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REVIEW

Challenging the effectiveness of green tea in primary and tertiary cancer prevention

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Abstract
Purpose Drinking green tea daily is part of Japanese culture, and various studies have revealed that green tea is a cancer preventive. We here review our progress in cancer prevention with green tea on 12 main topics, from basic to clinical level.
Topics and methods Biochemical and biological studies of green tea catechins, a prospective cohort study, preclinical safety trials with tablets of green tea extract, double-blind randomized clinical phase II prevention trial for recurrence of colorectal adenomas, and synergistically enhanced inhibition by the combination of green tea catechins and anticancer drugs. All results were significant, including human studies with informed consent.
Results Drinking 10 Japanese-size cups of green tea per day delayed the cancer onset of humans 7 years for females. For tertiary cancer prevention, consuming 10 cups of green tea per day fortified by green tea tablets, 50 %, significantly prevented the recurrence of colorectal adenomas. A minimum effective amount of green tea catechins for cancer prevention was found in humans. In addition, the combination of green tea catechins and anticancer drugs engendered a new cancer therapeutic strategy.
Conclusion The consumption of 10 Japanese-size cups of green tea per day is a significant factor in primary cancer prevention for the general population, and the preventive effect on recurrence of colorectal adenomas in patients is vital evidence in tertiary cancer prevention.

Keywords Apoptosis · Delayed cancer onset · GADD153 · Green tea tablet · Phase II prevention trial · Prospective cohort study · TNF-α

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Introduction

Japanese have the longest life span in the world, 86 years for females and 79 years for males. Even so, cancer mortality increases after age 60 and is the highest for age groups around 80 years of age for both males and females (Fig. 1). The multistage carcinogenesis study of B. Vogelstein revealed that the first neoplastic change in cells in human colon development starts many years earlier, probably 20–30 years earlier than the clinical appearance of cancer in patients (Vogelstein et al. 1988). M. Sporn coined the term "Cancer chemoprevention," and defined it as "prevention of the occurrence of cancer by administration of one or several compounds (Sporn et al. 1976)." Today, it is generally accepted that it is possible to delay the clinical appearance of cancer by slowing down the development of cancer. At present, for example, 50 % of breast cancer of high-risk group can be prevented by the administration of tamoxifen, in the United States (Fisher et al. 1998).

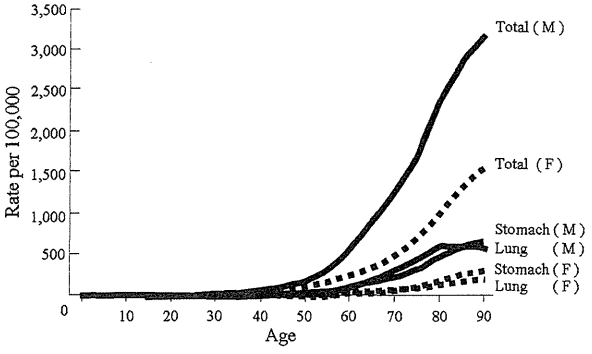


Fig. 1 Cancer mortality by age in Japan (1997). *M* male, *F* female

When research in cancer chemoprevention began in Japan in 1983, we were lucky to receive tannins or polyphenols isolated from medicinal plants and herbs from T. Okuda at Okayama University (Okuda et al. 1985). To screen for cancer preventive agents, we tried to find inhibitors of tumor promotion in two-stage carcinogenesis experiments on mouse skin, consisting of initiation and tumor promotion. The three diterpene ester tumor promoters discovered by E. Hecker were very useful for our further study (Hecker et al. 1984). From a total of 30 polyphenols, (−)-epigallocatechin gallate (EGCG) and penta-*O*-galloyl-*β*-D-glucose (5GG) showed binding to the phorbol ester receptor and inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced activation of protein kinase C (PKC). In 1987, we first reported, in the British journal Phytotherapy Research, that EGCG prevented tumor promotion of teleocidin, one of the TPA-type tumor promoters on mouse skin (Yoshizawa et al. 1987). Later, we also confirmed that repeated applications of 5 mg EGCG completely prevented tumor promotion of okadaic acid (Yoshizawa et al. 1992), a potent tumor promoter working through the inhibition of protein phosphatases 1 and 2A. The tumor-promoting activity by okadaic acid is as potent as TPA (Suganuma et al. 1988).

After we published the results in 1987, the Journal "New Scientist" soon introduced our results with EGCG in an article entitled "Green tea cuts cancerous growths" (November 12, 1987). In winter of 1987, an Australian TV scientific series entitled "Beyond 2000" visited us at the National Cancer Center Research Institute in Tokyo to make a TV film about our research. This 8-min-long TV film was shown all over the world in 1988 and 1989, and many overseas scientists took greater interest in cancer prevention with green tea than Japanese scientists. A putative carcinogenic potential for tannins or polyphenols suspected previously was eliminated by establishing the non-mutagenicity of EGCG and green tea catechins (Okuda et al. 1984), and by EGCG inhibition of TPA-induced activation of PKC (Yoshizawa et al. 1987). Moreover, EGCG and green tea extract are non-toxic for rodents and humans (Japanese). These findings encouraged us to intensively study cancer prevention by green tea (Fujiki and Suganuma 2002; Fujiki 2005).

Results and discussion

Green tea and green tea extract

Green tea is made from steamed fresh tea leaves and is a non-oxidized non-fermented product containing at least four green tea catechins—EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (−)-epicatechin gallate (ECG)—and caffeine on high-performance liquid chromatography (HPLC). EGCG is the main constituent (Fujiki and Okuda 1992). In addition to EGCG, we used for our experiments green tea extract, the dried green tea infusion containing all green tea catechins, a condensed form of green tea, and Japanese have been drinking green tea for 800 years. Green tea catechins are active in preventing carcinogenesis in a wide range of target organs, including the digestive tract, lung, liver, pancreas, breast, bladder, prostate and skin in rodents (Fujita et al. 1989; Yamane et al. 1991, 1995; Conney et al. 1992; Wang et al. 1992; Narisawa and Fukaura 1993; Yang and Wang 1993; Nishida et al. 1994; NCI et al. 1996; Fujiki et al. 1996; Gupta et al. 2001). Moreover, drinking 0.05 and 0.1 % EGCG solutions significantly prevented the spontaneous metastasis of B16-BL6 cells from foot pad to the lungs of male C57BL/6 mice (Taniguchi et al. 1992). It is interesting to note that green tea catechins are active in most of these organs.

Tissue distribution of ^{3}H-EGCG

To prove the systemic effects of EGCG, ^{3}H-EGCG was intubated into the stomach of mice. The microautoradiography of the lungs showed silver grains of radioactive EGCG in some cells, but not all (Suganuma et al. 1998), apparent confirmation that EGCG was incorporated from the digestive tract into the lungs, one of the target organs. Microautoradiography of human lung cancer cell line PC-9 cells treated with ^{3}H-EGCG showed silver grains in the membrane, cytosol and nuclei, and that ^{3}H-EGCG had been incorporated from culture medium into the cells (Okabe et al. 1997). Using cold spray ionization-mass spectrometry and surface plasmon resonance assay (Biacore), the direct binding of EGCG to single-strand 18 mers of DNA and RNA was demonstrated (Kuzuhara et al. 2006). The incorporation of ^{3}H-EGCG into target organs is shown in Table 1. Various amounts of the total administered radioactivity were found in the digestive tract, liver, brain, kidney, lung, pancreas and skin 24 h after intubation

Table 1 Incorporation of ^{3}H-EGCG into target organs

Organs	% of total administered radioactivity (24 h after)	Reduction in tumor incidence
Stomach	3.93	62.0 → 31.0
Duodenum	0.35	63.0 → 20.0
Small intestine	5.69	ND
Colon	4.52	77.3 → 38.1
		67.0 → 33.0[a]
Liver	0.89	83.3 → 52.2
Brain	0.32	ND
Kidney	0.28	ND
Lung	0.16	96.3 → 65.5
Pancreas	0.07	54.0 → 28.0
Skin	1.9×10^4/100 mg[b]	65.0 → 28.0[a]

ND, not determined

[a] Green tea extract

[b] dpm

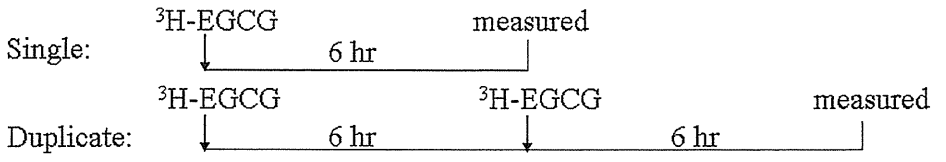
(Suganuma et al. 1998). The right column shows the reduction in tumor incidence in carcinogenesis by EGCG and green tea extract in rodents (Table 1) (Fujiki et al. 1996). Thus, radioactivity was found in the organs where EGCG and green tea extract had previously been shown to inhibit carcinogenesis.

Significance in multiple administrations of EGCG

Japanese drink green tea throughout the day. To study the effects of frequent consumption, ^{3}H-EGCG was intubated into the stomach of mice at 6-h intervals. Duplicate administrations enhanced incorporation of ^{3}H-EGCG 4- to 9-fold in most organs compared with a single administration, suggesting the accumulation of EGCG in cells. Radioactivity in blood and urine increased as well. We named this synergistic enhancement by EGCG the "Fujiki-Suganuma Effect," which is supported by frequent drinking of green tea (Table 2) (Suganuma et al. 1998). To further study the effects on enhanced incorporation of EGCG by multiple administrations, the induction of *growth arrest and DNA damage-inducible 153 (GADD153)* and *p21* gene expressions in human lung cancer cell line A549 cells was determined at 6-h intervals. *GADD153* (*CHOP*) is an apoptosis-regulating gene. The overexpression of *GADD153* gene induces apoptosis of the cells and leads to antiproliferative effects (Novoa et al. 2001), and EGCG was reported to induce apoptosis of the cells (Okabe et al. 1997; Ahmad et al. 1997; Yang et al. 1998; Okabe et al. 1999). *p21* is an inhibitor of *cyclin-dependent kinase* gene. The upregulation of *p21* gene inhibits proliferation by blocking the cell cycle (El-Deiry et al. 1994). And multiple treatments with EGCG induced enhancement of *GADD153* and *p21* gene expressions (Fig. 2) (Kuzuhara et al. 2007a).

