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Original Article

Growth Environment Affects Colorectal Cancer Cell Phenotype

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Defining the regulatory mechanisms of colorectal cancer cell growth, senescence, and death will help us to develop treatment strategies for colorectal cancer. In this study, we explored the biological activities and stimulation of colorectal cancer cells in different growth environments. Specifically, we observed the growth statuses of SW480 colorectal cancer cells cultured in Leibovitz L-15 medium (L-15) and Dulbecco's modified Eagle medium (DMEM), respectively. Interestingly, SW480 cells maintained in L15 (SW480/L-15) were larger and flatter with lower proliferation rate than SW480 cells maintained in DMEM (SW480/DMEM). The expressions of P21 and P16, the regulatory proteins related to senescence and cell cycle, were significantly induced in SW480/DMEM. However, the expression of P53 was stable in SW480/L-15 and SW480/DMEM. The cell senescence-associated β-galactosidase activity was undetectable in SW480/L-15 and SW480/DMEM. These results showed that different growth conditions affect the morphology and proliferation of SW480 cells, not through cellular senescence but, perhaps, via cell cycle alteration or other signaling pathway.

Keywords: Phenotype, Colorectal Cancer, Growth Environment, P21, P16, Senescence

Introduction

Colorectal cancer is among the top three malignancies in Taiwan in terms of incidence and mortality. Genetic factors and diet are the main causes. Daily dietary intake impacts on the health of individuals. Similarly, the growth environment affects the proliferation and signaling activities of cells. Most of the current biomedical research uses cell lines as models. However, cells grown in different culture environments may have various responses to the same stimulus. In 2019, Mikael demonstrated that the composition of cell culture medium greatly affects cell growth and sensitivity to selenium cytotoxicity. In another study, changes in phenotype of A549 cells maintained under different culture conditions were noted [1].

Cellular senescence is an irreversible state in which cells stop growing or there is cell cycle arrest. American scientist Leonard Hayflick proposed the "Hayflick Limit" for human cell growth. Cultured cells stop dividing after an average of 50 cumulative population doublings (CPDs). Senescence-related cellular stress includes oxidative stress, mitochondrial dysfunction, irradiation, and chemotherapeutic drugs. Expression of P53 is pivotal for the establishment of senescence, mainly following P53 activation through intrinsic DNA damage response (DDR) [2,3]. Initiation of cellular senescence not only involves P53/P21, but also P16/pRB signaling activation, usually in response to an endogenous factor, such as shortening telomeres, cell proliferation-related signals, or

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activation of tumor suppressor molecules [4-10]. In addition to the regulation of cellular senescence, the expression of P53 downstream mediator P21 leads to transient cell cycle arrest [3,11,12]. P21 binds to and inhibits the activity of CDK2 or CDK4 cell cycle regulatory complexes and, thus, functions as a regulator of cell cycle progression in G1 phase. P16 is also known to function as an inhibitor of CDK4 kinase to modulate cellular senescence and cell cycle arrest in response to a variety of stress stimuli [13,14]. The characteristic phenotype of cancer cells includes continuous cell proliferation signaling, cell homeostasis, and metabolism. Therefore, senescence may be a powerful physiological defense which can antagonize tumor activity, thereby counteracting carcinogenesis and revealing its potential for use in cancer therapy [3,4,8].

We cultured SW480 colorectal cancer cells in Leibovitz L-15 (L-15) medium according to the American Type Culture Collection (ATCC) suggested cell culture formula. However, laboratories also use other media such as Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, and RPMI to culture SW480 cells [1,15-22]. Different nutrients provide different cell growth environments to trigger different cellular activities. Therefore, it is interesting to observe whether the cellular responses of SW480 colorectal cancer cells cultured in L-15 medium and DMEM differ.

Materials and Methods

Cell culture and cell counting

The colorectal cancer cell line SW480 (CCL-228TM) was cultured in L-15 medium, based on ATCC (Manassas, VA, USA) suggestions, or DMEM. To both media were added 10% fetal bovine serum (FBS) at 5% CO_2 and 37°C. The growth of cultured cells was observed and images were captured with Olympus biological system microscope CX-41 under visible light. Cell counts were obtained following trypan blue staining.

Western blot analysis

SW480 cells were cultured with L-15 medium or DMEM and protein lysates were collected. Cells were washed twice with PBS and lysed in sample buffer, then boiled at 95°C for 10 min. Total proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Then, the membrane was blocked and incubated with indicated antibodies, followed by detection using HRP chemiluminescence method, during which the membranes were exposed to LAS-3000 (Fujifilm, Tokyo, Japan).

