorter-wavelength UV-C than currently available UV-A-LED (http://www.nitride.co.jp/). However, substantial technical advances and some related time would be essentially required before practical application of UV-C for colon cancer management. Moreover, further studies are required to determine whether these effects of UV-C safely occur in vivo.

In summary, we found that UV-C induces EGFR downregulation via p38 MAPK-mediated EGFR phosphorylation at Ser1046/7. Moreover, our results strongly suggest that UV-C irradiation induces the removal of EGFR from cell surface that can evade colon cancer cells from oncogenic stimulation of EGF, resulting in cell cycle arrest in colon cancer cells.

**Competing interests**

No conflict of interests exists in this manuscript.

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FOOTNOTES

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The abbreviations used are: UV-C; ultraviolet-C, EGF; epidermal growth factor, EGFR; EGF receptor, MAPK; mitogen activated protein kinase, EGCG; (-)-epigallocatechin gallate, MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Rb; retinoblastoma protein

FIGURE LEGENDS

Fig. 1. UV-C as well as EGFR kinase inhibitors inhibited cell survival and proliferation in colon cancer cells. (A) SW480 cells were exposed to UV-C for 48 h under medium containing 3% FCS and then the remaining cells were counted by MTT cell proliferation kit I. Results are expressed as percentage of growth with 100% representing untreated control cells. (B) The indicated cells (SW480, HT29 and DLD-1) were exposed to UV-C for 24 h under medium containing 3% FCS and the measurement of BrdU incorporation during DNA synthesis were performed using cell proliferation ELISA (BrdU). Results are expressed as the absorbance (OD 405nm-492 nm). (C) SW480 cells were treated with 1 μM of AG1478, 100 nM of PD153035 or vehicle for 4 days under medium containing 3% FCS and then the remaining cells were counted by MTT cell proliferation kit I. (D) The attached SW480 cells were exposed to 30 J of UV-C, 1 μM of AG1478 or 100 nM of PD153035, and incubated for 24 h. After trypsinisation, the counted cells (3 x 10^3) were re-seeded into new culture dishes and incubated for 7 days. The cells were then fixed with clonogenic reagent (see Experimental Procedures) and the average number of colony from 5 randomly chosen fields (x 80) were counted, respectively. (E) SW480 cells were treated with 1 μM of AG1478 or 100 nM of PD153035 for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against phospho-specific Rb, cyclin D1, and GAPDH. All assays were done in triplicate. Bars designate SD of triplicate assays. The asterisk indicates significant difference (p<0.05), compared with the control, respectively.

Fig. 2. UV-C caused downregulation of the EGFR and cell cycle arrest in colon cancer cells. (A) UV-C induced the decrease in the cell surface amount of EGFR in SW480 cells. Line graph shows quantification data for the cell surface amount of EGFR analyzed by ELISA (see in Experimental Procedures). Open circle (○): unstimulated SW480 cells. Closed circle (●): SW480 cells exposed to 30 J of UV-C. (B) The indicated cells (SW480, HT29 and DLD-1) were exposed to UV-C at a dose of 30 J and then incubated for the indicated periods. (C) SW480 cells were exposed to 30 J of UV-C for the indicated periods. (D) SW480 cells were exposed to UV-C at the indicated doses and then incubated for 24 h. Protein extracts were then harvested and examined by Western blotting using antibodies against EGFR, phospho-specific Rb, cyclin D1, and GAPDH. Representative results from triplicate independent experiments with similar results are shown.
Fig. 3. Effect of UV-C on the phosphorylation of EGFR at Ser1046/7 in colon cancer cells. (A) SW480 cells were exposed to UV-C at a dose of 30 J for the indicated periods. (B) SW480 cells were exposed to UV-C at the indicated dose and then incubated for 1 h. Protein extracts were then harvested and examined by Western blotting using anti-phospho-EGFR at Tyr1045, Tyr1068 and Ser1046/7, anti-EGFR and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

Fig. 4. Effects of p38 MAPK on the internalization and phosphorylation of EGFR at Ser1046/7 in colon cancer cells. (A) SW480 cells were exposed to UV-C at 30 J and then incubated for the indicated periods. Protein extracts were then harvested and examined by Western blotting using anti-phospho-p44/p42 MAPK, anti-p44/p42 MAPK, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-SAPK/JNK, anti-SAPK/JNK and anti-GAPDH antibodies. (B) SW480 cells were pretreated with or without 10 μM SB203580 for 1 h and then labeled for 15 min at 37°C with anti-EGFR antibodies which recognizes the extracellular domain of the EGFR. (C) SW480 cells were incubated with 100 nM of p38 MAPK-siRNA or negative control (NC)-siRNA at 37°C for 48 h in FCS-free opti-MEM and then labeled for 15 min at 37°C with anti-EGFR antibodies. They were then exposed to UV-C (30 J) and incubated for additional 1 h, followed by fixation with paraformaldehyde. After permeabilization of the cells with 0.1% Triton X-100, the cells were treated with Alexa 546 conjugated anti-mouse secondary antibody for EGFR (red signal) and DAPI (blue signal) for 1 h, and then examined by fluorescence microscope. (D) SW480 cells were pretreated with 10 μM of SB203580 or 1 μM of BIRB0790 for 1 h and then exposed to UV-C at 30 J and then incubated for additional 1 h. (E) SW480 cells were incubated with 100 nM of p38 MAPK-siRNA or negative control (NC)-siRNA at 37°C for 48 h in FCS-free opti-MEM, followed by exposure to UV-C (30 J) for 1 h. (F) HT29 and DLD-1 cells were pretreated with 1 μM of BIRB0790 for 1 h and then exposed to UV-C at 30 J and then incubated for additional 1 h. Protein extracts were then prepared and examined by Western blotting using antibodies against phospho-EGFR at Ser1046/7, EGFR, phospho-p38 MAPK and p38 MAPK, respectively. An antibody to GAPDH was used to control for protein loading. Representative results from triplicate independent experiments with similar results are shown.

