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Establishment of a multi-component dietary bioactive human equivalent dose to delete damaged Lgr5⁺ stem cells using a mouse colon tumor initiation model

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Abstract

Multi-component therapy has gained interest for its potential to synergize and subsequently lower the effective dose of each constituent required to reduce colon cancer risk. We have previously demonstrated that rapidly cycling Lgr5⁺ stem cells are exquisitely sensitive to extrinsic dietary factors that modulate colon cancer risk. In the present study, we quantified the dose-dependent synergistic properties of dietary n-3 polyunsaturated fatty acids (PUFA) and curcumin (Cur) to promote targeted apoptotic deletion of damaged colonic Lgr5⁺ stem cells. For this purpose, both heterogeneous bulk colonocytes and Lgr5⁺ stem cells were isolated from Lgr5-EGFP-IRES-CreER^{T2} knock-in mice injected with azoxymethane (AOM). Isolated cells were analyzed for DNA damage (γ H2AX), apoptosis (cleaved caspase-3) and targeted apoptosis (both γ H2AX and cleaved caspase-3) at 12 hr post AOM injection. Comparison of the percentage of targeted

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apoptosis in Lgr5⁺ stem cells (GFP^{high}) across a broad bioactive dose range revealed an ED₅₀ of 16.0 mg/d n-3 PUFA + 15.9 mg/d Cur. This corresponded to a human equivalent dose (HED) of 3.0 g n-3 PUFA + 3.0 g Cur. In summary, our results provide evidence that a low dose (n-3 PUFA + Cur) combination diet reduces AOM-induced DNA damage in Lgr5⁺ stem cells and enhances targeted apoptosis of DNA damaged cells, implying that a lower HED can be utilized in future human clinical trials.

Keywords

Lgr5⁺ stem cells; DNA damage; targeted apoptosis; curcumin; n-3 PUFA; human equivalent dose (HED)

Introduction

There is an increasing awareness that the use of synergistic drug and/or dietary bioactive combinations can lower the dose of each constituent and consequently minimize potential adverse effects [1, 2]. For example, there is a growing interest in drug combinations that might reduce cancer risk [3]. In a previous study, we demonstrated that azoxymethane (AOM)-induced DNA damaged Lgr5⁺ stem cells, the cells of origin of colon cancer [4], were preferentially deleted by targeted apoptosis via the enhancement of p53 signaling upon administration of a combinational chemo-protective diet. This resulted in the reduction in AOM-induced nuclear β -catenin levels in aberrant crypt foci (ACF) [5], a premalignant biomarker in mice [6–8]. The experimental combinatorial diet contained bioactive n-3 polyunsaturated fatty acids (PUFA), enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and curcumin (Cur) as compared to fish oil or curcumin alone [5]. This corresponded to a mouse daily dose of n-3 PUFA (46 mg / 30 g body weight) and curcumin (45 mg / 30 g body weight), which is equivalent to human daily dose of 8.6 g n-3 PUFA / 70 kg body weight and 8.5 g curcumin / 70 kg body weight [9]. With respect to toxicity, Cur is generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA) and in clinical studies, curcumin has been administered safely at doses of up to 12 g daily over 3 months [10]. With regard to n-3 PUFA, the FDA has established a GRAS level of 3 g of n-3 PUFA per day and 5 g in the European Food Safety Authority (EFSA). This creates a potential concern, because the HED of the n-3 PUFA (8.6 g/d) diet used in our previous preclinical study exceeds FDA and EFSA recommendations. Therefore, it is essential to assess the dietary threshold for phenotypically significant responses by using lower doses of n-3 PUFA and Cur.

In this study, we determined the dose-dependent combined chemo-protective effects of dietary Cur and n-3 PUFA on colonic Lgr5⁺ stem cells by quantifying the percentage of targeted apoptosis in the presence of a carcinogen using Lgr5-EGFP-IRES-CreERT² knock-in mice. As part of this effort, novel FlowSight™ image-based flow cytometry was used to increase sample throughput and reduce potential bias associated with traditional fluorescence microscopy. We also extrapolated the median effective animal dose (ED₅₀) required to remove DNA damaged Lgr5⁺ stem cells by targeted apoptosis to generate an HED. Our novel data provide evidence that Lgr5⁺ stem cells are highly responsive to a low

dietary dose of Cur and n-3 PUFA, which are capable of enhancing targeted apoptosis, a critical biomarker of colon cancer risk.

