Cell Culture Model for Colon Cancer Prevention and Therapy: An Alternative Approach to Animal Experimentation

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Summary
Mouse models for colon cancer that harbor a germ line mutation in the tumor suppressor gene Adenomatous polyposis coli (Apc) exhibit a primary genetic defect that predisposes to a high incidence of adenomatous polyps in the small intestine rather than in the colon. Colon cell culture models expressing quantifiable markers for carcinogenic risk may represent an alternative approach to reduce, refine or replace long-term animal experimentation. The newly developed colon epithelial cell lines 1638N COL-Cl1 (clonal derivative of the parental Apc mutant cell line 1638N COL) and 1638N COL-Pr1 (tumor derivative of the clone), established from an Apc1638N [+/-] mutant mouse, exhibit aberrant cell cycle progression, down-regulated apoptosis, enhanced carcinogenic risk and tumor formation, indicating that aberrantly proliferative preneoplastic1638N COL-Cl1 cells exhibit a quantifiable risk for carcinogenesis. Treatment of these preneoplastic Apc mutant cells with a combination of celecoxib and 5-fluorouracil at clinically achievable low concentrations produced a 2.1 fold to 5.5 fold higher efficacy for cytostatic growth arrest and a 40.2% to 52.4% higher efficacy for inhibition of carcinogenic risk, relative to that obtained by these agents used individually. Thus, a low dose combination of mechanistically distinct agents resulted in enhanced efficacy. These data validate a novel cell culture model and a rapid mechanism-based approach to prioritize efficacious drug combinations for animal studies and clinical trials on cancer prevention and, thereby, support the 3R concept by refining and/or reducing the use of animals in biomedical research relevant to prevention/therapy of colon cancer.

Zusammenfassung: Ein Zellkulturmodell für die Dickdarmkrebs-Vorsorge und Therapie: Eine Alternative zum tierexperimentellen Ansatz
Mausmodelle für Dickdarmkrebs, welche die Keimzell-Mutation für das Tumorsuppressorgen Apc (adenomatöse Polyposis coli) tragen, zeigen ein durch diesen primären Gendefekt bedingtes häufigeres Auftreten adenomatöser Polypen im Dünndarm eher als im Dickdarm. Dickdarm-Zellkulturmodelle, die messbare Marker für das Krebsrisiko exprimieren, könnten geeignet sein Langzeittierversuche zu reduzieren. Die neu entwickelten Dickdarm-Zelllinien 1638N COL-CL1 (klonale Ableitung der parentalen Linie 1638N COL) und 1638N COL-Pr1 (aus Tumoren entnommen), gewonnen aus der Mausmutante Apc 1638N [+/-], wiesen anormale Zellzyklen, verminderte Apoptose, erhöhtes Krebsrisiko und Tumorbildung auf. Die Behandlung von präneo-plastischen Apc Mutantenzellen mit einer Kombination aus Celecoxib und 5-Fluorouracil führte zu einer 2,1- bis 5,5- fach höheren Wirksamkeit der zytostatischen Wachstumshemmung und zu einer 40,2 bis 52,4% höheren Wirksamkeit bei der Unterdrückung des Krebsrisikos im Vergleich zum Einsatz der einzelnen Substanzen. Diese Daten belegen ein neues Zellkulturmodell und einen schnellen mechanistischen Ansatz zur Auswahl effektiver Therapiekombinationen für Tierversuche und klinische Versuche zur Krebsprävention. Das 3R Konzept wird damit durch eine Verminderung der Belastung und eine Reduktion der Tierzahlen in der biomedizinischen Forschung zur Vorsorge und Therapie des Dickdarmkrebses gefördert.