Fig. 5. The involvement of TAK-1 in UV-C-induced activation of p38 MAPK in colon cancer cells. (A) SW480 cells were exposed to UV-C at 30 J and then incubated for the indicated periods. (B) SW480 cells were pretreated with or without the 300 nM of (5Z)-7-oxozeaenol, as a TAK-1 inhibitor, for 2 h and then exposed to UV-C (30 J) and incubated for additional 30 min. Protein extracts were then harvested and examined by Western blotting using anti-EGFR, phospho-EGFR (Ser1046/7), phospho-TAK1, anti-MKK3/6, anti-p38 MAPK and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

Fig. 6. The inhibition of p38 MAPK restored UV-C-induced degradation of the EGFR in colon cancer cells. (A) SW480 cells were pretreated with or without 10 μM of SB203580 for 1 h and then exposed to UV-C at 30 J, followed by incubation for 8 h. (B) SW480 cells were incubated with 100
nM of p38 MAPK-siRNA or negative control (NC)-siRNA at 37°C for 48 h in FCS-free opti-MEM, followed by exposure to UV-C (30 J). Protein extracts were then prepared and examined by Western blotting using anti-EGFR and anti-cyclin D1 antibodies respectively. An antibody to GAPDH was used to control for protein loading. The lower bar graph shows quantification data for the relative levels of EGFR, after normalization with respect to GAPDH, as determined by densitometry. The asterisks indicate significant increase (*p<0.05 compared to lane 1 and **p<0.05 compared to lane 2). Representative results from triplicate independent experiments with similar results are shown.

Fig. 7. Effects of exposure to UV-C before EGF-stimulation on the phosphorylation of EGFR in colon cancer cells. (A) SW480 cells were pretreated with UV-C at the indicated doses of UV-C for 1 h and then exposed to 5 ng/ml of EGF for another 5 min. Protein extracts were then harvested and examined by Western blotting using anti-phospho-EGFR at Tyr1068 and Ser1046/7, anti-phospho-Akt and anti-EGFR antibodies. The bar graph shows quantification data for the relative phosphorylation levels of EGFR and Akt, after normalization with respect to EGFR, as determined by densitometry. *p<0.05, compared to the control (EGF-induced phosphorylation of EGFR at Tyr1068 in lane 2) and **p<0.05, compared to the control (EGF-induced phosphorylation of Akt in lane 2). (B and C) HT29 (B) and DLD-1 (C) cells were pretreated with UV-C at the indicated doses of UV-C for 1 h and then exposed to 5 ng/ml of EGF for another 5 min. Protein extracts were then harvested and examined by Western blotting using anti-phospho-EGFR at Tyr1068, anti-EGFR and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

Fig. 8. The involvement of DNA damage in UV-C-induced activation of p38 MAPK in colon cancer cells. (A) SW480 cells grown on coverslip-bottom dishes were pretreated with or without 1 μM of BIRB0790 and then exposed to UV-C (30 J) and incubated for another 3 h and the cells were examined by fluorescence microscopy. Red signals; γH2AX, Blue signals; DAPI. (B) SW480 cells were pretreated with or without 1 μM of BIRB0790 for 1 h and then exposed to 30 J of UV-C, followed by incubation for another 3 h. Protein extracts were then harvested and examined by Western blotting using anti-γH2AX, anti-phospho-p38 MAPK and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

Fig. 9. Schematic representation of the effect of UV-C on EGF-induced proliferation of colon cancer cells. After EGF binds to cell surface EGFR, it undergoes dimerization and autophosphorylation at tyrosine residues, and this triggers EGFR-related downstream signaling, leading to cell proliferation (7-10). By contrast, UV-C causes serial phosphorylation of TAK1, MKK3/6 and p38 MAPK, and subsequent phosphorylation of EGFR at S1046/7, but not tyrosine residues and the serine-phosphorylated EGFR are internalized and eventually degraded. Therefore, UV-C can escape colon cancer cells from oncogenic stimulation of EGF, since EGF hardly binds to the internalized EGFR caused by UV-C.
Figure 1

(A) Cell viability (% of control) vs. UV-C (J)

(B) BrdU incorporation (% of control) vs. UV-C (J)

(C) Cell viability (absorbance 550-550 nm) vs. Day

(D) Number of colony / hpf (x10)

(E) Western Blot for phospho-Rb, cyclin D1, GAPDH, AG1478 (1 µM), PD153035 (100 nM)
Figure 2

(A) Cell surface EGFR (\% of control) vs. Time post UV-C (min)

(B) EGFR and GAPDH expression in SW480, HT29, and DLD-1 cells at different time points post UV-C.