Materials and methods

Animals, diets and study design

The animal use protocol was approved by the University Animal Care Committee of Texas A&M University and conformed to NIH guidelines. Lgr5-EGFP-IRES-CreER^{T2} knock-in mice [4] (6 ~ 7 wk old, males and females) were acclimated for 1 wk and subsequently maintained on a semi-purified diet (Figure 1A) for 3 wk prior to injection with AOM (Sigma, St. Louis, MO, 10 mg/kg body weight). Mice (n = 7 ~ 8 per group, ~ 30 g body weight) were injected with a single dose of AOM and euthanized 12 hr later. Control mice (n = 5) received a single saline injection. Immediately after termination, the distal colon was rapidly removed, flushed with ice-cold saline and a longitudinal section of the distal colon was used for isolating colonocytes. Figure 1B shows the timeline of the treatments and the experimental design. Since fixation can alter the fluorescence signature, to avoid contamination of cell populations after the PFA fixing step (Supplemental Figure 1A and 1B), we initially sorted GFP^{high} and GFP^{neg} live cells using a MoFlow fluorescent cell sorter followed by fixation of cells with 4% PFA.

Diets

Diet details have been previously reported [5]. Briefly, complete diets containing 1.9~4.5% (w/w) corn oil (CO, containing n-6 PUFA) were used as baseline controls, i.e., contained no n-3 PUFA or curcumin (Figure 1A). Diets containing 1.4 ~ 4.0% (w/w) Menhaden fish oil (FO, containing n-3 PUFA) and 0.23~1.0% (w/w) Cur were used to determine the median effective dose (ED₅₀) required to promote targeted apoptosis in colonic Lgr5⁺ stem cells. All FO and Cur combinatorial diets contained 0.5% CO (w/w) to ensure that essential fatty acid requirements were met (Figure 1A). The four FO + Cur diets listed in Table 1 contained different levels of total fat (%), thus the level of total fat (%) in each CO diet was matched to serve as a control. In addition, the ratio of FO : Cur across all combination (FO + Cur) diets was maintained at 4:1 (w/w) (Figure 1A). Mice were provided with fresh diet (10 g) every day, which exceeded the average daily consumption (5 g) [11]. Animals had free access to food and water at all times. For quality control purposes, the fatty acid composition of the diets was analyzed by gas chromatography and the level of Cur was quantified by ¹H NMR [12]. To assess target cell incorporation of dietary bioactives in vivo, the fatty acid composition of isolated colonic crypts was quantified by gas chromatography [13] and the level of Cur was quantified by HPLC [12, 14].

DNA damage and apoptotic cell staining

To quantify targeted apoptosis in stem cells, isolated colonocytes from the distal colon were sorted based on GFP expression using a Beckman Coulter MoFlo Astrios as previously described [5, 15]. Dead cells were stained using propidium iodide (PI, 1 µg/mL) and excluded from sorting. GFP^{high} cells (Lgr5⁺ stem cells) and GFP^{neg} cells (includes all populations of colonocytes due to the mosaic nature of GFP expression [16]) were sorted and subsequently fixed in 4% PFA for 20 min at room temperature followed by incubation in

100 mM glycine for 30 min to quench paraformaldehyde autofluorescence. After washing with PBS, cells were permeabilized in 70% EtOH for 24 hr at -20°C followed by rehydration in PBS prior to immunostaining. Fixed and permeabilized cells were subsequently stained with phospho- γH2AX -Alexa Fluor 647 antibody (γH2AX -AF 647, Cell Signaling, 20E3, 4 μL Ab/ 0.5×10^6 cells), to detect DNA damaged cells, and cleaved caspase-3-Alexa Fluor 555 antibody (cleaved caspase-3-AF 555, Cell Signaling, D3E9, 2 μL Ab/ 0.5×10^6 cells) to detect apoptotic cells, for 24 hr at 4°C followed by washing with PBS. Because of the variable number of cells per sample, the antibody concentration varied proportional to the cell number. Stained cells were counterstained with DAPI and quantified by imaging flow cytometry.

Imaging flow cytometry

Samples were analyzed using a FlowSight® (Amnis®, EMD Millipore) imaging flow cytometer. Excitation lasers (403, 488 and 647 nm) were used for imaging single cells. On average, 18,000 GFP^{high} cell events and 434,000 GFP^{neg} cell events per sample were collected. Bright field (430–480 nm), nucleus (405 nm), cleaved caspase-3 (555 nm) and γH2AX (647 nm) channels were used for data collection. Color compensation was not necessary as no overlapping emission spectra were observed. IDEAS (Amnis®, EMD Millipore) version 6.0 software was used for analysis, and image based features and single cell gating control for each population was performed.

