PERGAMON

European Journal of Cancer 36 (2000) 1998–2006

European Journal of Cancer

www.ejconline.com

Differential growth inhibition by 5-fluorouracil in human colorectal carcinoma cell lines

E. Tokunaga [a], S. Oda [b,*], M. Fukushima [c], Y. Maehara [a,d], K. Sugimachi [a,d]

[a] *Cancer Center, Kyushu University Hospital, Fukuoka 812-8582, Japan*
[b] *Mammalian DNA Repair Laboratory, Clare Hall Laboratories, Imperial Cancer Research Fund, South Mimms, Herts EN6 3LD, UK*
[c] *Cancer Research Laboratories, Taiho Pharmaceutical Company, 1-27 Misugidai, Hanno 357-8527, Japan*
[d] *Department of Surgery II, Faculty of Medicine, Kyushi University, Fukuoda 812-8582, Japan*

Received 16 February 2000; received in revised form 11 April 2000; accepted 25 April 2000

Abstract

The effects of 5-fluorouracil (5-FU) on cell growth were investigated using a primary culture of human fibroblasts, MRC-5, and three established human colon cancer cell lines, DLD-1, LoVo and SW620. Detailed flow cytometric analyses revealed differential growth inhibition among these cell lines including three modes of cell growth modulation: (a) loss or accumulation of S phase cells; (b) G2/M block; and (c) G1-S arrest. From analyses on the amount of 5-FU incorporated into cellular RNA and the activity of thymidylate synthase (TS), suppression of TS and depletion of dTTP, a possible consequence of the former, was considered to be the major action of 5-FU in these cells. Differences in the cellular responses to the nucleotide pool imbalance appeared to make the cell growth modulation diverse. Loss of S phase cells and G1-S phase arrest were evident in p53 wild-type cells, MRC-5 and LoVo. Cells proficient in DNA mismatch repair, SW620 and MRC-5, showed marked modulations in S-G2/M progression. These findings suggest that multiple factors, including p53 and DNA mismatch repair, participate in diverse cell growth modulations in cells treated with 5-FU. Cellular resistance to 5-FU correlated well with a loss of modulations in S-G2/M progression, rather than with a defect of G1-S arrest, which suggests the significance of DNA mismatch repair as a factor affecting the sensitivity of cells to 5-FU.

Keywords: Thymidylate synthase; DNA damage; DNA mismatch repair; p53

1. Introduction

5-fluorouracil (5-FU) [1], a fluorinated pyrimidine analogue, has long been prescribed as an antineoplastic agent to treat patients with various malignancies. 5-FU is now included in many major chemotherapeutic regimens which have been statistically judged to be effective [2–5]. Despite numerous studies, detailed mechanisms by which 5-FU exhibits cytotoxicity and biological factors which determine cellular sensitivity against this agent are not completely understood. Thus far, the cytotoxic actions of 5-FU have been mainly attributed to three independent mechanisms. Once the base analogue, 5-FU, is taken up by cells, it is metabolised via two independent pathways; the ribonucleotide synthesis pathway and the deoxynucleotide synthesis pathway. In the former, 5-FU is converted to fluorouridine triphosphate (FUTP) via fluorouridine monophosphate (FUMP), and finally incorporated into RNA [6]. In the latter pathway, 5-FU is metabolised into fluorodeoxyuridine triphosphate (FdUTP) via fluorouridine diphosphate (FdUDP) or fluorodeoxyuridine monophosphate (FdUMP) [2]. The latter metabolite, FdUMP, serves as a substrate for thymidylate synthase (TS), a key enzyme in the synthetic pathway for deoxythymidine nucleotides, and forms stable enzyme–substrate intermediates with TS and 5, 10-methylenetetrahydrofolate [6–8]. Inhibition of TS is thought to lead to depletion of the final product of this pathway, deoxythymidine triphosphate (dTTP) [6–8], which may prevent completion of DNA synthesis. FdUTP was found to be incorporated into genomic DNA [6,9], but the significance of this mechanism in the cytotoxic action is unknown. The incorporation of fluorinated ribonucleotides into RNA has been shown to be a major fate of cellular 5-FU [10–13]. The contribution of mod-

* Corresponding author. Tel.: +44-171-269-3883; fax: +44-171-269-3801.
E-mail address: s.oda@icrf.icnet.uk (S. Oda).

0959-8049/00/$ - see front matter
PII: S0959-8049(00)00200-8

ified RNA to cytotoxicity is unclear, although disruption of splicing processes has been suggested [14,15]. How these actions of 5-FU: (a) FUTP incorporation into RNA; (b) TS inhibition; and (c) FdUTP incorporation into genomic DNA, lead to growth inhibitory or cell-killing effects remains to be elucidated.

Biological factors which determine cellular sensitivity against DNA damaging antineoplastic agents may be classified into five categories: (1) determinants of the intracellular drug concentration, e.g. pump mechanisms on cell membranes [16,17], detoxification mechanisms [18]; (2) determinants for drug–DNA interactions, e.g. HMG proteins [19,20]; (3) DNA repair mechanisms, e.g. nucleotide-excision repair [21,22]; DNA mismatch repair [23–26]; (4) proteins functioning in DNA damage-dependent signal transduction pathways, e.g. DNA-dependent protein kinase [27,28], poly(ADP-ribose) polymerase [28,29], p53; and (5) effector proteins for growth inhibition or cell death. Attention is currently focused on DNA repair and relevant cellular functions. Using established human colon cancer cell lines which included ones deficient in DNA mismatch repair and/or with mutant p53, we analysed the effects of 5-FU on cell growth.