(C) Western blot analysis of phospho-Rb, cyclin D1, and GAPDH in cells at different times post UV-C.

(D) EGFR, phospho-Rb, cyclin D1, and GAPDH expression in cells treated with different doses of UV-C.
Figure 3

(A) phospho-EGFR (Tyr1045)  
phospho-EGFR (Tyr1068)  
phospho-EGFR (Ser1046/7)  
EGFR  
GAPDH  
Time post UV-C (min) 0 1 5 10 20 30 60 120

(B) phospho-EGFR (Tyr1045)  
phospho-EGFR (Tyr1058)  
phospho-EGFR (Ser1046/7)  
EGFR  
GAPDH  
UV-C (J) (30 min) 0 10 20 30 50 70 100 200
Figure 4

(A) phopho-p44/p42
p44/p42
phospho-p38
p38
phospho-SAPK/JNK
SAPK/JNK
GAPDH
Time post UV-C (min) 0 1 5 10 20 30 60 120

(B) - UV-C + UV-C
- SB203580 1 2
- SB203580 3 4

(C) - UV-C + UV-C
negative control-siRNA

(D) - UV-C + UV-C
SW480
phospho-EGFR (Ser1046/7)
EGFR
phospho-p38
GAPDH
UV-C (30 J) DMSO SB203580 BIRB0790

(E) SW480
phospho-EGFR (Ser1046/7)
EGFR
p38 MAPK
GAPDH
UV-C (30 J) siRNA NC p38 MAPK

(F) HT29
phospho-EGFR (Ser1046/7)
EGFR
phospho-p38
GAPDH
UV-C (30 J) DMSO BIRB0790

DLD-1
phospho-EGFR (Ser1046/7)
EGFR
phospho-p38
GAPDH
UV-C (30 J) DMSO BIRB0790

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Figure 5

(A) 
phospho-TAK1
phospho-MKK3/6
GAPDH
Time post UV-C (min) 0 1 5 10 20 30 60 120

(B) 
EGFR
phospho-EGFR (S1046/7)
phospho-MKK3/6
phospho-p38
GAPDH
UV-C - + - +
(5Z)-7-oxozeaenol - - + +
Figure 6

(A) EGFR  
  cyclin D1  
  GAPDH

EGFR level (% of control)

| Lane | UV-C | SB203580 (μM) |
|------|------|--------------|
| 1    | -    | 0            |
| 2    | +    | 0            |
| 3    | -    | 10           |
| 4    | +    | 10           |

(B) EGFR  
  cyclin D1  
  GAPDH

EGFR level (% of control)

| Lane | UV-C | siRNA |
|------|------|-------|
| 1    | -    | NC    |
| 2    | +    | p38 MAPK |
| 3    | -    |       |
| 4    | +    |       |
Figure 7

(A) SW480

| Lane | UV-C (J) | EGF (5 ng/ml) |
|------|----------|---------------|
| 1    | 0        | -             |
| 2    | 0        | +             |
| 3    | 10       | +             |
| 4    | 30       | +             |
| 5    | 50       | +             |
| 6    | 100      | +             |
| 7    | 200      | +             |

(B) HT29

| Lane | UV-C (J) | EGF (5 ng/ml) |
|------|----------|---------------|
| 1    | 0        | -             |
| 2    | 0        | +             |
| 3    | 10       | +             |
| 4    | 30       | +             |

(C) DLD-1

| Lane | UV-C (J) | EGF (5 ng/ml) |
|------|----------|---------------|
| 1    | 0        | -             |
| 2    | 0        | +             |
| 3    | 10       | +             |
| 4    | 30       | +             |
| 5    | 50       | +             |
| 6    | 100      | +             |
Figure 8

(A) - BIRB0790
- UV-C + UV-C

+ BIRB0790

Blue: DAPI
Red: γH2AX

(B) γH2-AX
phospho-p38
GAPDH

BIRB0790 (1 μM)
Time post UV-C (h)
- - - + + + +
0 1 3 6 0 1 3 6
Figure 9

EGFR activation → cell proliferation

Ser1046/1047 → internalization → escape from EGF → cell cycle arrest

TAK1 → MKK3/6 → p38 MAPK
Ultraviolet irradiation can induce evasion of colon cancer cells from stimulation of epidermal growth factor

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