

Implication of necrosis-linked p53 aggregation in acquired apoptotic resistance to 5-FU in MCF-7 multicellular tumour spheroids

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Abstract. Three-dimensional (3D) multicellular tumour spheroids (MTS) have been used as an *in vitro* model of solid tumours for drug resistance studies because they mimic the growth characteristics of *in vivo* tumours more closely than *in vitro* two-dimensional (2D) culture of cancer cell lines. As observed in solid tumours, MTS exhibits a proliferation gradient with outer regions consisting of proliferating cells that surround inner quiescent cells. The innermost cells in core regions undergo cell death mostly by necrosis to form necrotic core due to insufficient supply of oxygen and nutrient such as glucose with increasing size of spheroids. Tumour necrosis is thought to indicate a poor prognosis and to contribute to acquisition of chemoresistance in solid tumours; however, the mechanism underlying necrosis-mediated chemoresistance remains unclear. In this study, we examined the chemoresistance to 5-Fluorouracil (5-FU) using MCF-7 breast cancer MTS. 5-FU (400 μ M) induced apoptosis in MCF-7 cell monolayer as determined by HO/PI staining, PARP cleavage, p53 induction, Bax induction, and Bcl-2 down-regulation. When MCF-7 breast tumour spheroids were cultured on agarose for 8 days, they reached \sim 700 μ m in diameter, with a necrotic core. We found that 5-FU-induced apoptosis is markedly reduced in spheroids that were cultured for 9 days and had necrotic core, compared with MCF-7 monolayer cells and spheroids that were cultured for 6 days and had no necrotic core, indicating that the formation of necrotic core may be linked to acquisition of chemoresistance to 5-FU. We also found that a specific set of cellular proteins including p53 was aggregated into a RIPA-insoluble form during MTS culture. Furthermore, most of p53 induced by 5-FU was aggregated in MTS with necrotic core. Our results suggest that necrosis-linked p53 aggregation may contribute to acquired apoptotic resistance to 5-FU in MTS model system.

Introduction

In vivo human cancer cells often fail to respond to chemotherapeutic agents. The chemoresistance is achieved by various factors including increased drug efflux due to enhanced expression of ATP binding cassette transporter proteins, alterations in drug activation/inactivation or drug targets, or inhibition of apoptotic pathways due to inactivation of the tumour suppressor p53 and/or activation of anti-apoptotic pathways such as PI3K and ERK1/2 (1-4). Most studies on drug resistance were usually conducted using two-dimensional (2D) monolayers. However, the 2D monolayer *in vitro* model does not exhibit the full range of drug resistance observed in *in vivo* solid tumours. For this reason, three-dimensional (3D) multicellular tumour spheroids (MTS) have been used as an *in vitro* model of solid tumours for drug resistance studies because they mimic the growth characteristics of *in vivo* tumours more closely than cancer cell line monolayers (5-9).

When grown as 3D MTS structures, tumour cells can acquire an additional resistance to apoptosis, referred to as multicellular resistance (MCR). The acquired MCR has been demonstrated to be linked to a limitation of drug diffusion, to the activation of survival pathways such as NF- κ B or mTOR, or to the upregulation of p27 (5-9). However, the molecular basis of MCR is not yet clear. MTS closely resembles avascular tumours. As observed in *in vivo* solid tumours (10-12), MTS exhibits a proliferation gradient with outer regions consisting of proliferating cells that surround inner quiescent cells (13). The innermost cells in core regions undergo cell death mostly by necrosis to form necrotic core due to insufficient supply of oxygen and nutrient such as glucose with increasing size of spheroids, similar with poorly vascularized solid tumours. Necrosis is characterized by the disruption of cellular membranes and the release of cellular components including HMGB1 which promotes cancer cell growth, invasion, and progression (14-18). Tumour necrosis has been shown to indicate a poor prognosis (19,20) and to contribute to acquisition of chemoresistance in solid tumours, including breast cancer, non-small cell lung cancer, and gastrointestinal stromal tumours (21). However, the mechanism underlying necrosis-mediated chemoresistance remains to be clarified.