Table 2 Enhanced incorporation of ^{3}H-EGCG by duplicate administrations: the "Fujiki-suganuma effect"

	Total radioactivity ($\times 10^4$ dpm)		Fold increase
	Single	Duplicate	
Blood (/ml)	25.13	149.40	×5.9
Brain	3.00	20.29	×6.8
Lung	3.47	16.14	×4.7
Liver	18.94	87.06	×4.6
Kidney	4.68	14.68	×3.1
Spleen	1.33	2.03	×1.5
Pancreas	0.77	3.29	×4.3
Uterus and ovary	2.04	7.23	×3.5
Bladder	0.14	0.74	×5.3
Mammary gland (100 mg)	0.29	0.72	×2.5
Bone (100 mg)	0.96	4.50	×4.7
Skin (100 mg)	3.41	4.91	×1.4



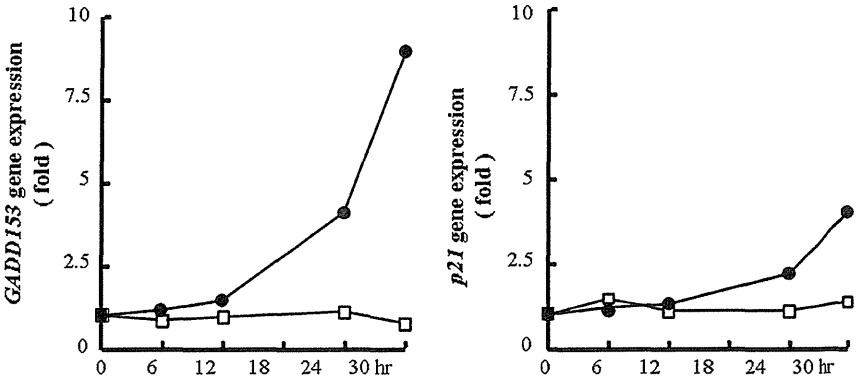


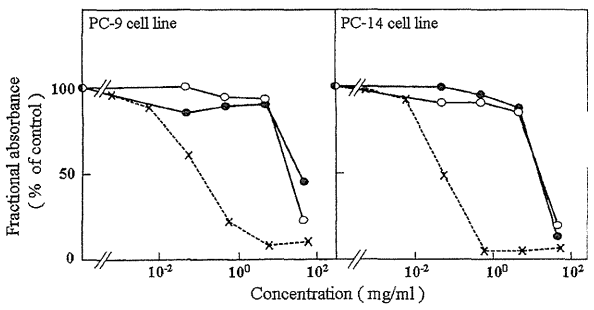
Fig. 2 The induction of *GADD153(L)* and *p21(R)* gene expressions in A549. Multiple treatments with EGCG (*filled circle*) at 6-h intervals and single treatment with EGCG (*open circle*)

We think that multiple treatments with EGCG induce synergistic effects on apoptosis in cells and that frequent drinking of green tea plays an important role in cancer prevention.

Inhibitions of in vitro cell growth and TNF-α release, and induction of apoptosis by green tea catechins

Various green tea catechins inhibited the growth of PC-9 cells, dose-dependently, with the order of potency being ECG, EGCG and EGC; EC was not effective (Okabe et al. 1997). Since the activity of EGCG was relatively weak, we compared the IC_{50} value of EGCG with that of the anticancer drug adriamycin for growth inhibition of two human lung cancer cell lines, PC-9 and PC-14. The IC_{50} value for adriamycin was 0.17 μM and that for EGCG was 41.5 μM (Fig. 3) (Komori et al. 1993a), which means that EGCG is approximately 250-fold less effective than adriamycin.

Although EGCG and green tea extract have very weak anticancer activity, they are unique in having multifunctions: inhibition of cell growth and TNF-α release from the cells induced by okadaic acid, a tumor promoter and



PC-9	IC_{50} (μM)	
EGCG	41.50	(244×)
Adriamycin	0.17	(1)

Fig. 3 Growth inhibition of two human lung cancer cell lines, PC-9 and PC-14 by EGCG (*open circle*), green tea extract (*filled circle*) and adriamycin (*times symbol*)

induction of apoptosis with green tea catechins (Table 3) (Suganuma et al. 1999a). EGCG, ECG and EGC were active in all three tests, while EC was inactive. However, cotreatment of EC with EGCG, ECG or EGC synergistically enhanced inhibition of TNF-α release from the cells (Table 4) (Suganuma et al. 1999a). Since we consider TNF-α to be an endogenous tumor promoter, EGCG inhibition of TNF-α release is a key mechanism in cancer prevention (Suganuma et al. 1999b; Fujiki and Suganuma 2011). Synergistic effects were also observed in the inhibition of cell growth and induction of apoptosis. EC obviously increased bioavailability of the catechins, since EC enhanced the incorporation of ^{3}H-EGCG into the cells (Suganuma et al. 1999a). In light of our evidence, we think that usual green tea infusion from the green tea leaves of sencha with warm water is the most appropriate and

Table 3 Three biochemical and biological effects with green tea catechins

Catechins	Inhibition of cell growth (% of control)	Inhibition of TNF-α release IC_{50} (μM)	Induction of apoptosis (A_{415} nm)
EC (100[a], 200[b] μM)	97.8[b]	>500[a]	0.01 ± 0.03[b]
EGCG (100 μM)	73.3	60	0.52 ± 0.22
ECG (50 μM)	62.2	30	0.27 ± 0.13
EGC (200 μM)	100.0	ND	0.14 ± 0.03

ND not determined

[a] 100 μM

[b] 200 μM

Table 4 Synergistic effects by cotreatment of EC with other green tea catechins on inhibition of TNT-α release

	Inhibition of TNT-α release IC_{50} (μM)	
	Without EC	With EC (100 μM)
		>500
EGCG (100 μM)	60	15
ECG (50 μM)	30	7

practical cancer preventive. Precisely how green tea catechins induce multifunctions favorable for cancer prevention is a significant question, and we have presented evidence showing that green tea catechins have potential to act as chemical chaperones (Kuzuhara et al. 2008).

Sealing effects of EGCG and green tea extract

It was exciting for us to find that repeated applications of EGCG inhibited the tumor promotion pathways of both TPA and okadaic acid, although their mechanisms of action are different: TPA and teleocidin bind to the phorbol ester receptor, whereas okadaic acid binds to protein phosphatases 1 and 2A. These two different receptors are present in the membrane and cytosol fractions of mouse skin. When mouse skin was treated with a single application of 5 mg EGCG, both the specific binding of ^{3}H-TPA and that of ^{3}H-okadaic acid decreased immediately, within 5–10 min; the levels of the specific binding gradually returned to normal. In the experiments with tumor promotion on mouse skin, EGCG was usually applied 15 min before each application of tumor promoter. Based on the evidence, we think that EGCG treatment inhibited the interaction of tumor promoters with their receptors and that EGCG can interrupt the interaction of ligand with its receptor on cell membrane: This was called the "Sealing Effects of EGCG" (Yoshizawa et al. 1992). Experimentally, EGCG caused reduction in detergent-insoluble membrane domain, that is, a decrease in lipid raft (Fujiki 2005; Adachi et al. 2007), and EGCG also inhibited the activation of specific receptor tyrosine kinases, such as epidermal growth factor receptor, insulin-like growth factor-1 receptor and vascular endothelial growth factor receptor 2 (Shimizu et al. 2011). The results indicate that treatment of cells with EGCG and green tea extract reduced kinase activity, one of the multifunctions of EGCG.

International response to cancer prevention with green tea

In 1991, the International Symposium on Physiological and Pharmacological Effects of Camellia Sinensis (Tea) was held at the American Health Foundation in New York. At a press conference, a US scientist said that since animal studies often do not have identical results in humans, more studies were needed before he could recommend green tea for its health effects on humans. However, one of our groups told the press that Japanese drink green tea every day and that green tea could be one of the most practical methods of cancer prevention available. The next day (August 27, 1991), his comments were on the first page of USA Today. We are convinced that his comments in 1991 are now more valid and widely accepted than ever. Furthermore, in 1994, the New York Times reported the cancer preventive effects of green tea in a story entitled "Green tea: more than just a soothing brew." This article was based on a report from Shangai that green tea may reduce the incidence of cancer of the esophagus in humans (June 15, 1994). And in 1995, one of us had the happy opportunity to give a 1-h lecture about our research under the title of "Inhaltsstoffe des grünen Tees, Ein Beispiel für Krebsprevention beim Menschen," within the framework of Teleakademie von Südwestdeutschen Fernsehen, Baden–Baden in Germany.

Green tea is a popular beverage in Asian countries, and cancer prevention with green tea is now a topic in Western nations. The cover of Cancer Research for September 1998 shows the leaves of the green tea plant and its small white flowers, and Molecular Carcinogenesis (February, 1999) introduced the results of T. Bowden, in which EGCG in a tea bag inhibited the activation of p38 mitogen-activated protein (MAP) kinase, resulting in the prevention of skin cancer (Fig. 4) (Chen et al. 1999). And in 2011, the 4th World Congress on Tea & Health was held at the Max Delbrück Center for Molecular Medicine in Berlin Buch, introducing new challenging applications of EGCG for neurodegenerative diseases.