2. Materials and methods

2.1. Chemical

5-FU was purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Cells

Human colorectal carcinoma cell lines, DLD-1 (p53-mutant/DNA mismatch repair-deficient) [30–32], LoVo (p53-wild type/DNA mismatch repair-deficient) [30,31,33,34] and SW620 (p53-mutant/DNA mismatch repair-proficient) [30–32] and a primary culture of human fibroblast, MRC-5, were obtained from the American Type Culture Collection (Manassas, VA, USA). MRC-5, DLD-1, LoVo and SW620 cells were maintained in Minimum Essential Medium (MEM), Roswell Park Memorial Institute (RPMI)1640, F12 and Leibovitz's L-15, respectively. For all the experiments, cells at the fifth to ninth passage were used. All the media used were purchased from Life Technologies Inc. (Rockville, MD, USA) and were supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA).

2.3. Growth inhibitory effects of 5-FU

Cells were seeded on to 6 cm dishes (Nunc A/S, Roskilde, Denmark) at an initial density of 5×10^4 cells per dish. After incubation for 24–36 h, cells in an exponential growth phase were treated with 5-FU, by replacing media with those containing various concentrations of 5-FU. At every time point, cells were recovered by trypsinisation, stained with trypan blue and counted using a haemocytometer. A time course of 72 h was used. The experiments were done in triplicate for each time point, and averages (means) with standard deviations (S.D.) were calculated.

2.4. Flow cytometry

Cells were harvested by trypsinisation and resuspended in 1.0 ml of the buffer containing 3.4 mM sodium citrate, 10 mM NaCl, 0.1–0.15% Nonidet (N)P-40 (0.1% for DLD-1, LoVo and SW620, 0.15% for MRC-5), and 50 mg/ml of propidium iodide. After incubation for 2–3 h at 4°C, samples were subjected to fluorescent activated cell (FAC)Scan (Becton Dickinson, Franklin Lakes, NJ, USA) [35]. For each sample, 10 000 cells were analysed, and the results were processed using analytical software, Cell FIT (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

2.5. 5-FU incorporated into RNA

Quantitation of the amount of 5-FU incorporated into cellular RNA was done as previously described [36,37]. Briefly, cells treated with 10 or 20 μM 5-FU for 36 h were collected by trypsinisation and washed twice with ice-cold phosphate-buffered saline (PBS). Cellular RNA was extracted from these cells, using RNeasy Mini Kits (QIAGEN GmbH, Hilden, Germany). Total RNA was digested at 100°C in 6.0 M HCl for 24 h. The amount for 5-FU was determined using gas chromatography (Models JGS-20kp, JEOL, Tokyo, Japan) combined with mass spectrometry (Models JMS-D 300, JEOL).

2.6. Western blotting analyses

Cells were washed twice with ice-cold PBS and counted. Cell pellets were kept at −80°C until use. Cells were lysed in Laemmli's sodium dodecyl sulphate (SDS) sample buffer [38] (0.125 M Tris–HCl; pH 6.8, 4% SDS, 20% glycerol, 4% 2-mercaptoethanol), boiled for 5 min and immediately cooled on ice. After centrifugation at 15 000 rpm for 15 min at 4°C, supernatants were collected. Lysates corresponding to 2×10^5 cells were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were electrotransferred onto nitrocellulose membranes, BA85 (Schleicher & Schuell Inc., Dassel, Germany), using Transblot SD (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 1×TBS (10 mM Tris–HCl; pH 7.4, 0.9% NaCl) solution

including 5% bovine serum albumin (BSA) and 0.05% Tween 20 at 52°C for 1 h, the membranes were reacted with appropriately diluted primary antibody solutions. The membranes were incubated with a horseradish peroxidase-conjugated protein A (Amersham Life Science Ltd, Little Chalfont, Bucks, UK) or anti-rabbit immunogloblin G secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and fluorescent signals were visualised using the ECL chemiluminescence system (Amersham Life Science) and subsequent exposure to ECL hyperfilms (Amersham Life Science). A rabbit polyclonal antibody raised against recombinant human TS has been described [39]. A mouse monoclonal antibody against human p53 (DO-1), which recognises an amino terminal epitope mapped between the 11th and 25th amino acids in both wild-type and mutant p53 proteins, was purchased from Santa Cruz Biotechnology.

2.7. TS activity

TS activity was determined in [6-^{3}H]FdUMP binding assays [40]. Cells collected after treatment with 20 μM 5-FU were homogenised in a Dounce homogeniser. After centrifugation at 105 000 *g*, supernatants were collected. To determine TS_f (free TS) activity, cell extracts were incubated with the [6-^{3}H]FdUMP in the presence of 5,10-methylenetetrahydrofolate for 20 min at 30°C, and radioactivity incorporated in the acid-insoluble fractions was measured, using a liquid scintillation counter. For the measurement of TS_t (total TS) activity, prior to assay, cell extracts were preincubated in buffer containing 0.6 M NH_4HCO_3; pH 8.0, 100 mM 2-mercaptoethanol, 100 mM NaF, 15 mM cytidylate, and 2% BSA, at 30°C for 3 h, to allow the ternary complexes to dissociate. The inhibition rate of TS activity (TSIR) was calculated, using the following equation; $TSIR\ (\%) = (1 - TS_f/TS_t) \times 100$.

2.8. Detection of single-stranded regions in genomic DNA

Cells treated with 20 μM 5-FU for 12 h were collected by trypsinisation and washed once with ice-cold PBS. Genomic DNA was extracted using QIAGEN Genomic-tip 20/G (QIAGEN GmbH). DNA samples are resuspended in 1×TE (10 mM Tris–Cl (pH 7.5), 1 mM ethylene diamine tetraacetic acid (EDTA)) and the concentration was determined, using a spectrophotometer. One microgram of DNA was reacted with 1 unit of Klenow/3′-exonuclease(−) enzymes (New England Biolabs. Inc., Beverly, MA, USA) in the presence of 2.5 μCi [α-^{32}P]dCTP. After purification by phenol/chloroform extraction and ethanol precipitation, samples were electrophoresed on agarose gels. Throughout the experiment, DNA samples were never vigorously shaken. Since intact genomic DNA forms a single band, the precise amount of DNA corresponding to each band can be determined, using the GEL DOC 100 SYSTEM (Bio-Rad Laboratories). After destaining overnight, gels were dried and autoradiography was done using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA, USA). Radioactivity corresponding to each band was estimated, using analytical software, ImageQuant (Molecular Dynamics). The radioactivity per unit mass DNA was determined, and the relative ratio to values obtained in control cells was calculated.