5-Fluorouracil (5-FU) is widely used in the treatment of breast, colon, and other cancers (22-24). It interferes with nucleoside metabolism and can be incorporated into RNA

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and DNA, causing cytotoxicity and cell death. 5-FU is converted intracellularly to several active metabolites: fluoro-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). FdUMP forms a stable complex with thymidylate synthase (TS), and thus inhibits production of deoxythymidine monophosphate (dTMP) that is essential for DNA replication and repair. Thus, dTMP depletion results in cytotoxicity. FdUTP and FUTP are incorporated into DNA and RNA, causing DNA and RNA damage, respectively. Chemoresistance to 5-FU is obtained by increased TS expression and by dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme in 5-FU catabolism to dihydrofluorouracil (DHFU) in the liver. The tumour suppressor p53 plays a critical role in cell cycle arrest and apoptosis in response to DNA damaging agents such as 5-FU (25,26). However, in many human cancers, p53 is found mutated, and its activity is reduced (27). For instance, colon cancers retain the mutant p53 allele in ~90% of the patients. p53 inactivation by specific mutations is a critical step in the development of many cancers, and reduces cellular sensitivity to chemotherapeutic drugs. However, certain cancer cells such as breast cancers contain the wild-type form of p53 protein; for instance, breast cancers retain the mutant allele in only <40% of the cases (28). However, breast cancer cells also exhibit resistance to 5-FU, indicating that there is an additional mechanism to overcome the wild-type p53 protein in these cancer cells. Some breast cancers have been shown to inactivate the tumour-suppressing activity of p53 by sequestering it in the cytosol, without mutation.

In this study, we examined the chemoresistance to 5-FU using MCF-7 breast cancer cell MTS. We found that 5-FU-induced apoptosis is markedly reduced in spheroids that were cultured for 9 days and had necrotic core, compared with MCF-7 monolayer cells and spheroids that were cultured for 6 days and had no necrotic core. We also found that most of p53 induced by 5-FU was aggregated into a RIPA-insoluble form in MTS with necrotic core. Our results suggest that necrosis-linked p53 aggregation may contribute to acquired apoptotic resistance to 5-FU in MTS model system.

Materials and methods

Monolayer and multicellular tumour spheroid (MTS) culture, and 5-FU treatment. Human breast adenocarcinoma cells MCF-7, MDA-MB-231, and MDA-MB-361 were obtained from American Type Culture Collection and cultured as two-dimensional (2D) monolayers in DMEM (WelGene) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin in a 37°C humidified incubator with 5% CO₂ (29,30). For three-dimensional (3D) MTS culture, 400 cells were seeded per well in 96-well plates previously coated with 1.2% agarose. Each well contained 200 µl of tissue culture medium, and the spheroids were fed every other day by carefully aspirating 100 µl of spent medium and replacing with the same quantity of fresh medium. 5-FU was purchased from Sigma. 5-FU was dissolved in dimethyl sulfoxide and then divided into aliquots and stored at -70°C until used. The stocks were freshly diluted in culture medium before experiment.

Hoechst 33258 and propidium iodide (HO/PI) double staining. To determine the cell death mode, HO (Invitrogen) and PI (Invitrogen) double staining was performed (29,30). In 2D culture, cells were seeded at a density of 2.5x10⁵ cells/ml in 35-mm dishes. After 24 h, the cells were treated with 5-FU for up to 72 h and stained with HO (1 µg/ml) and PI (5 µg/ml) for 15 min, and then dissociated with trypsin/EDTA. In 3D culture, equal numbers of spheroids were transferred to 1.2% agarose-coated 60-mm dishes at each of the days as indicated in the figure legends. The spheroids were treated with 5-FU and trypsinized and then stained with HO/PI. Additionally, 96-well plates of spheroids were harvested and fixed in 1% paraformaldehyde. After processing into wax blocks, the spheroids were sectioned and stained with H&E or HO/PI.

Western blotting. Protein lysates obtained from cells treated with 5-FU were measured using the Bradford assay. Equal amounts of protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels, and electrotransferred to NC membrane and analyzed by Western blotting with antibodies to p53, PARP, Bax, Bcl-2, p21, p27, CuZnSOD, MnSOD (Santa Cruz, CA, USA) and tubulin (Biogenex, CA, USA). After incubation with peroxidase-conjugated secondary antibody, protein expression was detected using ECL Western Blotting Detection Reagents (Amersham Biosciences).

RIPA-soluble and RIPA-insoluble fractionation. RIPA-soluble fraction was obtained by lysing cells in RIPA buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA) for 20 min on ice, followed by centrifugation at 14,000 rpm for 15 min and the RIPA insoluble fraction was suspended in PBS and boiled in 2X Laemmli sample buffer.

Spheroid selective dissociation. MTS were dissociated into subpopulations of cells from different locations in the spheroid as described previously (31). The spheroids were placed in a cylindrical chamber with a 75-µm nylon mesh. After washing the spheroids with PBS, they were treated with a dissociation solution containing 0.125% trypsin (Life Technologies, Inc.) in a phosphate buffer containing 1 mM EDTA and 25 mM HEPES (pH 7.4). Cells dissociated from the spheroids were collected into stirred tubes containing complete medium on ice, while the remaining aggregates remained in the chamber. Cell suspensions were stored on ice until dissociation was complete.