Senescence-associated β-galactosidase staining

SW480 cells were seeded onto 3.5cm dish with L-15 medium or DMEM and transfected with or without CMV-LacZ plasmid for 72hrs. Cells were washed with PBS and fixed with 1ml fixation buffer at room temperature for 10 min. Then, cells were stained with freshly prepared SA-β-Gal staining solution after washing with PBS, based on the protocol provided by the manufacturer (Beta-Galactosidase Staining Kit, Clontech #631780). Stained cell images were observed and captured with a 400X microscope.

Statistical analysis

Statistical analyses were performed using Student t-test with more than three independent experiments. Means are reported, with significance level set at $p < 0.05$ for all analyses.

Results

Differences in cell growth and morphology between media

To observe the effects of different growth conditions on colorectal cancer cells, we cultured SW480 colorectal cancer cells in L-15 medium or DMEM. Table 1 shows the various components required for cell growth in L-15 medium and DMEM. SW480 cells were cultured for 10 generations and cell growth density was recorded at 0, 24, 48, 72 and 96 hours after cell subculture, respectively. As shown in Figure 1, cell growth density of SW480 cells cultured in DMEM (SW480/DMEM) was significantly higher than that of SW480 cells cultured in L-15 medium (SW480/L-15) at 96 hours.

Changes in cell phenotypes were observed after 10 generations. As shown in Figure 2, SW480/L-15 cells were flatter and larger than SW480/DMEM cells. However, the growth rate of SW480/L-15

Table 1. Contents of L-15 medium and DMEM

L-15			DMEM (High Glucose)		
	mg/L	mmol/L		mg/L	mmol/L
$CaCl_2$ (anhydrous)	140	1.2615	$CaCl_2$ (anhydrous)	200	1.8021
KCl	400	5.3655	$Fe(NO_3).9H_2O$	0.1	0.0002
KH_2PO	60	0.4409	KCl	400	5.3333
$MgCl_2$ (anhydrous)	93.68	0.9839	$MgSO_4$ (anhydrous)	97.67	0.8139
$MgSO_4$ (anhydrous)	97.67	0.8112	NaCl	6400	110.345
NaCl	8000	136.8925	$NaH_2PO_4.H_2O$	125	0.9058
Na_2HPO_4 (anhydrous)	190	1.3384	L-Arginine HCl	84	0.3981
L-Alanine	225	2.5255	L-Cysteine 2HCl	62.57	0.2013
L-Arginine	500	2.87	L-Glutamine	584	3.996
L-Asparagine (anhydrous)	250	1.89	L-Histidine $HCl.H_2O$	42	0.2004
L-Cysteine	120	0.99	L-Isoleucine	104.8	0.8015
L-Glutamine	300	2.0527	L-Leucine	104.8	0.8015
Glycine	200	2.6642	L-Lysine HCl	146	0.7978
L-Histidine FB	250	1.611	L-Methionine	30	0.2013
L-Isoleucine	125	0.9529	L-Phenylalanine	66	0.3995
L-Leucine	125	0.95	L-Serine	42	0.3997
L-Lysine	75	0.51	L-Threonine	95	0.7983
L-Methionine	75	0.503	L-Tryptophan	16	0.0783
L-Phenylalanine	125	0.7567	L-Trysine $2Na.2H_2O$	103.79	0.3985
L-Serine	200	1.9031	L-Valine	93.6	0.8034
L-Threonine	300	2.5185	D-Ca Pantothenate	4	0.0084
L-Tryptophan	20	0.0979	Choline Chloride	4	0.0286
L-Trysine	300	1.6556	Folic Acid	4	0.0091
L-Valine	100	0.8536	Myo-Inositol	7	0.0389
D-Ca Pantothenate	1	0.0021	Niacinamide	4	0.0328
Choline Chloride	1	0.0072	Pyridoxine HCl	4	0.0195
Folic Acid	1	0.0023	Riboflavin	0.4	0.0011
Myo-Inositol	2	0.0111	Thiamine HCl	4	0.0119
Niacinamide	1	0.0082	D-Glucose	4500	24.9978
Pyridoxine HCl	1	0.0049	HEPES	0	0
Riboflavin-$5PO_4$-Na	0.1	0.0002	Phenol Red (Sodium)	15	0.0399
Thiamine PO_4-Cl-$2H_2O$	1.1	0.0026	Sodium Pyruvate	0	0
D(+)Glalactose	900	4.9956	$NaHCO_3$	3700	44.04762
Phenol Red (Sodium)	10.65	0.0283	Glycine	30	0.4
Sodium Pyruvate	550	4.9982			

was lower than that of SW480/DMEM. This may have been due to cellular senescence or cell cycle alteration. The most obvious senescence biomarkers are growth arrest and progressive morphological changes.