Statistics

One-way ANOVA followed by Sidak's multiple comparison test adjustment was used to determine the biological effect of dietary n-3 PUFA and Cur dose as compared to each respective CO control diet. The significant comparisons were obtained based on adjusted *P*-values <0.05 .

Results

Characterization of γH2AX -labeled DNA damaged cells and cleaved caspase-3-labeled apoptotic cells

To elucidate the dose response of colonic Lgr5⁺ stem cells to bioactive compounds in the presence of the carcinogen, we utilized mice expressing enhanced green fluorescent protein (EGFP) knocked into the Lgr5 genomic locus [17]. Mice were fed with different doses of the combination chemo-protective diet (n-3 PUFA + Cur) for 3 wks (Table 1), injected with AOM and euthanized 12 hr later. Since the colon contains a heterogeneous population of cells, Lgr5⁺ stem cells were sorted based on GFP expression and compared to bulk (GFP^{neg}) cells as a control (Figure 1B). Isolated cells were subsequently fixed in 4% PFA and % of DNA damage (γH2AX) and apoptosis (cleaved caspase-3) and targeted apoptosis (both γH2AX and cleaved caspase-3) at 12 hr post AOM injection were quantified. Focused single cells were identified using gradient root mean square of the bright field image and bright area and aspect ratio (Supplementary Figure 1C and 1D). Single cells not incubated with antibodies were used for establishing cells negative for γH2AX or cleaved caspase-3 (Supplementary Figure 1E and 1G). Supplementary Figure 1F and 1G shows representative images of γH2AX positive and negative cells and cleaved caspase-3 positive and negative

cells. To determine if signal from GFP^{high} cells (AF 488) interfered with the AF 555 channel (cleaved caspase-3), fixed GFP^{high} and GFP^{neg} cells were compared. As shown in Supplementary Figure 2, no GFP^{high} cells populated the AF 555 channel when compared to GFP^{neg} cells before or after compensation, indicating that the GFP signal did not interfere with the AF 555 channel.

To quantify the percentage of DNA damaged, apoptotic and targeted apoptotic cells, samples were prepared as shown in Figure 1B. To provide biological negative controls, colonic cells from saline injected mice (Figure 1C) were used to gate cells negative for γ H2AX-AF 647 and cleaved caspase-3-AF 555. As shown in Figure 1D & E, AOM-induced γ H2AX⁺ and/or cleaved caspase-3⁺ cells were detected both in mice fed FO + Cur and CO diets.

Representative images of Lgr5⁺ stem cells (GFP^{high}), apoptotic and/or DNA damaged Lgr5⁺ stem cell are shown in Figure 1F.

Lgr5⁺ stem cells respond to n-3 PUFA and curcumin in a dose-dependent manner in the presence of AOM

With respect to AOM-injected mice fed the control 4.5% CO diet, 23%, 20% and 35% of Lgr5⁺ stem cells (GFP^{high}) were DNA damaged (γ H2AX⁺), apoptotic (cleaved caspase-3⁺) and targeted apoptotic (γ H2AX⁺; cleaved caspase-3⁺) cells, respectively. In comparison, 5%, 14% and 69% of Lgr5⁺ stem cells were classified as DNA damaged, apoptotic and targeted apoptotic cells, respectively, in AOM-injected mice fed the 4% FO + 0.5% CO + 1% Cur diet (Figure 2A, B & C). This finding is consistent with previous results, where the chemo-protective effects of the 4% FO + 1% CO + 1% Cur diet were documented [5].