Prospective cohort study in Saitama Prefecture

The cancer preventive effect of green tea was first confirmed through a prospective cohort study with humans in Saitama Prefecture. In 1986, we first surveyed 8,552 individuals aged over 40 on their living habits, including their daily consumption of green tea. During the 10 years after 1986, a total of 419 cancer patients, 244 males and 175 females, were detected (Nakachi et al. 2000). Female and male cancer patients were divided into three groups based on consumption of green tea per day: under 3 cups, 4–9

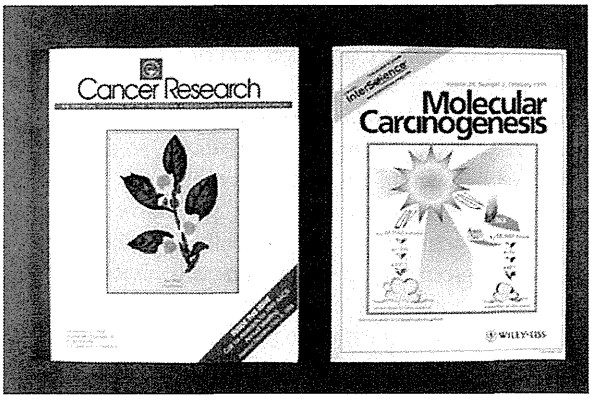


Fig. 4 The covers of Cancer Research (*L*) and Molecular Carcinogenesis (*R*)

Table 5 Average age at cancer onset and daily green tea consumption

Gender	Average age at cancer onset and consumption of green tea per day (cups)		
	≤3	4–9	≥10
Female (175)	67.0 ± 1.7 (28.0 %)	66.4 ± 1.3 (58.3 %)	74.3 ± 2.2 (13.7 %)
Male (244)	65.0 ± 1.5 (24.2 %)	67.2 ± 1.0 (46.7 %)	68.2 ± 1.1 (29.1 %)

cups and over 10 cups (Table 5). Next, the average age at cancer onset of all these cancer patients was obtained from clinical documents at hospitals. The average age at cancer onset and daily green tea consumption per patient were studied.

The cancer onset in female patients who had consumed over 10 Japanese-size cups (120 ml/cup) of green tea per day was 7.3 years later than that of female patients who had consumed less than three cups per day. The cancer onset in male patients who had consumed over 10 cups of green tea per day was 3.2 years later than that of patients who had consumed less than three cups per day (Table 5) (Nakachi et al. 2000). The difference between females and males is partly due to higher tobacco consumption by males. It is important to note that the delay of cancer onset is significant evidence of primary cancer prevention in humans. Key factors in primary cancer prevention include: tamoxifen for breast cancer (Fisher et al. 1998), cessation of smoking, diet modifications and physical activity. In addition, the cohort study first demonstrated that consumption of 10 cups of green tea per day is an additional significant factor in primary cancer prevention for the general population. Since the consumers of over 10 cups of green tea per day were only 13.7 % for female patients and 29.1 % for male patients, it would be possible to increase consumption to over 10 cups per day with green tea tablets, resulting in effective primary cancer prevention (Fujiki et al. 2002).

We next studied the reduced relative risk in human organs by high consumption of green tea in Saitama Prefecture. Green tea most significantly prevented lung cancer—a relative risk of 0.33 with over 10 cups of green tea per day. High consumption of green tea also prevented cancers of the colorectum, liver and stomach, in that order (Fig. 5) (Nakachi et al. 2000). It is important to note that consumption of less than ten cups is not effective as a preventive tool, suggesting that there is a minimum effective amount of green tea catechins for cancer prevention. In addition to the cancer preventive effects, the prospective cohort study provided other significant results. Increased green tea consumption was associated with decreased serum total cholesterol, decreased triglyceride levels and decreased atherogenic index. Moreover, the prevalence rates of cardiovascular disease and diabetes mellitus were significantly lower among the population consuming over 10 cups of green tea per day (Imai et al. 1997). Since green tea apparently prevents many major lifestyle-related diseases, including cancer, cardiovascular disease and diabetes mellitus, we reported that among women, the mean age at death for those consuming over 10 cups per day was 6 years later than that for those consuming less than 3 cups, suggesting that green tea reduces the risk of lifestyle-related diseases, resulting in a longer life span (Imai et al. 1997; Nakachi et al. 2000; Sueoka et al. 2001). All the results of this study indicate that the more green tea we drink, the higher cancer preventive activity we get in the target organs. Of course, cancer prevention for general population before cancer onset is a matter of individual responsibility, not the duty of clinicians. We call this the first stage of cancer prevention with green tea (Fig. 6).

Our cohort study was conducted in Saitama Prefecture, one of the main tea-producing areas in Japan. Other investigators have reported non-preventive effects of green tea against human cancers, such as no association between the consumption of green tea and the risk of gastric cancer

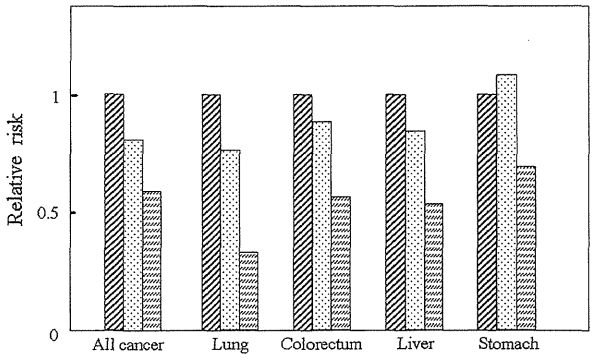


Fig. 5 Reduced relative risk of cancer in human organs with a high consumption of green tea. ▨ ≤3, □ 4–9, ▩ ≥10

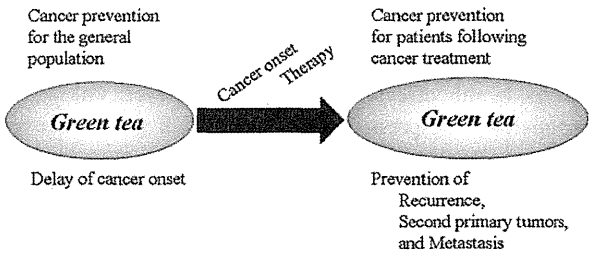


Fig. 6 Two stages of cancer prevention with green tea

in Miyagi Prefecture (Tsubono et al. 2001), and no association between green tea drinking and the risk of breast cancer in Japan (Iwasaki et al. 2010). It is important to note that various kinds of green tea are produced in Japan, such as sencha, hojicha and oolong tea. Sencha contains 13.6 % green tea catechins, hojicha made from roasted lowest grade of tea leaf contains only 2.9 %, and oolong tea 7.6 %. This clearly shows that the results of epidemiological study are dependent on the kinds of green tea consumed and the quality of green tea catechins. Thus, an ingested amount of green tea catechins is a limiting factor for demonstrating the preventive or non-preventive effects in a cohort study.

In addition, an interesting study reported that no association between green tea consumption and breast cancer incidence was found in the cohort studies of the United States, but that increased green tea consumption (more than three cups a day) was inversely associated with breast cancer recurrence (Pooled RR = 0.73, 95 % CI: 0.56-0.96) (Ogunleye et al. 2010). In 1998, we previously reported the decreased recurrence of human breast cancer with increased consumption of green tea, on the basis of results obtained from 472 cancer patients. Stages I and II cancer patients consuming over five cups of green tea per day (average 8 cups) showed a lower recurrence rate, 16.7 %, and a longer disease-free period, 3.6 years, than those consuming less than four cups (average 2 cups) per day, 24.3 % and 2.8 years (Nakachi et al. 1998). The results showed that drinking an average 8 cups of green tea per day prior to cancer onset results in more hopeful prognosis for breast cancer patients.

Preclinical safety trials for 10 cups of green tea

Next, we will think about cancer prevention for patients following cancer treatment, since surviving cancer patients are seriously looking for preventives. Our epidemiological results showed that the effective cancer preventive amount is also 10 Japanese-size cups of green tea per day, corresponding to 2.5 g green tea extract (Nakachi et al. 2000). To facilitate consumption, tablets of green tea extract are produced by the Tea Institute of Saitama Prefecture. A tablet dissolved in warm water becomes usual Saitama tea (Fujiki et al. 2001). 10 cups of green tea per day may seem to be a large amount, but the results of Phase I clinical trials with green tea extract conducted in USA reported that the maximum tolerated dose corresponds to 21–24 Japanese-size cups per day (Pisters et al. 2001), or even higher amounts (Laurie et al. 2005). Therefore, we think 10 cups of green tea is tolerable for most people.

Before going on to a clinical trial, we first asked some 102 healthy and voluntary citizens to consume this amount for 3 months, with informed consent. Group A of 51

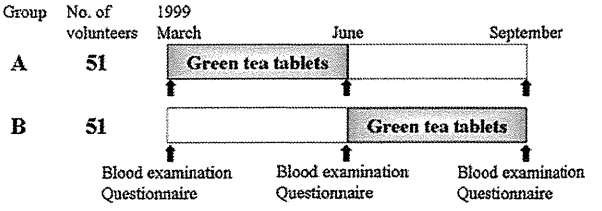


Fig. 7 Preclinical safety trials among 102 healthy and voluntary citizens of Saitama Prefecture for 6 months. 10 Cups of daily green tea beverage fortified by green tea tablets for 3 months

volunteers took 10 cups of daily green tea beverage supplemented with green tea tablets for the first 3 months, and group B took the same amount for the next 3 months. During the 6-month trial, all of the 102 volunteers answered questionnaires and their blood samples were biochemically examined three times (Fig. 7) (Fujiki et al. 2001). The blood examination did not show any serious side effects, so important answers to just two of the questions are presented. To the question, "Did you experience any discomfort from taking green tea tablets?", 93 % of the participants said that they were able to continue drinking green tea and taking green tea tablets. For another question, "Did you observe any changes in living habits or meals?", Over 90 % of the participants found their living habits or appetite unaffected by 10 cups of green tea daily.

However, about 50 % of the volunteers experienced very mild temporary disorders due to the caffeine in green tea extract. It is reported that the maximum tolerable dose of caffeine is about 1.0 g/day, and even though 10 cups do not contain total 1.0 g caffeine, the Tea Institute developed a new method to reduce the caffeine by washing the green tea leaves with warm water. They reduced the caffeine content from 5 % to less than 3 %, without using an organic solution. It is important to note that the tablets are a dried green tea infusion, not a powder of green tea leaves. The tablets are not controlled by the Pharmaceutical Affairs Act in Japan because they belong to the category of green tea beverage, not a drug.