Keywords: APC mutation, colon cancer risk, prevention

1 Introduction

Colon cancer is a multi-step, multi-factorial disease where genetic, environmental and dietary factors exert a profound effect on etiology, pathogenesis and preventive/therapeutic intervention (Potter, 1996). Traditionally, animal models such as azoxymethane (AOM)-induced rat colon cancer or in adenomatous polyposis coli (Apc) gene mutant mice have been utilized as preclinical approaches for the evaluation of toxicity and efficacy of potential preventive/therapeutic agents (Corpet and Pierre, 2003). Rational selection of relevant alternative approaches may reduce, refine or replace the need for

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extensive long-term animal experiments on colon carcinogenesis and its prevention.

The germ line mutation in the tumor suppressor Apc gene represents a primary genetic defect responsible for clinical familial adenomatous polyposis (FAP), a high-risk syndrome for early onset colon cancer (Fodde et al., 2001; Fearon and Vogelstein, 1990). Apc mutant mice, however, exhibit adenomas and carcinomas predominantly in the small intestine, rather than in the colon (Moser et al., 1990, Fodde et al., 1994). The predominance of carcinogenesis in the small intestine of Apc mutant mice represents a target site distinct from that for clinical colon cancer, and hence data generated from these models require extrapolation to determine their clinical relevance.

Reliable cell culture models established from the appropriate target organ site, that express a clinically relevant genetic defect, and exhibit quantifiable markers of risk for carcinogenesis are expected to offer alternative approaches that may reduce or refine animal experiments, minimize necessary extrapolation and, thereby, complement *in vivo* animal studies. Such cell culture studies may also identify a rapid mechanism-based approach to evaluate the potential efficacy of agents that inhibit, delay or reduce the incidence of colon cancer.

The Apc ^{Min}/+ mouse, which harbors a germ line mutation in the Apc tumor suppressor gene, exhibits a high incidence of intestinal adenomas (Moser et al., 1990) that are susceptible to growth inhibition by several mechanistically distinct chemopreventive agents used independently or in combination (Jacoby et al., 2000; Torrance et al., 2000; Swamy et al., 2006). It is also noteworthy that pharmacological (Saez et al., 1998; Paulson et al., 2001) or genetic (Rao et al., 2005; Sodir et al., 2006) manipulation leads to colon cancer in the Apc ^{Min}/+ mice, indicating that multi-step colon carcinogenesis is inducible in this model.

Recent cell culture studies using murine cell lines have demonstrated that histopathologically normal colon epithelial cells expressing mono-allelic mutations in the Apc gene (Katdare et al., 2002), or in Apc and Mlh_1 genes (Telang et al., 2006), exhibit aberrant proliferation and enhanced risk for carcinogenesis. Furthermore, these mutant cells are also susceptible to growth inhibition by several chemopreventive agents used either individually or in combination (Katdare et al., 2002; Telang et al., 2006; Telang and Katdare, 2007).

The present report summarizes recent experiments conducted on a newly developed colon epithelial cell culture model for FAP to: i) characterize the model with regard to the extent of aberrant proliferation and risk for spontaneous carcinogenic transformation and ii) evaluate the susceptibility of the model to inhibition of growth and reduction of carcinogenic risk by a combination of mechanistically distinct preventive/therapeutic agents. The outcome of the present study demonstrates that the clonally selected, aberrantly proliferative Apc mutant 1638N COL-Cl_1 cells display a defined risk for carcinogenesis and that combination of mechanistically distinct preventive/therapeutic agents given at low concentrations leads to enhanced efficacy of growth inhibition and to a greater reduction of the risk of carcinogenesis, relative to that produced by these agents used independently.

2 Materials and methods

2.1 Cell lines

Apc [+/+] C57 COL (Source: normal descending colon of C57BL/6J mouse), 1638N COL- Cl_1 (Source: Apc mutant clonal derivative from parental Apc [+/-] 1638N COL cell line) and 1638N COL-Pr_1 (Source: tumor-derived cells from transplantation of 1638N COL-Cl_1 cells) were grown in DME/F12 medium supplemented with 10% heat-inactivated fetal calf serum, 0.24 IU/ml (10 µg/ml) insulin and 1 µM dexamethasone. The culture medium also contained the antibiotic mixture (100 IU/ 100 µg/ml penicillin/ streptomycin +50 µg/ml fungizone +50 µg/ml gentamycin). Routinely, the stock cultures of all the cell lines were maintained at 37°C in an humidified atmosphere of 95% air: 5% CO_2, fed with fresh medium every 48 hr and sub-cultured at 1:10 split at about 70-80% confluency (Katdare et al., 2002).