Results and Discussion

5-FU-induced cell death. We examined the cytotoxic effects of 5-FU in MCF-7 monolayer cell cultures. MCF-7 cells were treated with 5-FU at different concentrations up to 400 µM. Treatment of 5-FU caused a dose-dependent decrease in the cell viability, with prominent inhibitory effect on cell viability observed at a concentration of 400 µM. HO/PI nuclear staining was performed to determine the amount and type of cell death. DNA-binding dyes HO that is known to cross the plasma membrane of all cells, whether they are damaged or not, causing a blue fluorescence of their nuclei and PI that only

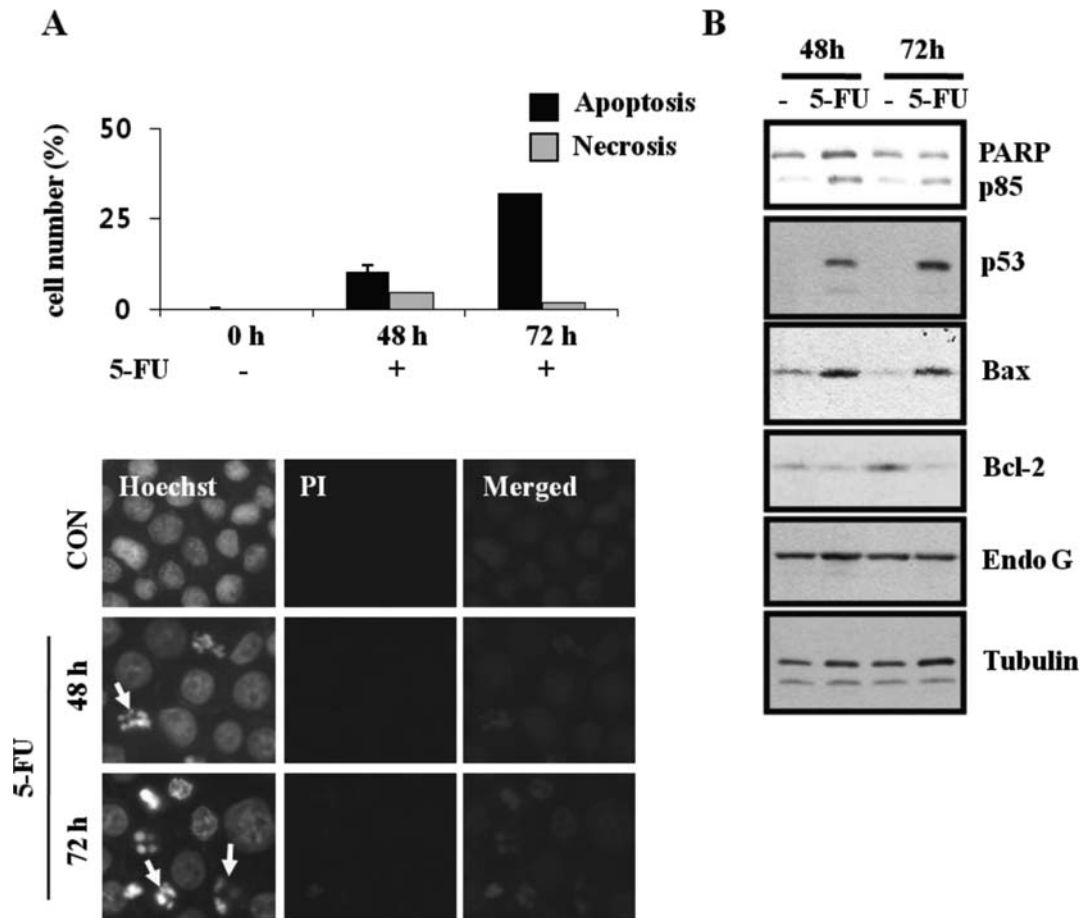


Figure 1. 5-FU induces apoptosis in MCF-7 monolayer cell cultures. (A) MCF-7 cells were treated with 400 μ M 5-FU for 48 and 72 h and then stained with HO/PI and observed with fluorescence microscopy (low panel) and apoptotic and necrotic cells were scored (upper panel). Results (500-800 cells in each group) are expressed as the means \pm SEM from three independent experiments. Arrow indicates apoptotic cells. (B) MCF-7 cells were treated with 400 μ M 5-FU for 48 and 72 h and the resulting cell lysates analyzed by Western blotting using antibodies to PARP, p53, Bax, Bcl-2, Endo G, and tubulin.