Next, we determined the dose-dependent effects of n-3 PUFA⁺ Cur on the percentage of DNA damaged, apoptotic and targeted apoptotic Lgr5⁺ stem cells derived from mice injected with AOM. As shown in Figure 2A, mice fed the 2.8% FO + 0.5% CO + 0.69% Cur and 1.9% FO + 0.5% CO + 0.46% Cur diets exhibited a marked suppression (20 ~ 25%) in DNA damaged (γ H2AX⁺) cells as compared to their respective control group. However, no statistically significant ($p > 0.05$) chemo-protective effect was detected in mice fed the 1.4% FO + 0.5% CO + 0.35% Cur diet as compared to their corresponding control. In terms of the number of stem cells exhibiting an apoptotic phenotype, no statistically significant chemo-protective diet effects were detected across all groups as shown in Figure 2B. With respect to targeted apoptosis of genetically compromised stem cells, the percentage of γ H2AX⁺ and cleaved caspase-3⁺ doublepositive cells was quantified. As shown in Figure 2C, mice fed 2.8% FO + 0.5% CO + 0.69% Cur and 1.9% FO + 0.5% CO + 0.46% Cur exhibited markedly increased numbers (1 ~ 1.5 - fold) of targeted apoptotic (γ H2AX⁺, cleaved caspase-3⁺) Lgr5⁺ stem cells as compared to its control group. However, no statistically significant ($p = 0.15$) chemo-protective effect was detected in mice fed the lowest level (1.4% FO + 0.5% CO + 0.35% Cur) of bioactives as compared to their respective control group of mice.

GFP^{neg} cells represent all populations of colonocytes including Lgr5⁺ stem cells, progenitor cells and differentiated cells due to the mosaic nature of GFP expression in Lgr5-EGFP-IRES-CreERT² knock-in mice [16]. In GFP^{neg} cells, only mice fed the 1.9% FO + 0.5% CO + 0.46% Cur diet exhibited a reduction in DNA damage as compared to mice in their control

group (Figure 2D), while only mice fed the 4% FO + 0.5% CO + 1% Cur diet exhibited enhanced apoptosis as compared to those controls (Figure 2E). This may reflect the dilution of DNA damaged and apoptotic cell populations in crypts, as the majority of AOM-induced DNA damaged/apoptotic cells are present in the crypt base where Lgr5⁺ stem cells and transit-amplifying cells reside [19]. With respect to the percentage of targeted apoptotic (γ H2AX⁺, cleaved caspase-3⁺) GFPneg cells, significant chemo-protective dietary effects were detected across all groups compared to control as shown in Figure 2F. These findings indicate that DNA damaged cell populations (predominantly Lgr5⁺ stem cells and transit-amplifying cells) are highly responsive to the combined FO + Cur treatment. We subsequently calculated the median effective dose (ED50) [18], i.e., the dietary bioactive dose that produces a chemo-protective targeted apoptosis effect in 50% of damaged (γ H2AX⁺) GFP^{high} stem cells. As shown in Figure 2G, the daily consumption of 16.0 mg of n-3 PUFA and 15.9 mg of Cur (1.4% FO + 0.5% CO + 0.35% Cur) resulted in targeted apoptosis of 50% of damaged stem cells. This corresponded to a daily HED of 3.0 g of n-3 PUFA (EPA + DHA) and 3.0 g of Cur.

Discussion

In a previous mouse study [5], we demonstrated that AOM-induced DNA damaged Lgr5⁺ stem cells, the cells of origin of colon cancer [4], were effectively deleted by targeted apoptosis upon feeding a diet containing the combination of 4% FO (equivalent to 1% n-3 PUFA) and 1% Cur, as compared to FO or Cur alone. This diet also synergistically reduced AOM-induced nuclear β -catenin levels in ACF [5], demonstrating the utility of using a combinatorial dietary regimen to delete damaged Lgr5⁺ stem cells and reduce colon cancer risk. However, the dose-dependent effects of a FO + Cur combination diet were not investigated. Therefore, in the present study, we further examined the potential synergistic chemo-protective effects of low dose Cur and n-3 PUFA combination in the presence of AOM. The daily dose of the experimental chemo-protective diet (4% FO + 1% Cur) in our previous study [5] was 46 mg of n-3 PUFA and 45 mg of Cur as described in Figure 1A. Comparison of the percentage of targeted apoptosis in (GFP^{high}) Lgr5⁺ stem cells across a broad dose range of bioactives (16 ~ 32 mg n-3 PUFA + 15.9 ~ 31.7 mg Cur) revealed an ED50 of 16.0 mg n-3 PUFA + 15.9 mg Cur. This corresponds to an HED of 3.0 g n-3 PUFA + 3.0 g Cur, which is generally recognized as safe (GRAS) by the FDA and EFSA. Analysis of the impact of the allometric exponent on the conversion of an animal dose to HED utilizes body surface area (BSA) for dose calculations [9]. The FDA has suggested that the extrapolation of animal dose to human dose is correctly performed only through normalization to BSA, which often is represented in mg/m² [9]. Accordingly, the approach of converting animal doses to an HED based on BSA is standard for estimating starting doses for initial studies in healthy volunteers. Thus, we extrapolated n-3 PUFA and Cur dosages in the mouse diet to an HED based on BSA [9] as shown in Figure 1A.