Significant factors for studying cancer prevention with humans

Before going on to human studies, we had to clarify the following factors. (1) It is necessary to have evidence that green tea is non-toxic for humans and that there is bioavailability in rodents (Suganuma et al. 1998). (2) We obtained the minimum effective amounts of green tea per day for humans from the results of human epidemiological study (Nakachi et al. 2000). (3) Collaboration of healthy volunteers with informed consent was established (Fujiki et al. 2001). (4) Clinicians developed an established clinical biomarker for target organ (Shimizu et al. 2008).

(5) Green tea tablets should be quality controlled, and the absence of pesticides and herbicides confirmed (Fujiki et al. 2001). (6) We were fortunate to be able to collaborate with the Tea Institute of Saitama Prefecture, which provided green tea extract and tablets. (7) Results should be published in reviewed international journals (Nakachi et al. 2000; Shimizu et al. 2008).

Double-blind randomized clinical phase II prevention trial for recurrence of colorectal adenomas

Table 6 shows the protocol for phase II prevention trial of colorectal adenoma recurrence with green tea extract, with informed consent, which was conducted at Gifu University, Department of Medicine and its related hospitals (Shimizu et al. 2008). One green tea tablet (GTE, 500 mg) contains 52.5 mg EGCG, 34.6 mg EGC, 11.1 mg ECG, 12.3 mg EC and 15.7 mg caffeine, approximately equivalent to two Japanese-size cups of green tea. Colorectal adenomas were removed by endoscopic polypectomy at the first colonoscopy, and 12 months later, the absence of polyps was confirmed by second colonoscopy. The patients were then double-blind randomized into two groups: one group maintained their usual daily consumption of green tea beverage without a placebo, and the other group (GTE group) took 10 cups of green tea, supplemented with six green tea tablets, daily for 12 months. The incidence of recurrent adenomas was determined by end-point colonoscopy 12 months later. The recurrence rate of the control group was 31 % and that of the GTE group was 15 % (Shimizu et al. 2008). The chi-square test, p was <0.05 (Table 6). In addition, the size of recurrent adenomas was smaller in the GTE group than in the control group. Thus, drinking 10 Japanese-size cups of green tea, supplemented with green tea tablets, 50 %, significantly prevented recurrence of colorectal adenomas in patients (Shimizu et al. 2008).

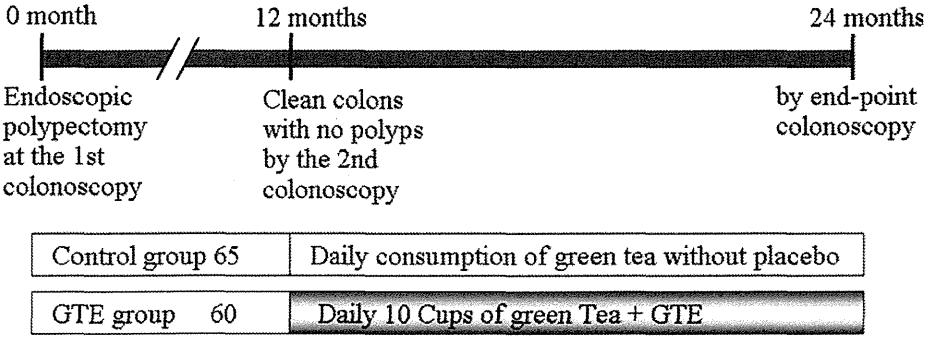
It is important to note that we did not use placebo in our phase II prevention trial because green tea is a daily beverage of Japanese, so, we aimed to raise the consumption to a target level, with supplemental tablets of green tea extract. The patients in the trial were told to take a minimum effective amount of green tea catechins. A randomized, placebo-controlled multicentre trial was recently begun to investigate the effects of diet supplementation with green tea extract containing 300 mg EGCG on the recurrence of colon adenomas for patients who have undergone polypectomy for colon polyps in Germany (Stingl et al. 2011).

Moreover, the prevention of prostate cancer development in patients with high-grade prostate intraepithelial neoplasia (PIN) using capsules of green tea catechins was confirmed in Italy. In the group using green tea capsules, only one tumor was diagnosed among 30 patients, whereas nine cancers were found among 30 placebo-controlled patients. Green tea catechins reduced incidence of prostate cancer from 30 to 3 % (Bettuzzi et al. 2006). In the United States, a phase I trial of green tea extract was first conducted at two Institutions (Pisters et al. 2001), and a phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions revealed that the two higher doses (750 and 1,000 mg/m^2) of green tea extract produced a trend toward a greater clinical response in association with a high baseline level and downregulation of angiogenic stromal vascular endothelial growth factor (Tsao et al. 2009).

Necessity of cancer prevention in our daily lives

Tumor promotion in human cancer is now understood as a disease affected by TNF-α upregulation and NF-κB

Table 6 Phase II prevention trial of colorectal adenoma recurrence with green tea extract in patients, with no polyps after polypectomy



Groups (cases)	Recurrence rate (%)	Average no. of polyps/patient
Control (20/65)	31.0	0.43
GTE (9/60)	15.0 ($p < 0.05$)	0.20

activation (Komori et al. 1993b; Suganuma et al. 1999b; Ben-Neriah and Karin 2011), induced by pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Suganuma et al. 2002). Pro-inflammatory cytokines are easily induced in humans by bacterial focal infections (Fujiki et al. 2004; Kuzuhara et al. 2007b); Cytokines and chemokines are numerous in aged people; and so, humans are always at risk of tumor promotion. With these physiological conditions, we need to establish our own cancer prevention strategy, based on reduction in TNF-α and inactivation of NF-κB. For this purpose, we believe cancer prevention with green tea is the most appropriate preventive, since it is effective on a wide range of target organs and has no toxic effects. Green tea can be ingested by everybody in the world.

Due to advancements in diagnosis and treatment, there are many healthy surviving cancer patients in Japan. They are seriously looking for drugs and preventives that will prevent recurrence, second primary tumors and metastasis. For these patients, green tea supplemented with green tea tablets is vital following cancer treatment. We call this the two stages of cancer prevention with green tea (Fig. 6) (Fujiki 1999; Fujiki et al. 2002). The left part of Fig. 6, the first stage, deals with primary cancer prevention, and the right part of Fig. 6, the second stage, shows tertiary cancer prevention.

New cancer treatment strategy using the combination of green tea catechins and anticancer drugs

Considering the cancer prevention for patients following cancer treatment (Fig. 6), anticancer drugs may be administered to Japanese cancer patients who consume green tea every day. So we raised the following questions: Is it advantageous for Japanese cancer patients to take cancer preventive green tea catechins and anticancer drugs together? Does the combination have the potential to enhance efficacy and decrease adverse effects of anticancer drugs? What kinds of anticancer drugs and compounds can work with green tea catechins? The names of the compounds are presented: gefitinib, 5-fluorouracil, taxol, doxorubicin, erlotinib, antiestrogen, COX inhibitors, sulindac and celecoxib, retinoids and curcumin.

We had two significant results. The combination of green tea extract with sulindac enhanced the inhibition of tumor development in multiple intestinal neoplasia (Min) mice (Suganuma et al. 2001). Min mice have a germline mutation of *APC* gene and develop intestinal tumors similar to those of familiar adenomatous polyposis, FAP patients. Intestinal tumors are seen as blue spots on the organ by staining with methylen blue. The combination reduced the number of tumors per mouse from 72.3 to 32.0, a decrease of 55.7 %, $p < 0.05$ (Suganuma et al. 2001). The second result was as follows: the combination of green tea extract with celecoxib enhanced the inhibition of tumor development in the lungs of A/J mice induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a carcinogen. The combination reduced the number of tumor-bearing mice from 100 to 73.3 %, a decrease of 26.7 %, $p < 0.05$ (Suganuma et al. 2011).

To understand the molecular mechanisms involved in synergistic enhancement by the combination of EGCG with sulindac, we first studied the upregulation of two gene expressions, *GADD153* and *p21* genes, and as previously reported, their inductions of gene expression were enhanced to be about 12-fold and 3-fold, respectively, (Okabe et al. 2001; Fujiki and Suganuma 2002). These gene expressions were not affected by treatment with either EGCG alone or sulindac alone. The combination of EGCG with sulindac induced apoptosis in PC-9 cells 10.5-fold stronger than EGCG alone or sulindac alone, and the combination of EGCG with celecoxib induced apoptosis in PC-9 cells 14.9-fold stronger than EGCG alone or celecoxib alone (Table 7) (Suganuma et al. 2006). The results show that the combination additionally induces new gene expressions that are not induced by EGCG alone or COX inhibitors alone. A schematic illustration of the combination is shown in Fig. 8. The combinations indicate the presence of a new therapeutic activity that will greatly expand cancer treatment and also bring successful tertiary cancer prevention for patients with good prognosis (Suganuma et al. 2011).

Our definition of cancer prevention with green tea

The administration of cancer preventives delays the carcinogenic processes in humans, no matter when the carcinogenesis starts, thereby blocking the appearance of clinical symptoms (Fujiki et al. 2002).