3. Results

3.1. Differential growth inhibition by 5-FU in human colorectal carcinoma cell lines

We analysed the growth inhibitory effects of 5-FU on three human colon carcinoma cell lines, DLD-1, LoVo and SW620 and a primary culture of human fibroblasts, MRC-5. First, we examined changes in the numbers of viable cells over 72 h by direct counting. As shown in Fig. 1(a), there was no significant difference in the growth rates of these four cell lines under normal conditions of culturing. However, when treated with 5-FU at a concentration of 20 μM, growth-inhibitory effects were evident in all the cell lines and the extent of growth inhibition differed depending on the cell line (Fig. 1b). We next determined changes in the numbers of cells in each phase of the cell cycle using flow cytometry. The cell cycle profile observed in each cell line treated with 5-FU was also distinctive (Fig. 2). While cells in S phase disappeared in MRC-5 and LoVo cells, DLD-1 and SW620 showed a marked retardation in S phase progression. This delay in S phase progression was transient and apparently shorter in DLD-1 cells than in the SW620 cells. G1/S phase arrest was markedly induced in the MRC-5 and LoVo cells, but not did occur in the DLD-1 and SW620 cells. In addition, MRC-5 and LoVo cells showed differences in G2/M progression. The G2/M fraction in MRC-5 cells did not change, implying that these cells had a block in G2/M progression and that a major fraction of the cells in S phase disappeared. In contrast, in the LoVo cells the G2/M fraction markedly decreased, hence G2/M progression was apparently not blocked. These distinctive profiles in the cell cycle analyses are highly compatible with the changes in the numbers of viable cells over the same time course (Fig. 1b). In MRC-5 cells, in which both a G1-S arrest and a G2/M block were induced by 24 h, the number of cells remained unchanged. The number of LoVo cells doubled once due to the absence of G2/M block, but did reach a plateau because of G1-S arrest. In contrast, DLD-1 and SW620 cells showed a gradual increase in number, possibly due to the retardation in S phase progression. However, in SW620 cells, increase in

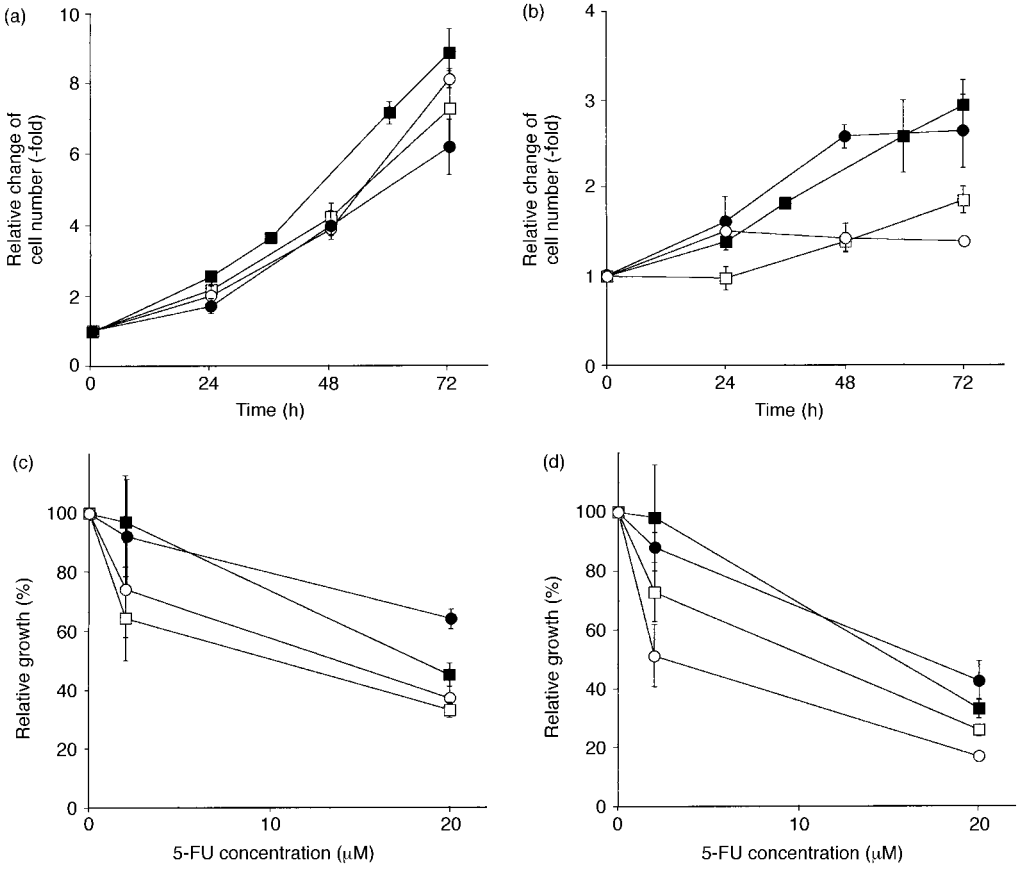

Fig. 1. Differential growth inhibition by 5-fluorouracil (5-FU) in human colorectal carcinoma cell lines and a primary culture of human fibroblasts. Growth profiles under the normal culture conditions (a) and growth-inhibitory effects of 20 μM 5-FU (b) are shown. Relative growth at 48 h (c) and 72 h (d) in the time course following varying doses of 5-FU are shown in the lower graphs. ■, DLD-1; □, SW620; ●, LoVo; ○, MRC-5.

cell number was delayed, since the S phase progression is more prolonged in SW620 than in DLD-1 cells. As shown in Fig. 1(c) and (d), results in dosimetrical analyses of cell survival at 48 and 72 h following different doses of 5-FU suggested that the transit from S phase or G2/M progression is a factor influencing cellular sensitivity against 5-FU. MRC-5 cells, in which both a G1-S arrest and a G2/M block were induced, were consistently sensitive to 5-FU. However, LoVo cells lacking the G2/M block were more resistant. Similarly, SW620 cells, in which S phase progression was prolonged, were relatively sensitive, compared with DLD-1 cells which had a slightly shorter delay in S phase progression.