penetrates cells with damaged membranes and leads to nuclear fluorescence. Thus, intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei were considered viable, early apoptotic, late apoptotic (secondary necrotic), and necrotic cells, respectively. As shown in Fig. 1A, MCF-7 cells treated with 5-FU at concentrations of 400 μ M for 72 h showed the characteristic features of apoptotic cell death, namely nuclear chromatin condensation and the appearance of typical apoptotic bodies. 5-FU is known to induce apoptosis through induction of the tumour suppressor p53 that induces expression of pro-apoptotic Bax, and suppresses anti-apoptotic Bcl-2. 5-FU (400 μ M) appeared to induce p53 and Bax and to downregulate Bcl-2 (Fig. 1B). 5-FU also induced cleavage of poly(ADP-ribose) polymerase (PARP), a hallmark of apoptosis.

Formation of MCF-7 multicellular tumour spheroids. To generate spheroid as solid tumour model, we seeded 400 MCF-7 cells to each well of a 96-well plate pre-coated with 1.2% agarose and the morphology of spheroids *in vitro* was monitored by light microscopy (Fig. 2A). As MCF-7 cells have an adhesion molecule, E-cadherin, on cell surface, they form tightly packed, rounded spheroids of a homogeneous size that required trypsin treatment and physical strength to

disintegrate the spheroids. Multicellular spheroid is known to develop a proliferation gradient, with proliferating cells at the periphery, cell cycle arrested cells in inner regions, and necrotizing cells in core regions (1-3).

To confirm a quiescent state in MTS, the spheroids were selectively dissociated to yield cells from four discrete regions within the spheroid, with each fraction representing \sim 25% of the total spheroid cell number. We found that increased levels of CKI family proteins, p21waf1/cip1 and p27Kip1 that are primarily involved in the regulation of G1-S progression, were detected in the second and third fractions, but their lower levels were detected in the outermost fraction (first fraction) and in the innermost fraction (fourth fraction) (Fig. 2B). The levels of other proteins such as tubulin were relatively constant throughout all fractions of the spheroids. p53 that is responsible for p21waf1/cip1 induction in cell cycle control was also observed in the second fraction (Fig. 2B).

Since p27 is known to be increased by hypoxia-activated HIF-1 α , the second and third fractions are likely to represent a hypoxic region. When MCF-7 breast tumour spheroids were cultured on agarose for 8 days, they reached \sim 700 μ m in diameter (Fig. 1A). As observed previously (unpublished data), necrotic core was observed in spheroids when their size reached $>$ 700 μ m (Fig. 2C). The necrotic core in tumour spheroids was observed using H&E staining (Fig. 2C) and