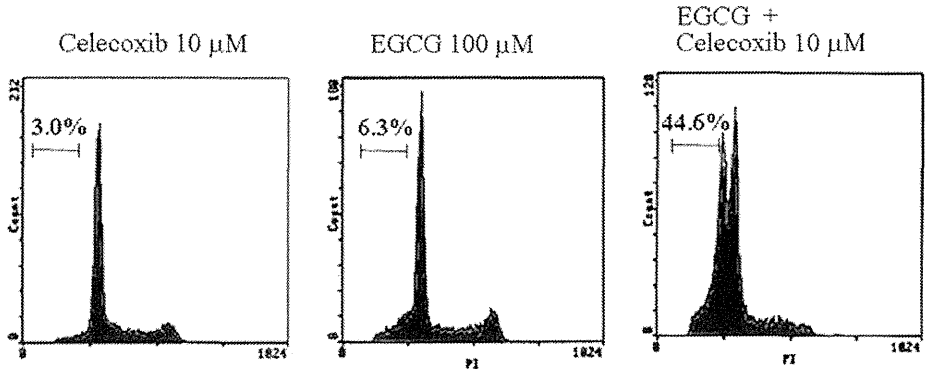
Afterword

Strong support by Professor James Watson

In May 2002, the International Conference on Green Tea and Cancer, A Critical Review, was held at the Banbury Center, Cold Spring Harbor Laboratory in New York. This Conference was strongly supported by the Director, J. Watson. After the Conference at the Banbury Center, Dr. Watson listened closely while one of the organizers noted the success of the Conference. The organizer told him that green tea clearly prevented cancer in rodents, whereas human epidemiological studies had proved inconclusive. Dr. Watson immediately answered that since green tea prevented carcinogenesis in rodents, it should prevent it in humans as well, since humans and mice have

Table 7 Synergistic induction of apoptosis by combination of EGCG with COX inhibitors

	Induction of apoptosis (% of apoptotic cells)	
	Without	With EGCG
Human lung cancer cell line PC-9		
Control	2.0	6.3
Sulindac	4.1	42.9 (×10.5)
Celecoxib	3.0	44.6 (×14.9)



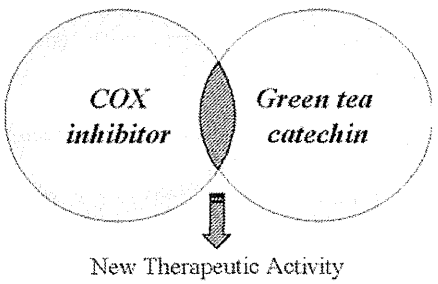


Fig. 8 Combination of green tea with COX inhibitor

similar genomes. It was wonderful encouragement for green tea scientists like us, because we got the impression that a world-famous molecular biologist now believes in the cancer preventive effects of green tea for humans. Seven years later, we successfully confirmed the prevention of colon adenomas in humans by green tea supplemented with green tea tablets.

The history of green tea in Japan

Drinking green tea is really part of Japanese culture. The Japanese Zen priest Eisai returned to Japan after 4 years training as Zen priest in China, carrying some seeds of green tea, as a medicine. When the third Shogun became ill, Eisai wrote a book entitled "Kitusa yohjohki" (Maintaining Health by Drinking Green Tea), which was presented to the Shogun together with green tea in the year 1211. The Shogun's illness, probably a disorder of the digestive tract, was cured soon, and this is the first case of therapeutic effects with green tea in Japan.

Numerous illnesses of course existed 800 years ago, but cancer and aging-associated diseases did not, because the average life span was only 40 years at that time. Thus, our study for the first time demonstrated that green tea prevents a lifestyle-related disease. Eisai concluded his book this way: I hope an excellent doctor will elucidate the mechanism of green tea action in the near future. We believe our research project with green tea is a present from Eisai.

Acknowledgments To Prof. Erich Hecker, we express gratitude for his fruitful discussion. We thank Prof. Takuo Okuda for providing numerous polyphenols including (−)-epigallocatechin gallate (EGCG) and penta-*O*-galloyl-β-D-glucose (5GG), Prof. Takashi Sugimura for his encouragement during the course of this work, and our Japanese colleagues cited in our references for their research. This work was supported by Japan Society for the Promotion of Science: Scientific Research on Priority Areas for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Comprehensive Research on Aging and Health; and Cancer Research from the Ministry of Health, Labor, and Welfare, Japan; Selective Applied and Developed Research, and Green Tea Extracts Research Development for Cancer Prevention by the Department of Agriculture and Forests and the Department of Health and Human Services of Saitama Prefecture, Japan; and the Smoking Research Fund.

Conflict of interest We declare that we have no conflict of interest.

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MOLECULAR CANCER

RESEARCH Open Access

Cisplatin and ultra-violet-C synergistically down-regulate receptor tyrosine kinases in human colorectal cancer cells

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Abstract

Background: Platinum-containing anti-cancer drugs such as cisplatin are widely used for patients with various types of cancers, however, resistance to cisplatin is observed in some cases. Whereas we have recently reported that high dose UV-C (200 J/m^2) induces colorectal cancer cell proliferation by desensitization of EGFR, which leads oncogenic signaling in these cells, in this study we investigated the combination effect of low dose cisplatin (10 μM) and low dose UV-C (10 J/m^2) on cell growth and apoptosis in several human colorectal cancer cells, SW480, DLD-1, HT29 and HCT116.

Results: The combination inhibited cell cycle and colony formation, while either cisplatin or UV-C alone had little effect. The combination also induced apoptosis in these cells. In addition, the combination caused the downregulation of EGFR and HER2. Moreover, UV-C alone caused the transient internalization of the EGFR, but with time EGFR recycled back to the cell surface, while cisplatin did not affect its localization. Surprisingly, the combination caused persistent internalization of the EGFR, which results in the lasting downregulation of the EGFR.

Conclusions: The combination of low dose cisplatin and low dose UV-C synergistically exerted anti-cancer effect by down-regulating RTK, such as EGFR and HER2. These findings may provide a novel strategy for the treatment of patients with colorectal cancer.

Keywords: Cisplatin, UV-C, EGFR, HER2, Down-regulation, Cell growth inhibition

Introduction

Among the receptor tyrosine kinases (RTKs), the ErbB family, such as epidermal growth factor receptor (EGFR; ErbB1) or human epidermal growth factor receptor-2 (HER2; ErbB2) plays a pivotal role in regulating a number of cellular processes including cell proliferation, survival and migration [1], and dysregulation of EGFR activity leads to tumorgenesis [2]. Mechanisms leading to oncogenic signaling behind EGFR are thought as follows: 1) increased EGFR 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 [3].

We have previously reported that the blockade of EGF stimulation significantly suppressed colorectal cancer cell growth, suggesting that the EGFR pathway plays an important role in proliferation of these cells [4]. Thus, EGFR downregulation is a critical target for therapy against colorectal cancer that is highly dependent on EGFR. As for HER2, their expression has been first reported to be amplified in breast cancer [5]. Since clinical and experimental evidences show a role for overexpression of the HER2 protein in the progression of human breast, ovarian, non-small cell lung [6] and colorectal cancer [7], HER2 may be a candidate target for receptor-targeted therapeutics.

Cis-diamminedichloroplatinum (CDDP) or cisplatin is one of the most effective DNA-damaging anti-tumor

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agent and is used for the treatment of various human cancers [8-10]. However, resistance to cisplatin arises in some cases and many compounds combined with platinum-based drugs are now ongoing clinical trials [11]. Increasing evidences show that cisplatin directly influences EGFR signaling. Cisplatin reportedly induces EGFR internalization [12], phosphorylation at Thr1045 mediated via a ubiquitin ligase, c-Cbl [13] and phosphorylation at Thr669, at a site which is phosphorylated by p38 MAPK [14], while activation of stress-activated protein kinase/*c-Jun*-N-terminal kinase or p38 MAPK by cisplatin has been reported to promote apoptotic cell death [15]. In addition, in many studies researchers have used cisplatin at relatively higher doses (30 μM or more), which is impractical *in vivo*.

Ultra-violet (UV) radiation is divided into three bands: UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm). Most of the UV-C and approximately 90% of UV-B are absorbed while passing through the atmospheric layers. UV-A and UV-B are recognized harmful for humans, while UV-C is used for studying DNA damage and cellular DNA repair process [16]. So far, the possibility of application rather for treatment of human cancer has been demonstrated [17,18]. In a series of papers, Petersen et al have investigated the photophysical consequences of illuminating the aromatic residues of proteins with UV-C [19-25]. In particular, they demonstrated that 280 nm UV illumination of aromatic residues in proteins causes the disruption of nearby disulphide bridges, where EGFR are excessively populated, leading to the suppression of the proliferative potential in human cancer cell lines [17].

Whereas we recently reported the availability of UV-C alone (30 J/m^2 and more) in human colorectal cancer cells, in which we showed that UV-C can evade these cells from oncogenic stimulation of EGF by decreasing the EGFR protein level [26], we herein investigated the combination use of low dose cisplatin and low dose UV-C on cell growth in human colorectal cancer cells (SW480, HT29, DLD-1 and HCT116) and found that the combination has synergistic effect on cell growth inhibition by down-regulating receptor tyrosine kinases, such as EGFR and HER2.

Results

Effects of cisplatin and/or UV-C on cell proliferation in human colorectal cancer cells

Bromodeoxyuridine (BrdU) is a synthetic thymidine analog that gets incorporated into DNA during cell division. Therefore, the measurement of BrdU incorporation reflects the ability of cell growth. We first investigated the effects of cisplatin and/or UV-C on cell proliferation using BrdU. Whereas either 10 μM of cisplatin or 10 J/m^2 of UV-C hardly affected BrdU incorporation in SW480 and DLD-1 cells (Figure 1A, lanes 2 and 3, respectively), the combination caused a marked inhibition in BrdU incorporation (Figure 1A, lane 4, respectively). While it has previously been reported that cisplatin induces cell cycle arrest at the G2-phase [27], cell cycle analysis using flow cytometry revealed that the combination of cisplatin and UV-C increased the population at G2/M phases (28.2 ± 1.35%), compared with cisplatin (21.9 ± 0.68%; $p = 0.0014$) or UV-C (15.2 ± 0.76%; $p = 0.0004$) (Figure 1B). Moreover, we examined the protein level of phospho-Rb and cyclin D1, both of which direct cells toward proliferation by controlling progression through the restriction point of cell cycle (Figure 2A) [28]. In SW480 cells, cisplatin by itself had little effect on phosphorylation level of Rb. However, when the cells were first exposed to UV-C and then incubated in the presence of cisplatin, the protein level of phospho-Rb was decreased in a time-dependent manner after 12 h (Figure 2). Since we have recently reported that 10 J/m^2 of UV-C did not cause the decrease in the protein level of Rb [26], these results suggest that the combination of cisplatin and UV-C exerts synergistic effect on the suppression of cell cycle. We also verified the combination effect in DLD-1, HT29 and HCT116, other human colorectal cancer cell lines (Figure 2).