2.2 Population doubling time, anchorage-independent colony formation and tumorigenecity

These end points were measured using previously optimized assays (Telang et al., 1990; Jinno et al., 1999; Katdare et al., 2002 and Telang et al., 2006). Population doubling time (PDT) was calculated from the exponential growth phase. Viable cell counts were obtained at 24, 48, 72 and 96 h post-seeding of $1x10^5$ cells. These time points cover the exponential growth phase of the cell lines.

For the anchorage independent colony formation (AICF) assay, cells were suspended at the initial seeding density of 100 cells/well in 0.33% agar made up in DME/F12 medium. This cell suspension in agar was overlaid on a basement layer of 0.6% agar in six well cluster plates. The cultures were maintained at 37°C, and the numbers of anchorage independent colonies per well were determined at day 14 post-seeding (Telang et al., 1990; 1991).

For the tumorigenicity assay $1x10^6$ cells suspended in 0.1 ml of the culture medium were injected sub-cutaneously in the flank region of C57BL/6J mice. The transplant site was palpated at weekly intervals, and palpable tumors were excised at 1 cm diameter. The primary tumor cells were expanded in culture to establish the tumor-derived 1638N COL-Pr_1 cell line using previously optimized culture conditions (Telang et al., 1990).

2.3 Cell cycle analysis

The status of cell cycle progression was determined by monitoring percent distribution of cells in sub G_0, G_0/G_1, S, and G_2/M phases of the cell cycle (Jinno et al., 1999; Katdare et al., 2002). The cell cycle data was expressed as $S+G_2/M$ (proliferative, P): sub G_0 (apoptotic, A) ratio.

2.4 Preventive/therapeutic test compounds

The selective COX-2 inhibitor celecoxib (CLX) and selective thymidylate synthase inhibitor 5-fluorouracil (5-FU) were used as prototypical test compounds because of their documented preclinical and clinical efficacy in prevention/therapy of colon cancer (Jacoby et al., 2000; Steinbach et al., 2000 and Dia-

sio and Harris, 1989). The stock solutions (100 mM) were made up in 100% ethanol (EtOH), and were serially diluted in the culture medium to obtain 0.01, 0.1, 1, 10 and 100 µM concentrations. The growth inhibitory effects were determined by obtaining viable cell counts at day 5 post-seeding of $1x10^5$ cells (Katdare et al., 2002; Telang et al., 2006). The primary cell count data were expressed as inhibitory concentration (IC_{50}) values and were compared with the reported clinically or experimentally achievable mean plasma levels of the two compounds.

2.5 Combinatorial efficacy

The efficacy of low concentration combinations of the mechanistically distinct CLX and 5-FU was evaluated using each compound independently and in combination at less than its respective individual IC_{20} concentration. Viable cell number and cell cycle progression at day 5 post-seeding and AICF at day 14 post-seeding represented the quantitative end points.

3 Results

3.1 Aberrantly proliferative Apc mutant cells exhibit an enhanced risk for carcinogenesis

Relative to the wild type Apc [+/+] C57 COL cells, the Apc [+/-] mutant clonal derivative 1638N COL-Cl_1 and the tumor derived 1638N COL-Pr_1 cells exhibited a progressive 55.9% and 64.7% decrease in population doubling time (PDT). Furthermore, the Apc mutant cell lines also exhibited a 100% incidence of anchorage-independent colony formation (AICF), and a 100% incidence of tumor formation (Tab. 1). It is noteworthy that the 1638N COL-Cl_1 cell line, representing a clonal derivative obtained from a single anchorage-independent colony of the parental Apc [+/-] 1638N COL cell line, exhibited persistent anchorage-independent colony formation *in vitro* and tumorigenicity upon *in vivo* transplantation. Thus, 1638N COL-Cl_1 cells exhibit pre-requisites for a preneoplastic phenotype.