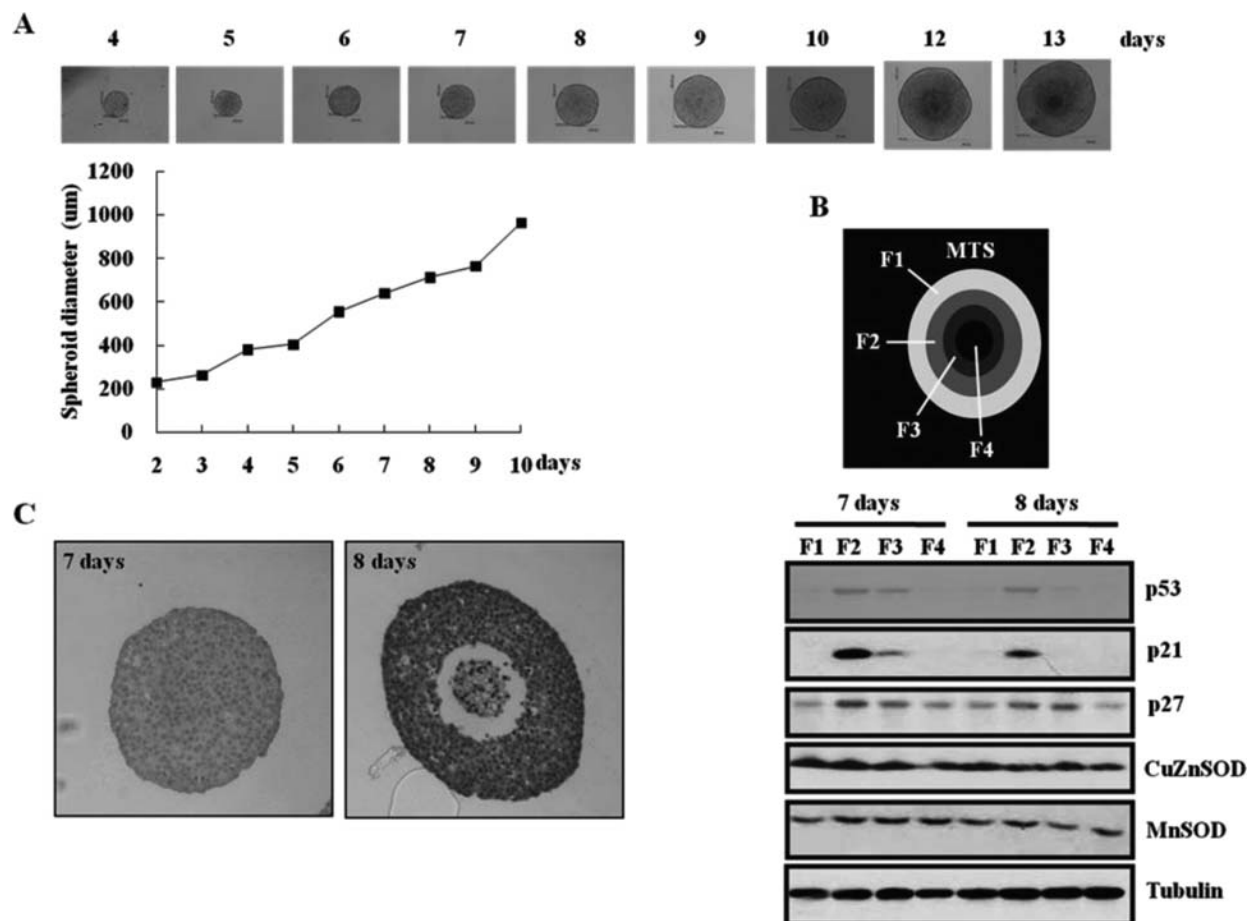


Figure 2. Formation of the necrotic core during MTS culture. (A) A growth curve of MCF-7 cells in MTS culture. MCF-7 cells were seeded into 1.2% agarose-coated 96-well plates at a density of 400 cells per well and cultured for up to 13 days. To calculate MTS size, diameters of 5 spheroids were measured every day. (B) After 7 and 8 days of MCF-7 MTS culture, MTS were dissociated into subpopulations of cells from different locations in the spheroids as described in Materials and methods. The cells isolated from different locations within MCF-7 spheroids were analyzed by Western blotting using antibodies to p53, p21, p27, CuZnSOD, MnSOD, and tubulin. (C) After 7 and 8 days of MCF-7 MTS culture, MTS were sectioned and stained with H&E.

HO and PI double staining (data not shown) of paraffin section. The cells in core regions is likely to die by necrosis due to insufficient supply of oxygen and nutrient such as glucose.

Acquisition of chemoresistance in MCF-7 spheroids after emergence of necrotic core. We next examined 5-FU-induced apoptosis using two different MCF-7 MTS model systems: 1) the spheroids that were collected on 4-7 days and did not contain necrotic core and 2) the MTS that were collected on 8-9 days and contained necrotic core. The MTS were treated with 5-FU in agarose-coated 60-mm dishes and the cells dissociated from the spheroids were stained with the HO/PI. As shown in Fig. 3, the cytotoxicity of 5-FU in 6-day spheroid was similar to that in monolayers. The similar cytotoxicity of 5-FU in monolayers and spheroids has been reported in MGH-U1 cells growing as monolayers or spheroids (32). In addition, in Chinese hamster V79 cells, identical amounts of damage were produced in monolayers and spheroids by 5-FU (33). However, 5-FU-induced apoptosis was markedly reduced in spheroids that were cultured for 9 days and had a necrotic core, compared with that of monolayers and spheroids that were cultured for 6 days and had no necrotic core (Fig. 3).

These results demonstrated that the formation of necrotic core in tumour spheroids may be linked to drug resistance to 5-FU.

A specific set of proteins including p53 was aggregated during MCF-7 MTS culture. In the course of this study, we found that the amount of RIPA-insoluble proteins was increased during MTS culture (Fig. 4). RIPA-soluble protein and RIPA-insoluble protein patterns were quite different, indicating that a specific set of proteins forms RIPA-insoluble aggregates during MTS culture. While cytosolic CuZnSOD and mitochondrial MnSOD were found only in RIPA-soluble fraction, p53 and tubulin was found in both RIPA-soluble and RIPA-insoluble fractions (Fig. 4). The levels of p53 and tubulin in RIPA-insoluble fractions were increased in MTS that were cultured for 8-9 days and had necrotic core, compared with 6-day MTS without necrotic core.