Effects of cisplatin and/or UV-C on colony formation in human colorectal cancer cells

We next performed colony formation assay, which is a microbiology technique for studying the effectiveness of specific agents on the survival and proliferation of cells (Figure 2B) [29]. The combination synergistically suppressed colony formation of SW480 cells, although cisplatin or UV-C alone did to a lesser extent. Similarly, the combination synergistically decreased the number of colony formation in DLD-1 and HCT116 cells, whereas UV-C alone slightly affected them in these cells. As for HT29 cells, while cisplatin or UV-C alone has no effect, the combination synergistically suppressed colony formation. As a whole, these results suggest that the combination has cytocidal effects on several colorectal cancer cells.

Effects of cisplatin and/or UV-C on the apoptosis in human colorectal cancer cells

We next investigated the combination effect of cisplatin and UV-C on apoptosis by observing PARP cleavage, since PARP is a family of proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death, indicating cell apoptosis [30]. While cisplatin or UV-C alone had little effect on PARP, the combination caused PARP cleavage in SW480, DLD-1, HT29 and HCT116 cells (Figure 3A).

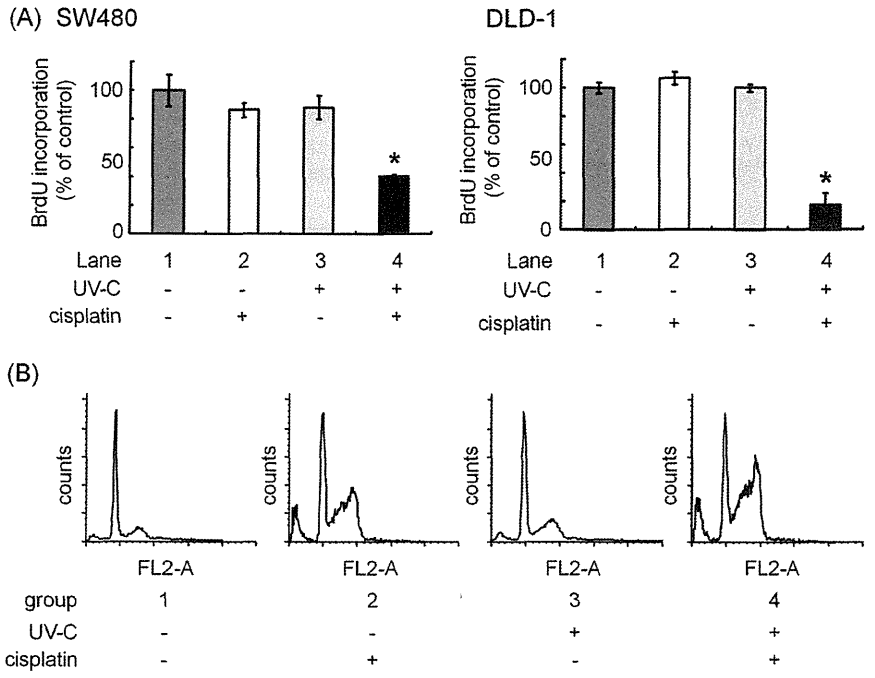

Figure 1 (A) Effects of cisplatin and/or UV-C on cell proliferation in human colorectal cancer cells. SW480 and DLD-1 cells were either exposed to 10 J/m^2 UV-C (lanes 3), treated with 10 μM cisplatin (lanes 2), or received both (lanes 4). Twenty four h later, the measurement of BrdU incorporation was performed using cell proliferation ELISA (BrdU). Results are expressed as percentage of incorporation with 100% representing that by untreated control cells (lanes 1). (**B**) SW480 cells were treated with 10 μM cisplatin (group 2), 10 J/m^2 UV-C (group 3) or combination of 10 μM cisplatin and 10 J/m^2 UV-C (group 4). The cells were then stained with propidium iodide (PI) to analyze progression of cell cycle. The distribution of cells in the apoptosis and each phase of cell cycle were calculated in each group. Bars designate SD of triplicate assay. The asterisks (*) indicate significant decrease ($p < 0.05$) as compared to the corresponding control, respectively.

While Hoechst33258 are used to stain DNA and easily detect such DNA fragments, we next examined the effect of combination of cisplatin and UV-C on DNA fragmentation utilizing this dye and found that the combination increased the number of Hoechst 33258-positive apoptotic cells in SW480 and HT29 cells (Figure 3B), which are consistent with our results shown in Figure 3A.

Effects of cisplatin and/or UV-C on the protein level of EGFR and HER2 in human colorectal cancer cells

As described in Introduction, EGFR downregulation is the most prominent regulatory system in signal attenuation and involves the internalization and subsequent degradation of the activated receptor in the lysosomes. As well, HER2 is frequently overexpressed in colorectal cancer when compared with normal colonic mucosa, and the extent of overexpression seems to correlate with increasing disease stage and poorer patient survival [31]. Therefore, therapies that target the EGFR and/or HER2 may be effective in the chemoprevention and/or therapy of colorectal cancer [32]. Whereas we recently reported that EGFR signaling plays a critical role in proliferation of colorectal cancer cells [26], we next focused on the expression level of EGFR as well as HER2 in several colorectal cancer cells including SW480, DLD-1, HT29 and HCT116, since we observed that the combination use of cisplatin and UV-C synergistically exerts suppressive effect on cell proliferation and apoptosis (Figures 1 and 3). As depicted in Figure 4, 10 μM cisplatin alone did not affect these levels even after a longer treatment in SW480 (Figure 4, lanes 1–4). As well, while UV-C at a dose over 30 J/m^2 caused a marked decrease in the EGFR protein level [26], in this study we observed that 10 J/m^2 of UV-C did not affect (Additional file 1). Interestingly, the combination use of 10 μM cisplatin and 10 J/m^2 UV-C clearly induced the decrease in the protein levels of EGFR as well as HER2 in SW480 cells, which were appeared at 12 h after treatment with cisplatin and UV-C (Figure 4, lanes 5–8). Similar results were observed in other colorectal cancer cells, DLD-1, HT29 and HCT116. Together, the combination effect of cisplatin and UV-C on the suppression of cell growth seems to be due to the down-regulation of EGFR and/or HER2.

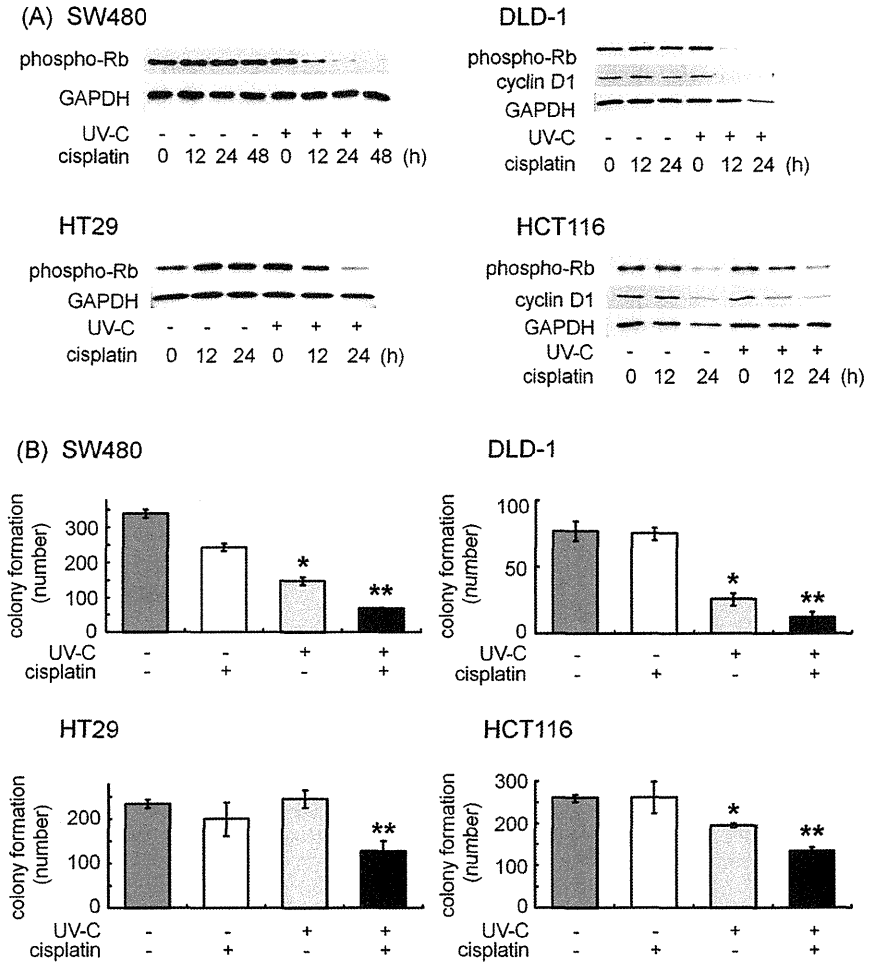

Figure 2 (A) Effects of cisplatin and/or UV-C on cell proliferation markers, phospho-Rb and cyclin D1 in human colorectal cancer cells. SW480, DLD-1, HT29 and HCT116 cells were first exposed to 10 J/m^2 of UV-C or not, and then treated with 10 μM of cisplatin for the indicated periods. Protein extracts were harvested and examine by Western blotting using anti-phospho-Rb and anti-cyclin D1 antibodies. (**B**) Effects of cisplatin and/or UV-C on colony formation in SW480, DLD-1, HT29 and HCT116 human colorectal cancer cells. The attached human colorectal cancer cells were first exposed to the indicated doses UV-C (0 or 10 J/m^2), just after the aspiration of the growth medium. The cells were then incubated in normal growth media with/without 10 μM cisplatin for 24 h. After trypsinization, 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 Materials and methods) and the average number of colony from 5 randomly chosen fields (x 20) were counted, respectively. Bars designate SD of triplicate assay. The asterisks (* and **) indicate significant decrease ($p < 0.05$) as compared to the control and UV-C alone, respectively.