3.2. TS activity was uniformly suppressed by 5-FU in these cell lines

To determine which type of cellular damage leads to these differential responses in cell growth, we next examined the incorporation of 5-FU into cellular RNA and the suppression of TS activity in these cell lines, since these two mechanisms are thought to be the major pathways through which 5-FU exhibits its cellular toxicity. We first quantified the amount of 5-FU in cellular RNA recovered from cells treated with 5-FU, using gas chromatography after acid digestion of the RNA (Fig. 3). No fluorinated base was detected in RNA of any of these cell lines under normal conditions of culturing. Following treatment with an increased concentration of 5-FU, the amount of fluorinated RNA was markedly elevated. However, in cells treated with 20 μM 5-FU the level of fluorinated RNA did not differ widely among these cell lines, although relatively high and low levels were noted in DLD-1 and MRC-5 cells, respectively. Our data on cell survival indicate that sensitivity against 5-FU is high in MRC-5 cells and that LoVo cells were the most resistant among these cell lines (Fig. 1c and d). There was no correlation between growth inhibition and fluorinated RNA. It is unlikely that incorporation into RNA is a major mechanism for the growth inhibition noted in these cell lines. We next examined the extent of suppression of TS activity. Firstly, the amount of TS and of ternary complexes which are stable enzyme-substrate intermediate composed of FdUMP, TS and folate, was determined using Western blotting (Fig. 4a).

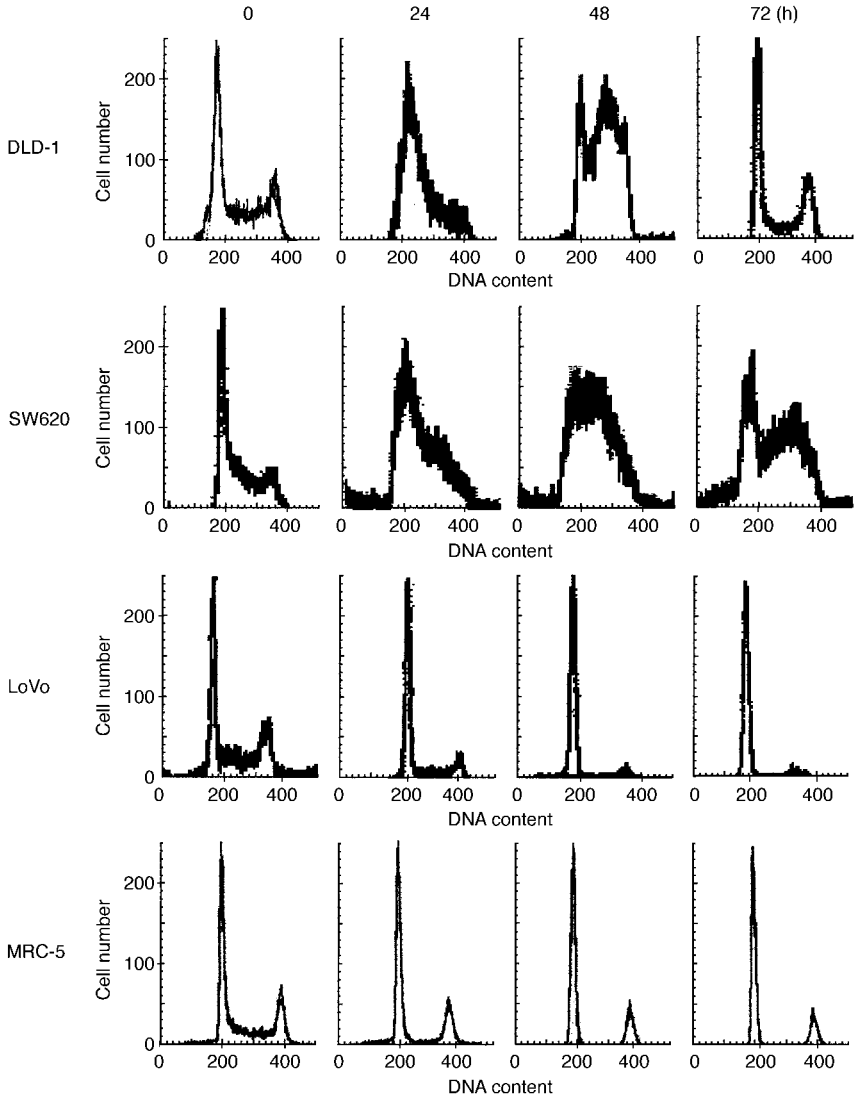

Fig. 2. Flow cytometric analyses of changes in the cell cycle profiles after 5-fluorouracil (5-FU) treatment.

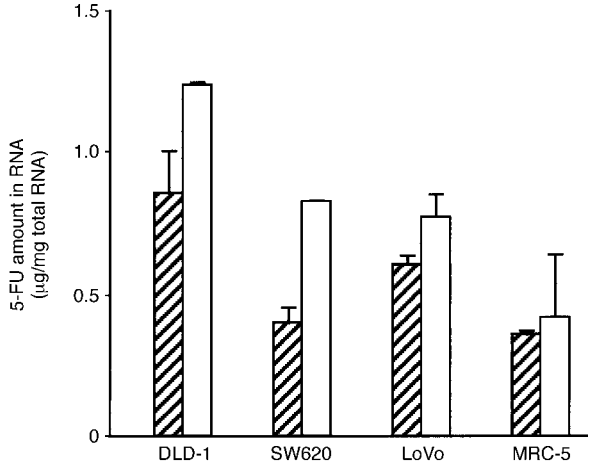

Fig. 3. 5-Fluorouracil (5-FU) incorporated into cellular RNA. Cells were treated with 10 μM (▨) or 20 μM 5-FU (□) for 36 h.