Inductions of P21 and P16 are independent of P53 in DMEM

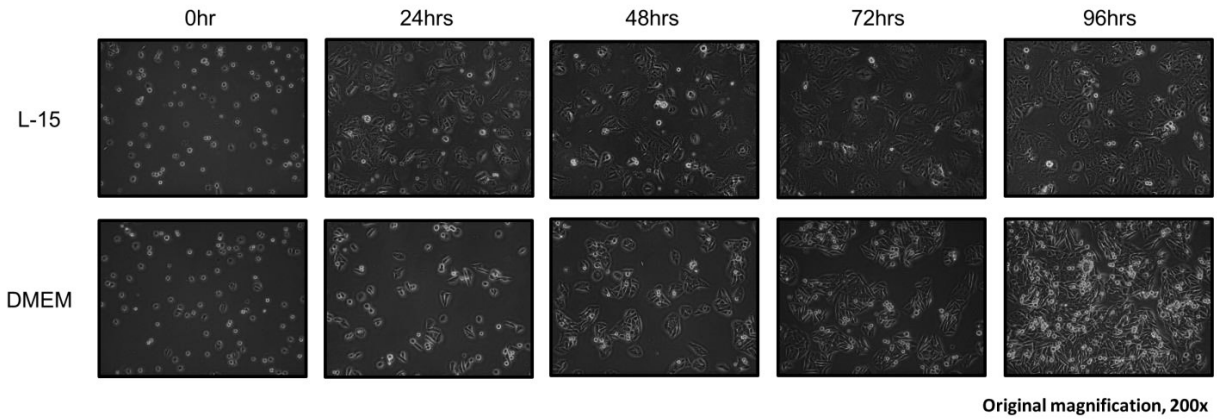

Figure 1. Differences in cell growth density in two media.
SW480 colon cancer cells (1.5 x 10^5) were seeded onto 3.5cm dish and incubated with L-15 medium or DMEM. Cells were observed and recorded at different times (0hr, 24hrs, 48hrs, 72hrs, 96hrs) under 200X microscope. The growth density of SW480/L-15 was lower than that of SW480/DMEM.