3.2 Apc mutant cells exhibit altered cell cycle progression

The aberrant proliferation in the Apc mutant cells was associated with an increase of the cell population in the S+G_2/M phase of the cell cycle and a decrease in the sub G_0 (apoptotic) population. Thus, relative to that observed in the C57 COL cells, the 1638N COL-Cl_1 and 1638N COL-Pr_1 exhibited an 89.4% and 152.8% increase in the cell population in the S+G_2/M phase of the cell cycle. In addi-

Tab. 1: Aberrant proliferation and carcinogenic transformation in Apc mutant colon epithelial cell lines

Cell Line	PDT [a] (hr)	AICF [b] (%)	Tumour Incidence [c] (%)
C57 COL	34	0/12 (0%)	0/10 (0%)
1638N COL-Cl_1	15	12/12 (100%)	10/10 (100%)
1638N COL-Pr_1	12	12/12 (100%)	5/5 (100%)

[a] Population doubling time (PDT) determined from the exponential growth phase.
[b] Anchorage independent colony formation (AICF) determined from the number of colonies at day 14 post-seeding. Number of wells with colonies/Total wells.
[c] Tumour incidence determined after sub-cutaneous transplantation of $1x10^6$ cells in C57BL/6J mice.

Tab. 2: Aberrant cell cycle progression in Apc mutant colon epithelial cell lines

Cell Line	Biomarker		
	S+G_2/M [a] (% P)	Sub G_0 [a] (% A)	P:A Ratio
C57 COL	24.6±3.3[b]	4.3±1.1[e]	5.7
1638N COL-Cl1	46.6±1.9[c]	0.6±0.4[f]	77.7
1638N COL-Pr1	62.2±3.0[d]	0.3±0.2[g]	207.3

[a] Mean ± SD, n=6 per cell line.
[b - c, e - f] p=0.02,
[c - d, e - g] p=0.01.

Tab. 3: *In vivo* pharmacokinetics and *in vitro* dose response of preventive/therapeutic compounds

Agent	Plasma Levels (range, µM)		1638N COL-Cl_1 (IC_{50}, µM) [a]
	Clinical	Experimental	
CLX [b]	2.5-10.0	3.0-5.7	5.2±0.5
5-FU [c]	3.0-10.0	5.0-10.0	0.3±0.1

[a] Determined from the concentration range of 0.01-100 µM. Mean ± SD, n=6.
[b] Jacoby, R. .F et al., *Cancer Res. 60*, 5040-5044, 2000.
[c] Takimoto, C. H. et al., *Clin. Cancer Res. 5*, 1347-1352, 1999.

tion, the Apc mutant cells exhibited an 86.0% and 93.0% inhibition in confluency-dependent spontaneous apoptosis. The cell cycle data, expressed as P:A ratio, revealed a progressive increase in favor of proliferation (Tab. 2).

3.3 Apc mutant cells are susceptible to growth inhibition by a combination of preventive/therapeutic compounds

The experiments presented in Table 3 compared the efficacy of growth inhibition by CLX and 5-FU in the Apc mutant clonally derived preneoplastic 1638N COL-Cl_1 cells, determined by the IC_{50} values, to the published *in vivo* clinical and experimental pharmacokinetic parameters that were determined by measuring the plasma levels of the two agents. These comparative data demonstrate that the IC_{50} values are within the pharmacologically achievable range.

The experiments presented in Table 4, examined the effects of CLX and 5-FU independently and in combination on the clonally derived Apc mutant preneoplastic 1638N COL-Cl_1 cells. The status of the viable cell population and AICF represented the quantitative end points. A low dose combination of CLX+5-FU at IC < 20 exhibited 2.1 to 5.5 fold increased efficacy for growth arrest, relative to that obtained by these compounds used independently. In addition, the data obtained from the AICF assay demonstrated that the CLX+5-FU combination was 40.2% to 52.4% more efficacious than the two compounds used independently.