Previously we demonstrated that glucose depletion, one of the stresses that cause metabolic stress in tumours, induces necrosis in cancer cell lines including A549, HepG2, and MDA-MB231 cells *in vitro* (29,30) and that during metabolic stress-induced necrosis, many cellular proteins including p53 are aggregated in insoluble forms while some cellular proteins including CuZnSOD, HMGB1, and LDH were released (30).

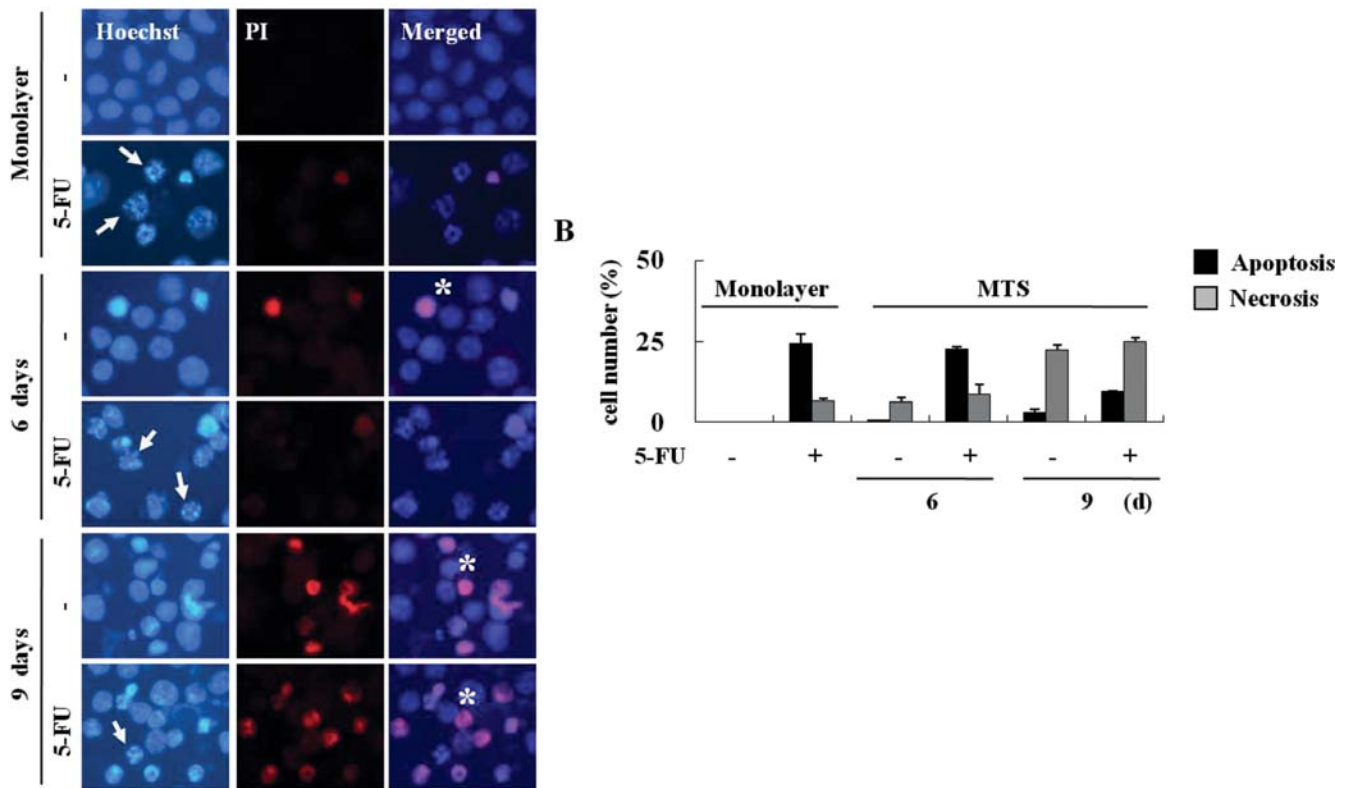


Figure 3. MCF-7 MTS acquire resistance to 5-FU-induced apoptosis. MCF-7 monolayer cells and spheroids cultured for 6 and 9 days were exposed to 400 μ M 5-FU for additional 72 h. Then the cells were dissociated and stained with HO/PI and observed with fluorescence microscopy (A) and apoptotic and necrotic cells were scored (B). Results (500-800 cells in each group) are expressed as the means \pm SEM from three independent experiments. Arrow indicates apoptotic cells; * indicates necrotizing cells.