Effects of cisplatin and/or UV-C on the internalization of EGFR in SW480 cells

It has previously been reported that UV irradiation (100 J/m^2) induces rapid and persistent internalization of EGFR [33]. As well, we have recently reported that UV-C at a dose over 30 J/m^2 caused the internalization and subsequent down-regulation of the EGFR in SW480 cells [26]. In order to elucidate the mechanism underlying combination effect of cisplatin and UV-C, we next examined whether cisplatin (10 μM) and/or UV-C (10 J/m^2) induces changes in the subcellular localization of EGFR in SW480 cells. Whereas antibody-tagged EGFR remained on the cell surface (Figure 5A, panels 1, 6 and 11), 0.5 h incubation after the treatment of the cells with UV-C alone (10 J/m^2) resulted in the distribution of the EGFR to cytosol beneath the plasma membrane, thus indicating that UV-C indeed induced the internalization of the EGFR (Figure 5A, panel 7). By contrast, cisplatin (10 μM) by itself did not affect the localization of the EGFR (Figure 5A, panels 2–5). Interestingly, when the cells were first exposed to UV-C and then incubated in the absence of cisplatin for 6 h and more, the antibody-

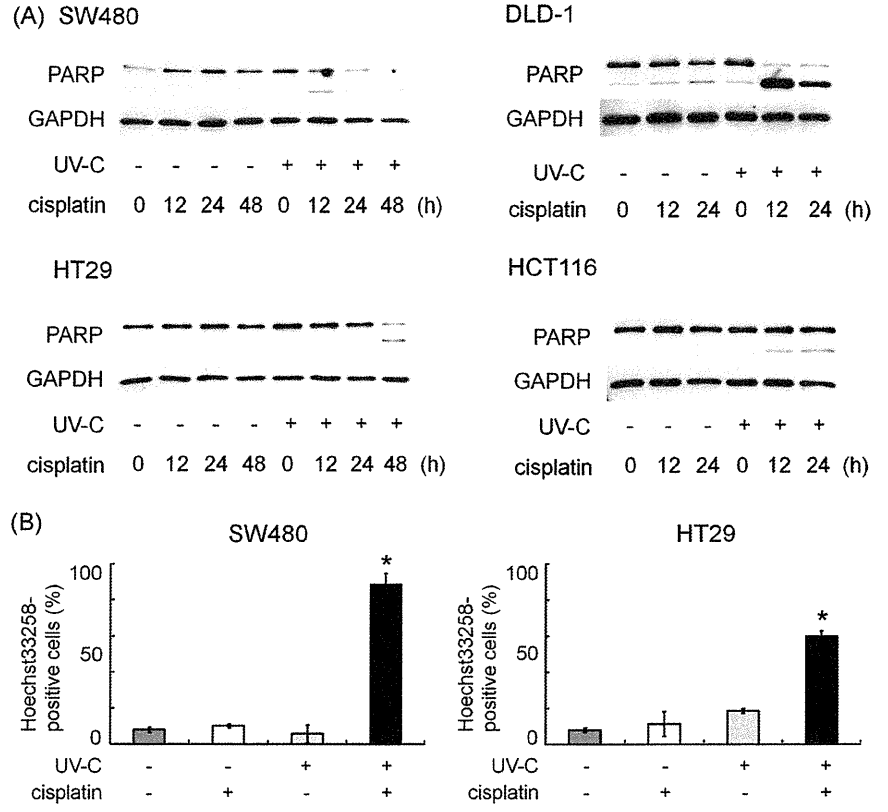

Figure 3 (A) Effects of cisplatin and/or UV-C on PARP cleavage and DNA fragmentation in human colorectal cancer cells. SW480, DLD-1, HT29 and HCT116 cells were first exposed to the indicated doses of UV-C (0 J/m^2 or 10 J/m^2), and then treated with/without 10 μM of cisplatin for the indicated periods. Protein extracts were then harvested and examine by Western blotting using anti-PARP and anti-GAPDH antibodies. (**B**) SW480 and HT29 cells were first exposed to the indicated doses of UV-C (0 J/m^2 or 10 J/m^2), and then treated with/without 10 μM of cisplatin for the indicated periods. They were then treated with Hoechst 33258 and were examined by fluorescence microscopy. The numbers of Hoechst33258-positive cells (apoptotic nuclei) from 5 randomly chosen fields (x 40) were counted, respectively. Bars designate SD of triplicate assay. The asterisks (*) indicate significant increase ($p < 0.05$) as compared to the corresponding controls, respectively.

tagged EGFR reappeared on the cell surface, thus suggesting that internalized EGFR recycled back to the cell membrane (Figure 5A, panels 8–10). However, the EGFR remained to be internalized when the cells were treated with the combination of cisplatin and UV-C (Figure 5A, panels 12–15).

To verify these results, we measured the amount of cell surface EGFR by enzyme-linked immunosorbent assay (ELISA). Whereas UV-C alone decreased the amount of cell surface EGFR within 0.5 h (Figure 5B, lane 3). However, they were gradually recovered 3 h after treatment with UV-C (Figure 5B, lanes 3, respectively). On the contrary, cell surface EGFR in the cells treated with the combination of cisplatin and UV-C remained to be decreased (Figure 5B, lanes 4, respectively). Taken together with our results obtained from fluorescence study, we strongly suggest that the treatment with cisplatin after UV-C exposure blocks the recycling of the EGFR which are internalized by UV-C.

Discussion

Platinum-containing anti-cancer drugs, including cisplatin, inhibit DNA replication [34,35] and RNA transcription [36], and induce cell cycle arrest at the G2-phase and apoptosis [27,37]. However, cisplatin at a higher dose concomitantly raises severe adverse effects, such as myelo-supression, nausea, anorexia, diarrhea and liver dysfunction. Therefore, many trials have made effort to minimize the dose of cisplatin in cancer patients. In the present study, we examined the combination effect of low dose cisplatin (10 μM) and low dose UV-C (10 J/m^2) on human colorectal cancer cells, while we recently reported the potential availability of UV-C in these cells [26].

We herein demonstrated that the combination use synergistically inhibited the cell proliferation by BrdU assay (Figure 1A), flow cytometry (Figure 1B), Western blotting (Figure 2A) and colony formation assay (Figure 2B). We also unveiled that the cisplatin and

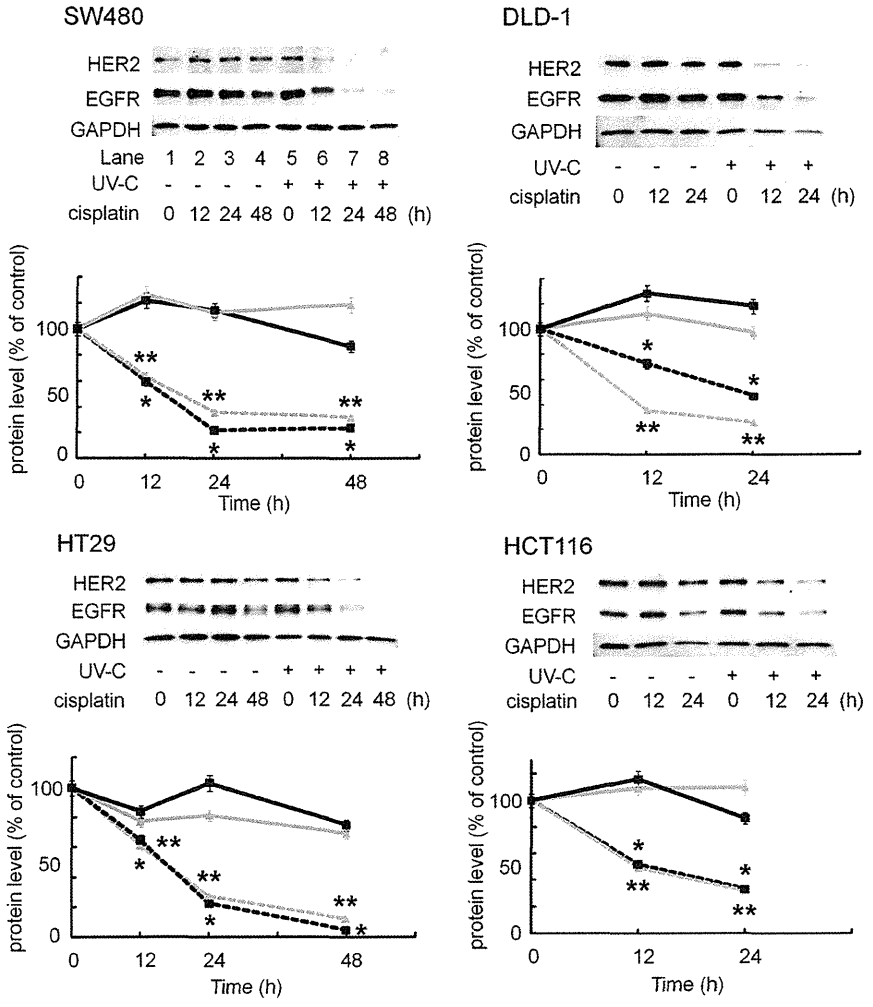

Figure 4 Effects of cisplatin and/or UV-C on HER2 and EGFR in human colorectal cancer cells. SW480, DLD-1, HT29 and HCT116 cells were first exposed to the indicated doses of UV-C (0 J/m^2 or 10 J/m^2), and then treated with/without 10 μM of cisplatin for the indicated periods. Protein extracts were then harvested and examine by Western blotting using anti-HER2, anti-EGFR and anti-GAPDH antibodies. The lower line graphs show quantification data for the protein levels of HER2 and EGFR, after normalization to GAPDH, respectively. Bars designate SD of triplicate assay. The asterisks (* and **) indicate significant decrease ($p < 0.05$) as compared to the corresponding controls, respectively.

UV-C have synergistic effect on apoptosis, while cisplatin or UV-C alone had little effect (Figure 3). They were accompanied by downregulation of RTKs, such as EGFR and HER2 (Figure 4), both of which reportedly play a critical role in cell proliferation in many types of cancers including colorectal cancer [7,38].