3.4 Combination of preventive/therapeutic compounds alters cell cycle progression

The experiments presented in Table 5 examined the status of cell cycle progression in clonally derived Apc mutant preneoplastic 1638N COL-Cl_1 cells in response to treatment with low dose CLX and 5-FU independently and in combination. The CLX+5-FU combination induced an at least 80.7 to 105.7% increase in the cell population at G_1 phase and 38.1 to 41.4% decrease in the cell population at the S+G_2/M phase of the cell cycle relative to that observed with CLX and 5-FU used independently.

Tab. 4: Inhibition of aberrant proliferation in Apc mutant colon epithelial 1638N COL-Cl_1 cell line

Agent	Concentration (μM)	Inhibition (% of Control)	
		Viable Cell Number [a]	AICF [b]
CLX	1.0	7.9	52.9
5-FU	0.1	16.5	57.5
CLX+5-FU	1.0+0.1	51.4	80.6

[a] Number of viable cells in solvent controls on day 5 post-seeding: 49.5±3.1x10^5. Mean ± SD, n=6 per treatment group.

[b] Anchorage independent colony formation (AICF). Number of anchorage independent colonies in solvent controls on day 14 post-seeding: 18.1±1.1. Mean ± SD, n=12 per treatment group.

Tab. 5: Regulation of cell cycle progression in Apc mutant colon epithelial 1638N COL-Cl_1 cell line

Agent	Concentration (μM)	Phase of Cell Cycle [a] (%)		
		G_1	S+G_2/M	Ratio
CLX	1.0	32.1	67.9	0.4
5-FU	0.1	28.2	71.8	0.4
CLX+5-FU	1.0+0.1	58.0	42.0	1.4

[a] Determined from at least 10^4 fluorescent events at day 5 post-seeding. Mean n=6 per treatment group.

4 Discussion

In an effort to minimize indiscriminate use of animal testing, the 3R concept strives to replace, reduce or refine the existing *in vivo* animal models (Russell and Burch, 1959). In the area of colon cancer prevention the rat model for chemically induced colon cancer and the Apc mutant mouse model for spontaneous intestinal cancer remain the most extensively used preclinical models (Corpet and Pierre, 2003). However, unlike the clinical FAP syndrome for colon cancer, the Apc mouse models exhibits carcinogenesis predominantly in the small intestine rather than in the colon (Moser et al., 1990; Fodde et al., 1994), thus, requiring extrapolation of the data to determine their clinical relevance. These aspects emphasize a need to develop alternative models that express clinically relevant genetic defects (Apc mutation) at the appropriate target organ site (colon), and exhibit a quantifiable risk for carcinogenesis.

Most of the existing cell culture models relevant to genetically predisposed syndromes of colon cancer are developed from clinical colon cancer cell lines that exhibit genetic defects in Apc (Ilyas et al., 1997) or DNA mismatch repair genes (Meyers et al., 2001). Fully transformed tumor cell phenotype of these models offers only a limited advantage in studies focused to identify early occurring molecular/genetic events that predispose to the multi-step carcinogenic process by enhancing the risk of cancer development. Existing animal models for the genetic predisposition exhibit carcinogenesis predominantly in the small intestine, rather than in the colon (Moser et al., 1990, Fodde et al., 2001), thus differing in the target organ site from that in the early onset clinical colon cancer. In contrast, the present cell culture model ex-

presses a clinically relevant genetic defect (Apc mutation) in cells established from the appropriate target organ site (histopathologically normal colon), and exhibit a quantifiable risk for carcinogenesis (AICF *in vitro* and tumorigenicity *in vivo*). Thus, these observations strongly suggest that the present cell culture model uniquely facilitates identification of clinically translatable mechanistic leads with minimal extrapolation.