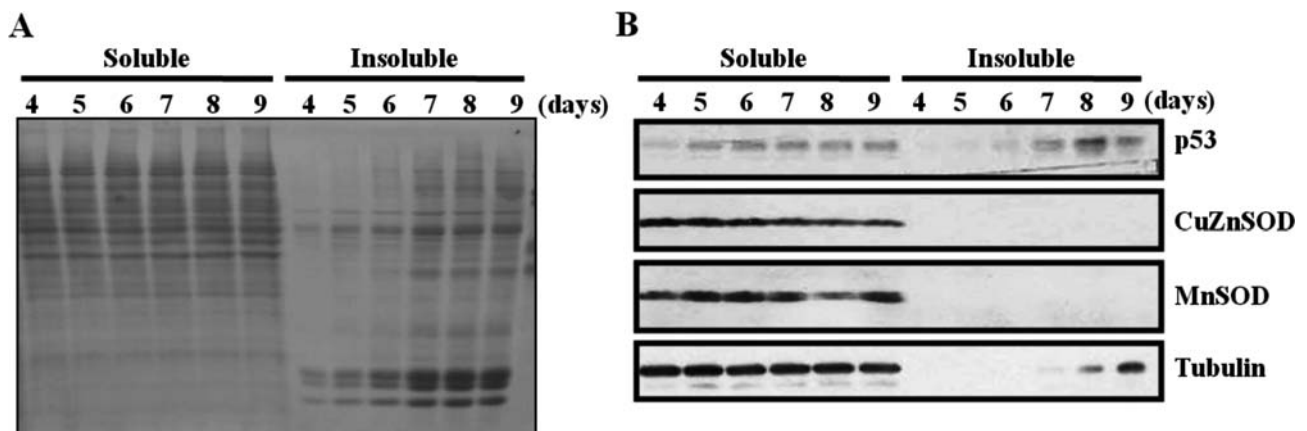


Figure 4. A specific set of proteins including p53 was aggregated during MCF-7 MTS culture. (A and B) MCF-7 cells were seeded into 1.2% agarose-coated 96-well plates at a density of 400 cells per well and cultured for 4-9 days. MTS were treated with RIPA buffer and RIPA-soluble and RIPA-insoluble fractions were obtained and analyzed by SDS-PAGE and Ponceau S staining (A) and Western blotting with antibodies to p53, CuZnSOD, MnSOD, and tubulin (B).

Intracellular H_2O_2 and O_2^- and mitochondrial ROS play a crucial role(s) in metabolic stress-induced necrosis (29). Since ROS could trigger the formation of oxidatively modified proteins that tend to aggregate, metabolic stress-induced protein aggregation is thought to be mediated by ROS (unpublished data). In MTS system, ROS may be increased in inner regions of MTS as results of metabolic stress due

to hypoxia and glucose depletion and may cause protein aggregation and necrosis. In general, necrotic cell death has been suggested to release most cellular proteins due to cell membrane rupture. However, our results show that many cellular proteins form insoluble aggregates, while certain cellular proteins including CuZnSOD, HMGB1, and LDH was selectively released. The molecular mechanism for such