The basal levels of the 35 kDa antigen corresponding to TS were similar among the four cell lines. In cells treated with 20 μM 5-FU, TS was slightly induced at the later time points and its migration was retarded to the position corresponding to 38 kDa. More than 12 h after treatment, almost all the detectable TS was trapped in the 38 kDa complexes in all the cell lines. Since the 38 kDa complex represents the inactive TS, the enzyme is unlikely to be functional in cells treated with 20 μM 5-FU for more than 12 h. Furthermore, in parallel, changes in TS activity in each cell line were examined. In agreement with the Western blotting analysis, over 80% of TS activity was inhibited in each cell line by 12 h after 5-FU treatment (Fig. 4b). During the time course, there was no significant change in the total TS activity for any of these cell lines used (data not shown). These two complementary approaches indicate that the activity of TS is almost completely suppressed in all these cell

lines under the conditions we used. This strongly suggests depletion of dTTP in these cells and the extent of dTTP depletion is likely to be similar among these cell lines, since it has also been shown that the contribution of the salvage pathways for nucleotide synthesis is minor in cells treated with 5-FU [41]. Thus, the differential growth inhibition in these cell lines appeared to derive mainly from differences in cellular responses to dTTP depletion.

3.3. Single-stranded DNA regions in the genome were increased by 5-FU treatment of SW620, but not DLD-1 cells

Mammalian cells have finely regulated signal transduction pathways responding to DNA damage [42–44]. p53 proteins are induced in response to DNA damage and play a major role in these regulatory pathways [45,46]. We therefore examined the induction of p53 in these cell lines using western blotting. As shown in Fig. 5, in p53 wild-type cell lines, MRC-5 and LoVo cells 53 kDa antigens were markedly induced after treatment with 5-FU. In contrast, p53 was constitutively expressed at a high level in the p53-mutant cell lines, DLD-1 and SW620, as has been reported elsewhere [47]. These findings indicate that cells treated with 5-FU may undergo DNA damage. An imbalance in the deoxynucleotide pool, i.e. loss of dTTP, may prevent the completion of DNA synthesis. Indeed, p53-mutant cells, DLD-1 and SW620, showed a marked retardation in S phase progression, and in p53 wild-type cells, MRC-5 and LoVo cells, S phase cells disappeared (Fig. 2). We then examined the length of single-stranded DNA regions remaining on the genome to be synthesised, using Klenow/3′-exonuclease(−) enzymes [48] and radiolabelled nucleotides. Genomic DNA was recovered from the cells treated with 20 μM 5-FU for 12 h and was then incubated with Klenow/3′-exonuclease(−) enzymes in the presence of radiolabelled dCTP. After purification, the samples were electrophoresed on agarose gels. Since throughout the experiment DNA samples were never vigorously shaken, each DNA sample formed an intact single band on an agarose gel. The precise amount of DNA corresponding to each band was determined by digital scanning. Radioactivity was estimated using a PhosphorImager. The radioactivity per unit mass DNA in each cell line was calculated and the relative ratios to the values obtained in control cells are shown in Fig. 6. SW620 cells showed a significant increase in the incorporation of radiolabelled nucleotides into genomic DNA. Intriguingly, in DLD-1 cells, which showed a delay in S phase progression, the level of incorporation was unchanged. In LoVo cells and MRC-5 cells, there was no increase in the incorporation, possibly due to a paucity of S phase cells (Fig. 2). Thus, single-stranded DNA regions on the genome were increased by 5-FU treatment of SW620, but not DLD-1 cells. This event may relate to the depletion of dTTP.

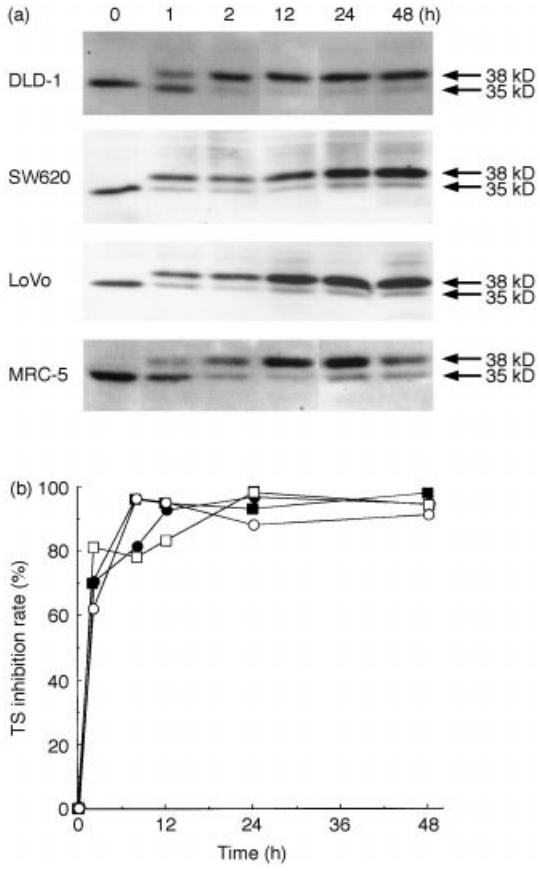

Fig. 4. Suppression of thymidylate synthase (TS) activity by 5-fluorouracil (5-FU). Formation of the ternary complexes, composed of fluorouridine monophosphate (FdUMP), TS and folate, was analysed using Western blotting (a). Cells treated with 20 μM 5-FU were harvested and lysates corresponding to 2×10^5 cells were subjected to Western blotting using a rabbit polyclonal anti-human TS antibody. 35 and 38 kDa antigen correspond to free TS and ternary complexes, respectively. Assessing TS enzyme activity directly, the inhibition rate of TS in cells treated with 20 μM 5-FU was also estimated (b). TS enzyme activity was determined using [6-^{3}H]FdUMP binding assay [35] and the TS inhibition rate was calculated. ■, DLD-1; □, SW620; ●, LoVo; ○, MRC-5.