An anti-EGFR monoclonal antibody inhibits EGFR activation, resulting in the enhancement of the anti-cancer effect of cisplatin [39,40]. Indeed, chemotherapy with cetuximab or panitumumab, both of which are also anti-EGFR monoclonal antibodies, can prolong survival period of colorectal cancer patients by nearly twenty-four months [41-43]. On the contrary, it has recently been reported that EGFR inhibition can protect EGFR from cisplatin-mediated phosphorylation and subsequent ubiquitination and degradation, indicating that treatment with an EGFR inhibitor before cisplatin would be antagonistic [13]. Thus, the efficacy of the combination of cisplatin and EGFR targeting drugs remains to be elucidated. In this study, low dose UV-C (10 J/m^2) induced EGFR internalization, but these receptors recycled back to the cell surface, whereas the combination use of cisplatin and UV-C induced persistent EGFR internalization (Figure 5). It has previously been reported that if cisplatin-bound EGFRs remain on the cell surface, they catalytically inhibit cell death [33]. Therefore, we speculate that pretreatment with UV-C helps cisplatin to induce degradation of EGFR, since

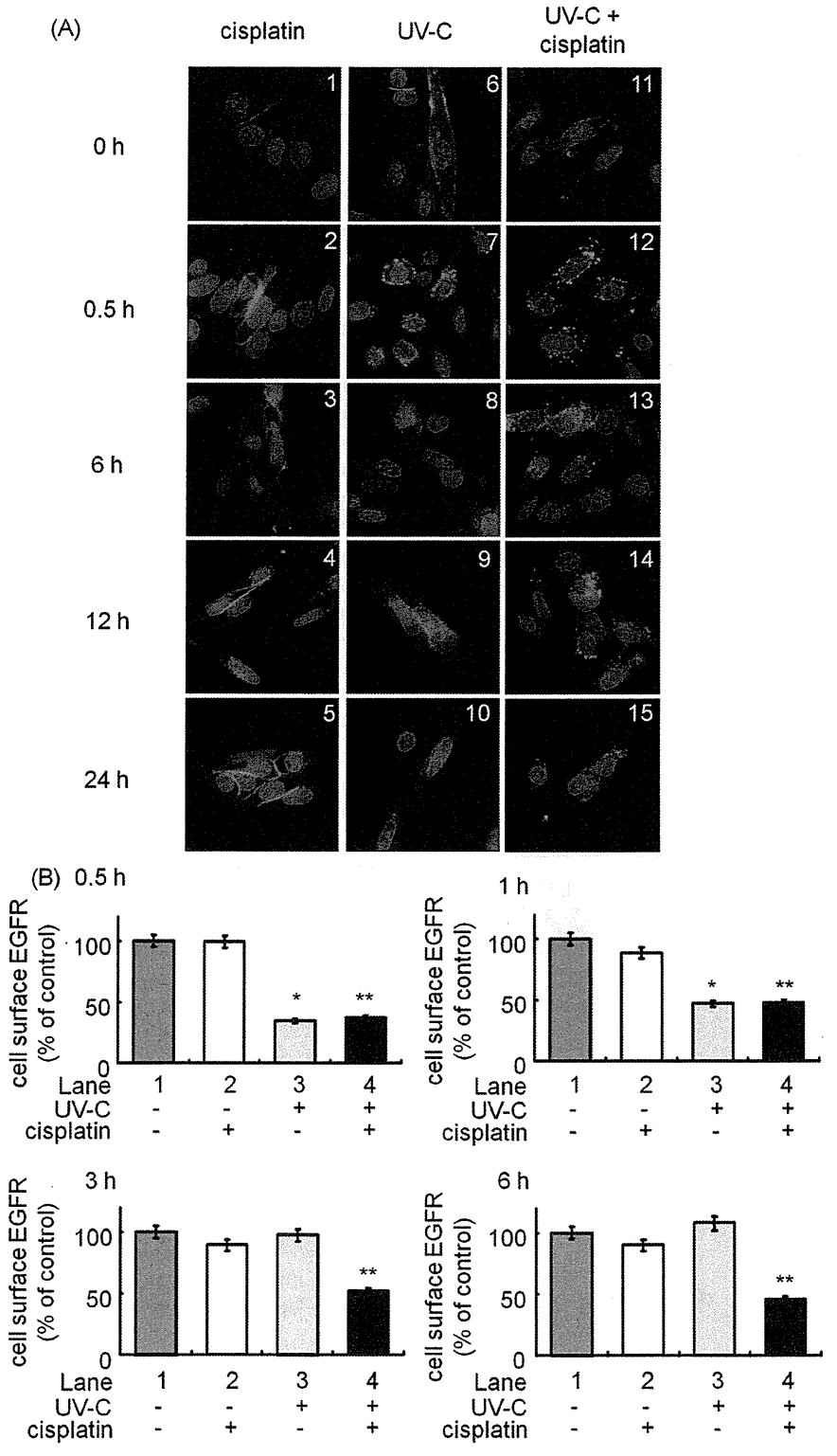

Figure 5 (See legend on next page.)

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Figure 5 (A) Effects of cisplatin and/or UV-C on the localization of EGFR in SW480 cells. SW480 cells were first labeled for 15 min at 37°C with anti-EGFR antibodies. They were then exposed to 10 J/m^2 of UV-C (panels 6–10 and 11–15, respectively) or not (panels 1–5, respectively), followed by the treatment with (panels 1–5 and 11–15, respectively) or without (panels 6–10, respectively) 10 μM of cisplatin for the indicated periods at 37°C. After fixation and permeabilization, the cells were stained with Alexa 488® conjugated anti-mouse secondary antibody for EGFR (green signal) and DAPI (blue signal) for 1 h, and then examined by fluorescence microscope. (**B**) Effects of cisplatin and/or UV-C on the amount of cell surface EGFR in SW480 cells. SW480 cells were first labeled for 15 min at 37°C with an anti-EGFR antibody that recognizes the extracellular domain of the EGFR. They were then exposed to 10 J/m^2 UV-C (lanes 3 and 4) or not (lanes 1 and 2), followed by the treatment with (lanes 2 and 4) or without 10 μM cisplatin (lanes 1 and 3) for the indicated periods at 37°C. The amount of cell surface EGFR was then measured by ELISA. The asterisks (* and **) indicate significant decrease ($p < 0.05$) with respect to the control (lane 1, respectively). For additional details see Materials and methods.

UV-C alone caused EGFR internalization into the perinuclear area of the cells, where cisplatin might exert maximum effect on the donwregulation of EGFR (summarized in Figure 6). Nevertheless, further investigation is required to elucidate why UV-C causes EGFR internalization and why cisplatin induces EGFR degradation.

Regarding the mechanisms underlying EGFR down-regulation, they involve several important phosphorylation sites in EGFR, including Tyr1045, a docking site for the ubiquitin ligase c-Cbl, and Ser1046/1047, which are required for EGFR desensitization in EGF-treated cells [44,45]. We recently found that (−)-epigallocatechin-3-gallate as well as heat shock protein 90 inhibitors cause down-regulation of the EGFR via phosphorylation at Ser1046/1047 through p38 MAPK in human cancer cells [46,47]. However, we did not observe the phosphorylation of EGFR at these residues when the cells were treated with low dose cisplatin and/or low dose UV-C in colorectal cancer cells (data not shown). Therefore, it seems that EGFR degradation by the combination does not depend on Tyr1045 or Ser1046/1047. Moreover, it has previously reported that p38 MAPK plays an important role in 100 J/m^2 UV-induced EGFR internalization [33]. However in the present study, the combination did not influence the phosphorylation of p38 MAPK (data not shown). These results also suggest that the synergistic effect of cisplatin and UV-C also does not depend on p38 MAPK activation.

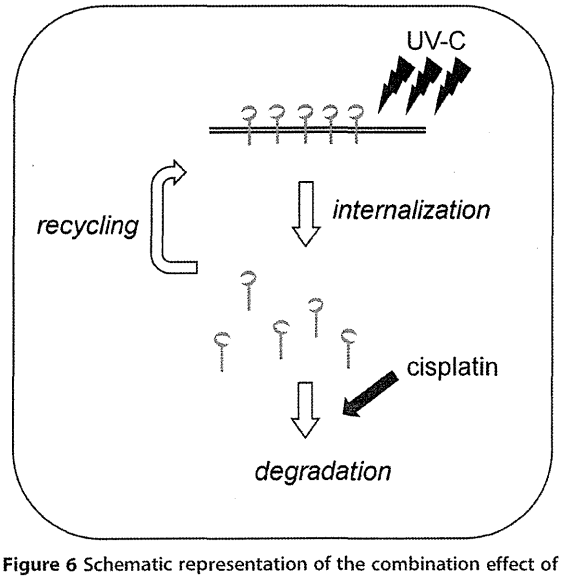

Figure 6 Schematic representation of the combination effect of cisplatin and UV-C in human colorectal cancer cells. After UV-C exposure even at a low dose, cell surface EGFR is internalized. With time the internalized EGFR by UV-C recycles back to the cell membrane, but cisplatin blocks this recycling and induces EGFR degradation, resulting in cell cycle arrest.

Initial platinum treatment is generally responsive, but the majority of cancer patients eventually relapse with cisplatin-resistance [10,48]. Several mechanisms of resistance to cisplatin are proposed; 1) reduced drug uptake, 2) increased drug inactivation, 3) increased DNA adduct repair, and 4) defective apoptotic response [10]. Importantly, a poor response of human cancers to cisplatin is associated with amplification and overexpression of HER2 found in some of breast and ovarian cancer patients [10,48]. Since we showed that the combination use of cisplatin and UV-C down-regulated HER2 (Figure 4), UV-C could alter the resistance to cisplatin in human colorectal cancer cells.

Conclusions

These results suggest that UV-C synergizes with cisplatin in the downregulation of receptor tyrosine kinases in human colorectal cancer cells. Our findings could provide a new aspect for the treatment of patients with colorectal cancer, although further investigation is required to develop devices that supply UV-C efficiently into human colorectal cancer, for example with endoscopic/laparoscopic approach.

Materials and methods

Materials

Antibodies against total EGFR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total HER2, cyclin D1, phospho-retinoblastoma