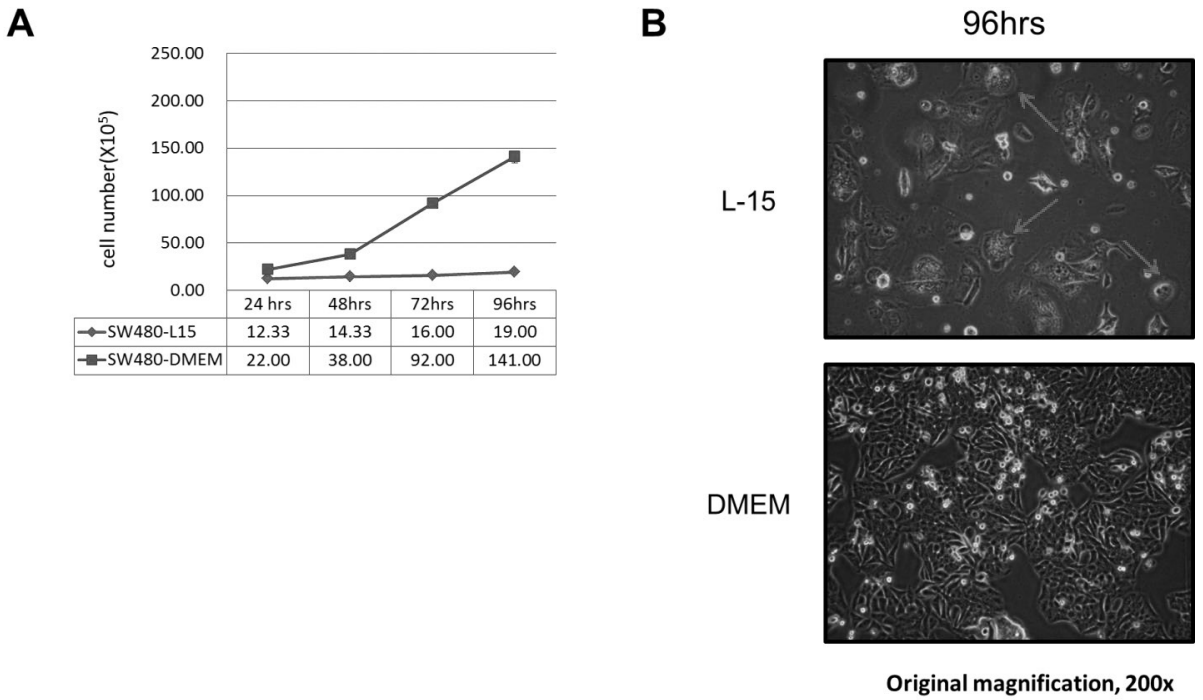

Figure 2. Differences in cell growth rate and morphology between the two media.
(A) 3 x 10^5 SW480 colon cancer cells were seeded onto 6cm dish and incubated with L-15 medium or DMEM. Quantitative results are the numbers of SW480 cells in different media at different times (24hrs, 48hrs, 72hrs, 96hrs). (B) Cells cultured in L-15 medium or DMEM at 96hrs were observed under a microscope (200X). SW480/L-15 cells were large and flat (red arrows).

As the critical marker of cellular senescence, P21, the downstream target of P53, has been shown to initiate cellular senescence. Another major pathway for senescence is P16/pRB signaling. Both P16 and P21 are cyclin-dependent kinase inhibitors that negatively regulate the cell cycle. We used Western blot to detect the expressions of P53, P21, and P16 in SW480/L-15 and SW480/DMEM. As shown in Figure 3, expressions of P21 and P16 were induced in SW480/DMEM. However, P21 and P16 protein expressions were P53-independent, as P53 was expressed stably and P53 expressions did

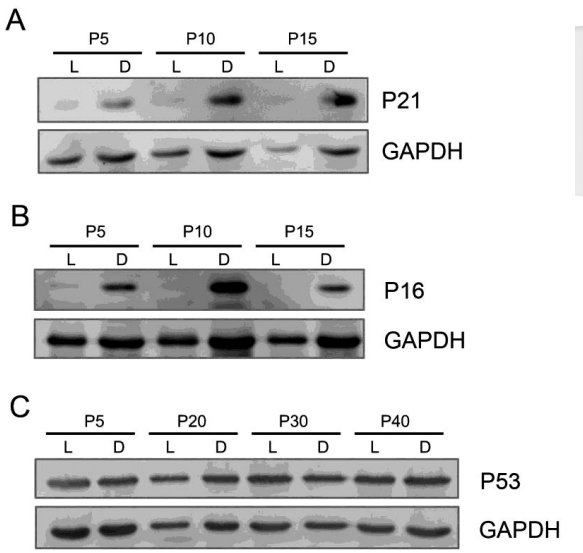

Figure 3. DMEM induces P21 and P16 expressions independent of P53.
SW480 cell lysates were prepared in different passages (P5, P10, P15) and protein expressions were analyzed by Western blot with the indicated P21 or P16 antibodies. (A) P21 protein expressions in SW480/L-15 and SW480/DMEM. (B) P16 protein expressions in SW480/L-15 and SW480/DMEM. All protein expressions of P21 and P16 were inducible in SW480/DMEM compared with L-15. (C) Cell lysates were prepared in different passages (P5, P20, P30, P40) and protein expressions were analyzed by Western blot with the indicated P53 antibodies. There were no significant differences in protein expression of P53 between SW480/L-15 and SW480/DMEM. (L: L-15, D: DMEM).

not change significantly in SW480/L-15 or SW480/DMEM.

Neither medium triggers cell senescence-associated β-galactosidase activity

Currently, a widely used senescence marker is senescence-associated β-galactosidase (SA-β-gal) activity. Therefore, we stained SW480/L-15 cells with SAβ-gal to detect whether cellular senescence is initiated in different culture environments. All SW480 cells were continuously cultured in L-15 medium or DMEM and senescence was detected by β-galactosidase activity at the 20th passage. The plasmid CMV-lacZ was transfected into SW480/L-15 or SW480/DMEM by lipofectamine plus as a positive control for β-galactosidase activity. After 48 hours of incubation, the cells were fixed and SA-β-gal activity was detected. The positive control transfected CMV-lacZ turned blue due to the presence of β-galactosidase activity, but SW480/DMEM and SW480/L-15 did not, which means that no cellular senescence-associated β-galactosidase was detected in either culture environment.