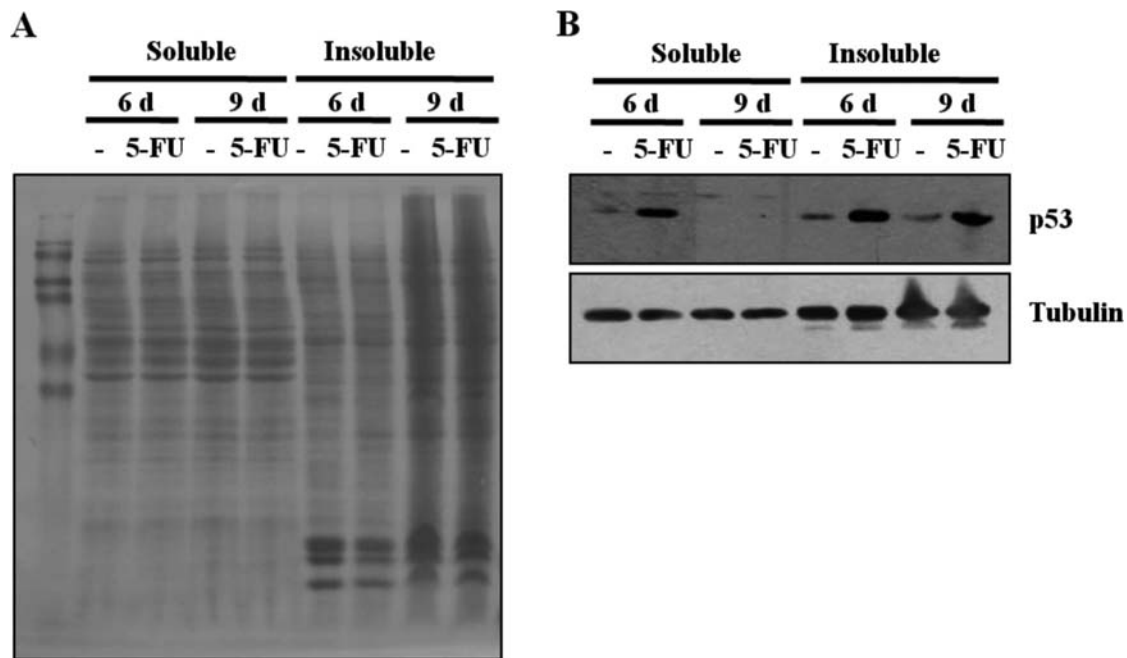


Figure 5. Most of p53 induced by 5-FU was aggregated in MTS with necrotic core. MCF-7 spheroids cultured for 6 and 9 days were exposed to 400 μ M 5-FU for additional 72 h. MTS were then treated with RIPA buffer and RIPA-soluble and RIPA-insoluble fractions were obtained and analyzed by SDS-PAGE and Ponceau S staining (A) and Western blotting with antibodies to p53 (B).

necrosis-linked protein aggregation found in both monolayers and MTS system remains to be elucidated.

Most of p53 induced by 5-FU was aggregated in MTS with necrotic core. When 5-FU was treated to MTS, p53 induction was detected (Fig. 5). However, while p53 induced in 6-day MTS in response to 5-FU was detected in both RIPA-soluble and RIPA-insoluble fractions, most of p53 induced in 9-day MTS was detected only in RIPA-insoluble fractions. Thus, p53 induced by 5-FU could be easily aggregated in spheroids with necrotic core. Insoluble p53 could be inactivated in terms of its ability to induce Bax and other target genes. Since 5-FU could induce p53 in MTS at 9 days, the apoptotic resistance to 5-FU at 9 days is not likely to be due to its limited diffusion. 5-FU is known to reach cells at all depths of the spheroid viable rim (32). In addition, examination on sensitivity profiles of cells from various regions within spheroids of Chinese hamster V79 multicell spheroids after treatment with doxorubicin, bleomycin, 5-FU, carmustine, cisplatin, chlorambucil, and mitomycin showed that penetration was a problem only for doxorubicin (34). Our results suggest that necrosis-linked p53 aggregation may contribute to acquired apoptotic resistance to 5-FU in MCF-7 MTS.

Biological relevance of this study. p53 negatively regulates cell proliferation and induces apoptosis in response to DNA damage and thus functions as a suppressor of tumorigenesis. In many human cancers, however, p53 is found mutated, and its activity is reduced, and thus, p53 inactivation by specific mutations is the most common mechanism to inhibit the tumour suppressive activities of p53 in the development of many cancers. However, recent studies showed that certain cancer cells such as breast cancers contain the wild-type form

of p53 protein (28). Nevertheless, breast cancer cells exhibit resistance to anti-cancer drugs including 5-FU, indicating that there is an additional mechanism to overcome the wild-type p53 protein in these cancer cells. These cancers are characterized by a loss of function of wild-type p53, which accumulates in intracellular aggregates. Actually, accumulation of wild-type inactive p53 has been described in various cancers including neuroblastoma, retinoblastoma, and breast and colon cancers (28,35-38). In these cells, p53 is aggregated in large cytoplasmic and/or nuclear deposits possibly arising from its conformational change. Our results show that in MTS system, ROS may be increased in inner regions of MTS as result of metabolic stress including hypoxia and glucose depletion and may cause protein aggregation including p53.

Necrosis have been shown to contribute to acquisition of chemoresistance in solid tumours (21). Our results suggest that necrosis-linked p53 aggregation may be linked to chemoresistance. In the process of acquiring resistance, the tumour may become cross-resistant to many different types of unrelated drugs. Many anti-cancer drugs have been shown to exert their tumour suppressive activities through inducing and activating p53. Thus, necrosis-linked p53 protein aggregation may contribute to tumour cell cross-resistance to other anti-cancer drugs that exert the tumour preventive activity through p53.

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