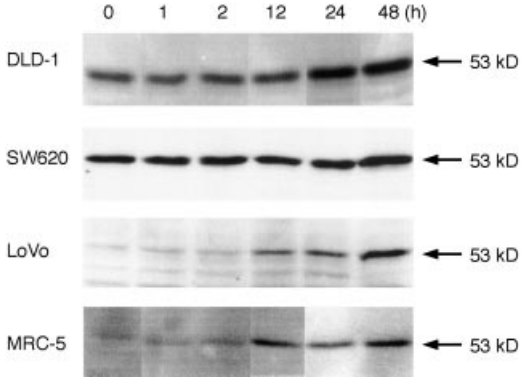

Fig. 5. Induction of p53 after 5-FU treatment. 2×10^5 cells treated with 20 μM 5-FU were analysed by Western blotting.

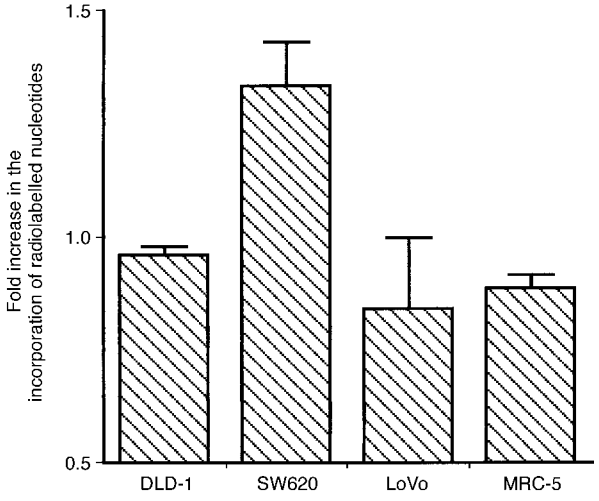

Fig. 6. Increase of single-stranded DNA regions by 5-FU. Cells treated with 20 μM 5-FU for 12 h were collected and genomic DNA was extracted and the relative increase of radiolabel incorporated by Klenow/3′-exonuclease(−) enzymes was determined.

Increase of single-stranded DNA regions may prevent the completion of S phase and, consequently, lead to a longer delay in S phase progression in SW620 cells.

4. Discussion

Detailed studies using flow cytometry revealed that cellular damage caused by 5-FU induces three different modes of cell growth modulations: (a) loss or accumulation of S phase cells; (b) G2/M block; and (c) G1-S arrest. Incorporation of 5-FU into cellular RNA and suppression of thymidylate synthase (TS) activity are thought to be major pathways through which 5-FU exhibits cellular toxicity. From our data, we concluded that suppression of TS is more likely to be the major action of 5-FU in the four cell lines we examined. Direct measurement of the intracellular deoxyribonucleotide pool remains technically difficult, although there are some reports in the literature [49,50]. Instead, we analysed the expressed amount and the activity of TS. These two complementary approaches revealed that inhibition of TS was uniformly observed in these cell lines. Suppression of TS strongly suggests depletion of dTTP in these cells, since the contribution of the salvage pathways is minor in cells treated with 5-FU [41]. The differential cell growth modulations noted in these cell lines appeared to derive from differences in cellular responses to the imbalance in the deoxynucleotide pool. In cells with wild-type p53, MRC-5 and LoVo, p53 proteins were induced by treatment with 5-FU. In these cells, cell growth modulations were evident; S phase cells disappeared and a marked G1-S arrest was induced. Therefore, the fate of S phase cells and G1-S arrest are considered depend on p53 status. Induction of p53 in p53 wild-type cells may also indicate the presence of DNA damage in cells treated with 5-FU. However, it was unclear which type of DNA damage was induced by depletion of dTTP. Deoxynucleotide pool imbalance has been reported to induce nucleosome laddering and cell death [50]. One may argue the possibility that in cells treated with 5-FU, arrest of DNA replication could be caused by a lack of this essential nucleotide. Indeed, in SW620 cells treated with 5-FU, which showed an extremely retarded S phase progression, there was an increase in the single-stranded DNA regions on the genome. However, in DLD-1 cells, which also showed a delay in S phase progression, there was no increase in these single-stranded DNA regions on the genome. DLD-1 and LoVo cells are deficient in DNA mismatch repair [30,51]. In DNA mismatch repair, the replicational polymerases are used for repair synthesis, which results in a long tract of DNA synthesis [52]. It appears likely that an imbalance in the deoxynucleotide pool affects the rate of misincorporation of nucleotides by DNA polymerases and that activation of DNA mismatch repair leads to an increase of DNA synthesis. In cells proficient in DNA mismatch repair, marked modulations in S-G2/M progression were noted; MRC-5 cells showed a G2/M block and SW620 cells exhibited an extremely delayed transit from S phase. Indeed, a connection between S-G2/M progression and DNA mismatch repair has been reported [53]. These findings suggest that, in addition to p53, DNA mismatch repair also participates in cell growth modulations observed in cells treated with 5-FU. In addition, the dosimetrical analyses suggest a connection between resistance to 5-FU and a loss of modulations in S-G2/M progression, rather than a defect of G1-S arrest. This may suggest DNA mismatch repair and p53 as a factor to participate in cell growth modulations induced by 5-FU treatment and, consequently, to influence cellular sensitivity against 5-FU.