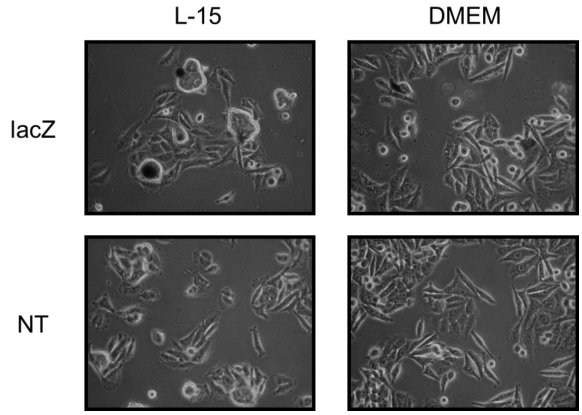

Figure 4. Senescence-associated β-galactosidase activity was negative in SW480/L-15 and SW480/DMEM.
Ten generations of SW480 cells were cultured in L-15 medium or DMEM medium and cellular senescence was detected by β-galactosidase activity stain. CMV-lacZ was transfected into SW480/L-15 or SW480/DMEM as positive control for β-galactosidase activity. After 24hr incubation, cells were fixed and SA-β-gal activity was detected. Shown here is a representative image following SA-β-gal staining (magnification 400X). (NT: non-transfection)

Discussion

In this study, the phenotype of SW480 colorectal cancer cells was affected under different culture conditions. We found that culture condition influences cellular morphology and proliferation. SW480 cells were larger and flatter with lower growth rate in L15 medium compared with SW480 cells in DMEM. P53-independent expressions of P21 and P16 were significantly induced in SW480/DMEM. Senescence-associated β-galactosidase activity was undetectable in both SW480/L-15 and SW480/DMEM.

Although changes in cell morphology and low growth rate are characteristic of cellular senescence, SA-β-gal activity was negative under both culture conditions. Most importantly, expression of cell senescence regulatory proteins P21 and P16 did not increase in the larger, flatter, and slower growing SW480/L-15 cells. In contrast, induction of P21 and P16 proteins was observed in the rapidly growing SW480/DMEM group. P21 and P16 are not only senescence regulatory proteins, but also cell cycle regulatory proteins. P21 binds to and inhibits the activity of CDK2 or CDK4 complexes and P16 inhibits CDK4 kinase [6,13,23]. The expressions of these cell cycle regulatory proteins may inhibit cell cycle progression and cause cell cycle arrest. However, we did not observe growth retardation or cell cycle arrest in SW480/DMEM cells expressing P21 and P16 proteins. Growth was faster than for SW480/L-15 cells, which were not induced to express P21 or P16 proteins.

P21 was first identified as a downstream target of P53 and an essential mediator of P53-dependent cell-cycle arrest. Subsequent evidence has demonstrated that the activity of P21 does not necessarily require the participation of P53. P21 acts either as a tumor suppressor or as an oncogene depending on whether P53 status is gain of function or loss of function. P21 also has a multifunctional role in cell biological activity, including cell proliferation, cell migration, apoptosis, transcription regulation, DNA repair, cell autophagy, and cell senescence [12,24-27]. To date, the functional role of P21 depends on cell type, environmental stimulation, subcellular localization, post-translational modification regulation, and even p53 status.

The proliferation of SW480/DMEM cells, in which the expressions of P21 and P16 were induced, was relatively fast. In contrast, there were only weak expressions of P21 and P16 in slow-growing SW480/L-15 cells. To clarify this, we compared the differences in the components of L-15 and DMEM. As shown in Table 1, the DMEM formula contains glucose, while the L-15 formula does not. This result is similar to our previous findings. P16 protein expression in and growth rate of SW480 cells increase in the presence of high concentration of glucose [28]. A previous study has shown that MCF7 breast cancer cells cultured in low-glucose medium enter senescence, inhibiting their growth [29].

Based on our results, P21 and P16 play different roles in regulating cell proliferation. Understanding their detailed regulatory mechanisms requires a more rigorous experimental design. Most importantly, the cell culture environment biologically stimulates the cells, which in turn affects cell proliferation and biological activity. This leads to different biological responses of the same cells to different culture environments. As cell models are widely used in experimental research in many life science fields, our findings provide scientific experimental evidence and a sufficient reference for research design and the impact of comparative analysis.

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