The outcome of experiments designed to characterize the present model clearly demonstrated that the clonally derived Apc mutant preneoplastic 1638N COL-Cl_1 and the tumor derived 1638N COL-Pr_1 cell lines exhibit a progressive decrease in PDT, increase of the cell population in the S+G_2/M phases of the cell cycle, and decrease in confluency-dependent spontaneous apoptosis. In this context it is noteworthy that, similar to the present data, a previously published study on the aberrantly proliferative parental 1638N COL cells (Katdare et al., 2002) has demonstrated that Apc mutant cells exhibit aneuploidy, increased population doublings and decreased G_0/G_1:S+G_2/M ratio. Similar alterations have also been reported in mammary epithelial cells transfected with ras, myc or Her-2/neu oncogenes (Telang et al., 1991; Telang et al., 1990; Katdare et al., 2003). Thus, these observations suggest that down-regulation of the tumor suppressor Apc gene expression or aberrant over-expression of ras, myc or HER-2/neu oncogenes may represent a primary genetic defect responsible for aberrant proliferation.

The experiments conducted to evaluate the risk of carcinogenesis utilized AICF *in vitro* and tumor incidence upon transplantation as the quantitative end points. The data generated from these experiments demonstrated that the Apc mutant cells exhibit a higher incidence of AICF *in vitro* and tumor formation *in vivo* in comparison with the wild type Apc [+/+] C57 COL cells that tested negative for both these end points. These data are consistent with those from previous studies on mammary epithelial cells where targeted over-expression of ras, myc or HER-2/neu oncogenes results in AICF *in vitro* and tumor formation *in vivo* (Telang et al., 1990; Telang et al., 1991; Zhai et al., 1993). It is noteworthy that clonally selected 1638N COL-Cl_1 cells exhibited a high incidence of tumors upon *in vivo* transplantation. Thus, the 1638N COL-Cl_1 represent a preneoplastic phenotype, and AICF, a modulatable surrogate end point biomarker for the risk of carcinogenic transformation, similar to that reported for mammary epithelial cells (Jinno et al., 1999; Katdare et al., 2002; Katdare et al., 2003).

In a previously published study the Apc mutant parental 1638N COL cells demonstrated susceptibility to growth inhibition by several mechanistically distinct chemopreventive agents used as single agents (Katdare et al., 2002). The mechanistically distinct preventive/therapeutic compounds CLX and 5-FU have documented efficacy as single agents in the clinical management of colon cancer (Steinbach et al., 2000; Takimoto et al., 1999; Diasio and Harris, 1989). Long-term high dose therapy with these compounds, however, is associated with adverse toxicity that compromises patient compliance (Solomon et al., 2005; Diasio and Harris, 1989; Takimoto et al., 1999). The IC_{50} values obtained in the present cell culture model compare favorably with clinically or experimentally achievable plasma levels, thereby providing clinical relevance for the responsiveness of the present cell culture model to CLX and 5-FU.

Preclinical animal studies with low dose combinations of mechanistically distinct compounds have demonstrated enhanced efficacy with minimal toxicity (Torrance et al., 2000; Swamy et al., 2006). Consistent with the above observations, data from the present experiments provide proof-of-principle evidence that combination of low dose CLX+5-FU exhibits greater efficacy for cytostatic growth arrest and for inhibition of AICF, relative to that exhibited by these compounds used independently. Furthermore, the higher efficacy of the CLX+5-FU combination is predominantly due to enhanced G_1 phase arrest and inhibition of S+G_2/M phase of the cell cycle. The molecular mechanisms responsible for the observed cell cycle modulation and synergistic/additive interactions of the CLX+5-FU combination, however, remain to be identified.

In conclusion, the data generated from this study demonstrate that aberrantly proliferative Apc mutant cells display an increased risk for carcinogenic transformation, and that the risk is modifiable by combination of mechanistically distinct preventive/therapeutic compounds. These data therefore validate a novel alternative cell culture approach to rapidly prioritize efficacious preventive/therapeutic agents and, thereby, reduce and refine traditional animal studies identifying interventional strategies for future clinical trials on prevention/therapy of colon cancer.

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