The finding that DLD-1 showed no increase in single-stranded DNA regions and yet a delay in S phase progression was observed may suggest that a different type of DNA damage is induced in DLD-1 cells. One of the intracellular metabolites of 5-FU, FdUTP, is known to be finally incorporated into the genome and removed by uracil–DNA glycosylase (UDG)-related mechanisms [9,54]. Incorporation of FdUTP into the genome may activate this base-excision repair mechanism and induce small pieces of DNA synthesis which will not be detected in an incorporation assay such as used in our study. In the presence of wild-type p53, this might lead to cell death of damaged S phase cells and G1/S phase arrest in the next cell cycle, as observed in LoVo cells. However,in p53-mutant cells, the same event might induce only modest and transient retardation in S phase progression, as noted in DLD-1 cells, possibly through p53-independent pathways. We did not address the incorporation of FdUTP

into the genome. The possibility that UDG-related activities also contribute to the altered sensitivity to 5-FU under some biological conditions is not excluded.

We used established human colon carcinoma cell lines. From the differential growth inhibition observed in these cell lines and their genotypes, we discussed DNA mismatch repair and p53 as factors that might participate in cell growth modulations induced by 5-FU treatment and, consequently, influence cellular sensitivity to 5-FU. However, such problems can be approached in a more direct and deductive manner, i.e. using knock-out mouse cells. Knock-out mice in which one of the genes functioning in DNA mismatch repair are homozygously disrupted have been reported [55–57]. Cells which are nullizygous for one of the DNA mismatch repair genes show a marked resistance to alkylating agents [24,25]. However, the sensitivity against 5-FU in such knock-out mouse cells has not been addressed. In our laboratories, analyses on the sensitivity to 5-FU in *Msh2*$^{-/-}$ [55] and *Mlh1*$^{-/-}$ [56] knock-out mouse cells are now underway.

Acknowledgements

We are most grateful to P. Karran and M. Sekiguchi for their helpful advice. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Heidelberger C, Chaudhuri NK, Danenberg P, *et al*. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature* 1957, **179**, 663–666.
2. Pinedo HM, Peters GF. Fluorouracil: biochemistry and pharmacology. *J Clin Oncol* 1988, **6**, 1653–1664.
3. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989, **7**, 890–899.
4. Poon MA, O'Connell MJ, Wieand HS, *et al*. Biochemical modulation of fluorouracil with leucovorin: confirmatory evidence of improved therapeutic efficacy in advanced colorectal cancer. *J Clin Oncol* 1991, **9**, 1967–1972.
5. Sobrero AF, Aschele C, Bertino JR. Fluorouracil in colorectal cancer — a tale of two drugs: implications for biochemical modulation. *J Clin Oncol* 1997, **15**, 368–381.
6. Parker WB, Cheng YC. Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol Ther* 1990, **48**, 381–395.
7. Santi DV, McHenry CS, Sommer H. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* 1974, **13**, 471–481.
8. Heidelberger C, Danenberg PV, Moran RG. Fluorinated pyrimidines and their nucleosides. *Adv Enzymol Related Areas of Mol Biol* 1983, **54**, 58–119.
9. Ingraham HA, Tseng BY, Goulian M. Nucleotide levels and incorporation of 5-fluorouracil and uracil into DNA of cells treated with 5-fluorodeoxyuridine. *Mol Pharmacol* 1982, **21**, 211–216.
10. Spiegelman S, Sawyer R, Nayak R, Ritzi E, Stolfi R, Martin D. Improving the anti-tumor activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulation. *Proc Natl Acad Sci USA* 1980, **77**, 4966–4970.
11. Kufe DW, Major PP. 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *J Biol Chem* 1981, **256**, 9802–9805.
12. Glazer RI, Lloyd LS. Association of cell lethality with incorporation of 5-fluorouracil and 5-fluorouridine into nuclear RNA in human colon carcinoma cells in culture. *Mol Pharmacol* 1982, **21**, 468–473.
13. Takimoto CH, Cadman EC, Armstrong RD. Precursor-dependent differences in the incorporation of fluorouracil in RNA. *Mol Pharmacol* 1986, **29**, 637–642.
14. Will CL, Dolnick BJ. 5-Fluorouracil augmentation of dihydrofolate reductase gene transcripts containing intervening sequences in methotrexate-resistant KB cells. *Mol Pharmacol* 1986, **29**, 643–648.
15. Doong SL, Dolnick BJ. 5-Fluorouracil substitution alters pre-mRNA splicing in vitro. *J Biol Chem* 1988, **263**, 4467–4473.
16. Gottesman MM, Pastan I, Ambudkar SV. P-glycoprotein and multidrug resistance. *Curr Opin Genet Dev* 1996, **6**, 610–617.
17. Bradshaw DM, Arceci RJ. Clinical relevance of transmembrane drug efflux as a mechanism of multidrug resistance. *J Clin Oncol* 1998, **16**, 3674–3790.
18. Tew KD, Monks A, Barone L, *et al*. Glutathione-associated enzymes in the human cell lines of the National Cancer Institute Drug Screening Program. *Mol Pharmacol* 1996, **50**, 149–159.
19. Pil PM, Lippard SJ. Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science* 1992, **256**, 234–237.
20. Huang JC, Zamble DB, Reardon JT, Lippard SJ, Sancar A. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc Natl Acad Sci USA* 1994, **91**, 10394–10398.
21. Moggs JG, Szymkowski DE, Yamada M, Karran P, Wood RD. Differential human nucleotide excision repair of paired and mispaired cisplatin–DNA adducts. *Nucl Acids Res* 1997, **25**, 480–491.
22. Reed E. Platinum–DNA adduct, nucleotide excision repair and platinum based anti-cancer chemotherapy. *Cancer Treat Rev* 1998, **24**, 331–344.
23. Branch P, Aquilina G, Bignami M, Karran P. Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature* 1993, **362**, 652–654.
24. Karran P, Bignami M. DNA damage tolerance, mismatch repair and genome instability. *Bioessays* 1994, **16**, 833–839.
25. Karran P, Hampson R. Genomic instability and tolerance to alkylating agents. *Cancer Surveys* 1996, **28**, 69–85.
26. Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. *Clin Cancer Res* 1998, **4**, 1–6.
27. Turchi JJ, Patrick SM, Henkels KM. Mechanism of DNA-dependent protein kinase inhibition by cis-diamminedichloroplatinum(II)-damaged DNA. *Biochemistry* 1997, **36**, 7586–7593.
28. Boulton S, Kyle S, Durkacz BW. Interactive effects of inhibitors of poly(ADP-ribose) polymerase and DNA-dependent protein kinase on cellular responses to DNA damage. *Carcinogenesis* 1999, **20**, 199–203.
29. Lehmann AR, Broughton BC. Poly(ADP-ribosylation) reduces the steady-state level of breaks in DNA following treatment of human cells with alkylatingagents. *Carcinogenesis* 1984, **5**, 117–119.
30. Branch P, Hampson R, Karran P. DNA mismatch binding defects, DNA damage tolerance, and mutator phenotypes in human colorectal carcinoma cell lines. *Cancer Res* 1995, **55**, 2304–2309.

31. Umar A, Koi M, Risinger JI, *et al*. Correction of hypermutability, N-methyl-N′-nitro-N-nitrosoguanidine resistance, and defective DNA mismatch repair by introducing chromosome 2 into human tumor cells with mutations in MSH2 and MSH6. *Cancer Res* 1997, **57**, 3949–3955.
32. Rodrigues NR, Rowan A, Smith ME, *et al*. p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990, **87**, 7555–7559.
33. Baker SJ, Preisinger AC, Jessup JM, *et al*. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* 1990, **50**, 7717–7722.
34. Oki E, Oda S, Maehara Y, Sugimachi K. Mutated gene-specific phenotypes of dinucleotide repeat instability in human colorectal carcinoma cell lines deficient in DNA mismatch repair. *Oncogene* 1999, **18**, 2143–2147.
35. Nakabeppu Y, Oda S, Sekiguchi M. Proliferative activation of quiescent Rat-1A cells by delta FosB. *Mol Cell Biol* 1993, **13**, 4157–4166.
36. Marunaka T, Umeno Y, Yoshida K, Nagamachi M, Minami Y, Fujii S. High-pressure liquid chromatographic determination of ftorafur [1-(tetrahydro-2-furanyl)-5-fluorouracil] and GLC-mass spectrometric determination of 5-fluorouracil and uracil in biological materials after oral administration of uracilplus ftorafur. *J Pharm Sci* 1980, **69**, 1296–1300.
37. Shirasaka T, Nakano K, Takechi T, *et al*. Antitumor activity of 1 M tegafur–0.4 M 5-chloro-2,4-dihydroxypyridine–1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. *Cancer Res* 1996, **56**, 2602–2606.
38. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, **227**, 680–685.
39. Okabe H, Tsujimoto H, Fukushima M. Preparation of the antibodies against recombinant human thymidylate synthase for the detection of its intratumoral levels and the application to sensitivity-study of 5-fluorouracil. *Oncol Reports* 1997, **4**, 685–690.
40. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. In vivo kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and-resistant murine colon adenocarcinomas. *Cancer Res* 1982, **42**, 450–456.
41. Kinsella AR, Smith D. Tumor resistance to antimetabolites. *Gen Pharmacol* 1998, **30**, 623–626.
42. Brugarolas J, Jacks T. Double indemnity: p53, BRCA and cancer. p53 mutation partially rescues developmental arrest in BRCA1 and BRCA2 null mice, suggesting a role for familial breast cancer genes in DNA damage repair. *Nature Med* 1997, **3**, 721–722.
43. Nurse P. Checkpoint pathways come of age. *Cell* 1997, **91**, 865–867.
44. Wang JY. Cellular responses to DNA damage. *Curr Opin Cell Biol* 1998, **10**, 240–247.
45. Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* 1993, **8**, 307–318.
46. Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol Cell Biol* 1994, **14**, 1815–1823.
47. Levine AJ, Momand J. Tumor suppressor genes: the p53 and retinoblastoma sensitivity genes and gene products. *Biochim Biophys Acta* 1990, **1032**, 119–136.
48. Derbyshire V, Freemont PS, Sanderson MR, *et al*. Genetic and crystallographic studies of the 3′,5′-exonucleolytic site of DNA polymerase I. *Science* 1988, **240**, 199–201.
49. Cross DR, Miller BJ, James SJ. A simplified HPLC method for simultaneously quantifying ribonucleotides and deoxyribonucleotides in cell extracts or frozen tissues. *Cell Prolif* 1993, **26**, 327–336.
50. James SJ, Basnakian AG, Miller BJ. In vitro folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells. *Cancer Res* 1994, **54**, 5075–5080.
51. Umar A, Boyer JC, Thomas DC, *et al*. Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. *J Biol Chem* 1994, **269**, 269.
52. Modrich P. Mechanisms and biological effects of mismatch repair. *Ann Rev Genet* 1991, **25**, 229–253.
53. Hawn MT, Umar A, Carethers JM, *et al*. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. *Cancer Res* 1995, **55**, 3721–3725.
54. Warner HR, Rockstroh PA. Incorporation and excision of 5-fluorouracil from deoxyribonucleic acid in *Escherichia coli*. *J Bacteriol* 1980, **141**, 680–686.
55. de Wind N, Dekker M, Berns A, Radman M, te Riele H. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* 1995, **82**, 321–330.
56. Baker SM, Plug AW, Prolla TA, *et al*. Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. *Nature Genet* 1996, **13**, 336–342.
57. Kawate H, Sakumi K, Tsuzuki T, *et al*. Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes. *Proc Natl Acad Sci USA* 1998, **95**, 5116–5120.