Although n-3 PUFA are well tolerated at levels approaching 20 g/day [19, 20], bioactivity in the 3 g/d range is noteworthy, because treatment of human subjects with 2 g of EPA for 6 mos was associated with a 22% reduction in polyp number [21]. In addition, 2.5 g of n-3 PUFA (corresponding to 1.4 g EPA + 1.1 g DHA) significantly reduced proliferative indices in patients with colonic polyps [22]. According to [ClinicalTrials.gov](https://clinicaltrials.gov), a number of clinical

trials utilizing curcumin are underway or have been completed [23]. Cur is known to be well tolerated when taken at high doses (12 g/d) [24, 25], and a 40% reduction in colonic ACF number was observed in a phase II clinical trial upon treatment with a 4 g/d dose of Cur [26]. Based on our findings, we propose that this promising clinical outcome may be attributed to the enhanced deletion of DNA damaged stem cells.

In summary, the present study provides cogent rationale for translating the observed synergistic chemo-protective effects of n-3 PUFA and Cur to a clinical human colon cancer risk trial. Our novel findings demonstrate that Lgr5⁺ stem cells are very responsive to low dose dietary cues following the induction of DNA damage, and provide a therapeutic strategy for eliminating damaged stem cells and reducing colon cancer risk in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental design and validation.

(A) Daily dose of 5 g of experimental diets and their corresponding HED values (left). Animal dose was extrapolated to an HED by applying a conversion based on body surface area (right). (B) Experimental procedure and schematic showing the gating strategy of the targeted apoptosis assay. (C) Fluorescence intensity of γ H2AX-AF 647 and cleaved caspase3-AF 555 of cells from mice injected with saline (control) used to identify double negative cells. Cells from mice fed (D) 4.5% CO control diet and (E) 4% FO + 0.5% CO + 1% Cur diet were used to identify DNA damaged (γ H2AX⁺), apoptotic (cleaved caspase3⁺) and targeted apoptotic (γ H2AX⁺, cleaved caspase3⁺) cells. (F) Representative images of a double negative cell (#1849), apoptotic cell (#147), DNA damaged cell (#963) and triple positive DNA damaged, apoptotic and GFP^{high} cell (#1830). FO, fish oil; Cur, curcumin; CO, corn oil; n-3 PUFA, n-3 polyunsaturated fatty acids.

Figure 2. Dose response of colonic GFP^{high} and GFP^{neg} cells to n-3 PUFA and curcumin in the presence of AOM during cancer initiation.

Comparison of the percentage of (A) γ H2AX⁺ (DNA damaged) GFP^{high} cells, (B) cleaved caspase3⁺ (apoptotic) GFP^{high} cells and (C) γ H2AX⁺; cleaved caspase3⁺ (targeted apoptotic, double positive) GFP^{high} cells from mice fed varying doses of FO + Cur diets relative to the CO (control) diet. Comparison of the percentage of (D) γ H2AX⁺ (DNA damaged) GFP^{neg} cells, (E) cleaved caspase3⁺ (apoptotic) GFP^{neg} cells and (F) γ H2AX⁺; cleaved caspase3⁺ (targeted apoptotic, double positive) GFP^{neg} cells from mice fed varying doses of FO + Cur relative to mice fed the CO (control) diet. (G) Dose response curve. ED₅₀ is indicated by the arrow.

Table 1.**Experimental diets.**

Experimental diet composition (FO, fish oil; CO, corn oil; Cur, curcumin).

Ingredients	Diets (g/100 g)							
	4.5% CO	4.0% FO + 0.5% CO + 1.00% Cur	3.3% CO	2.8% FO + 0.5% CO + 0.69% Cur	2.4% CO	1.9% FO + 0.5% CO + 0.46% Cur	1.9% CO	1.4% FO + 0.5% CO + 0.35% Cur
Sucrose	42.50	41.50	43.70	43.01	44.60	44.14	45.10	44.75
Casein	20	20	20	20	20	20	20	20
DL-methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
AIN-76 Mineral mix	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
AIN-76 Vitamin mix	1	1	1	1	1	1	1	1
Choline Chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Corn Starch	22	22	22	22	22	22	22	22
Cellulose	6	6	6	6	6	6	6	6
Curcumin	0	1.00	0	0.69	0	0.46	0	0.35
Fish oil	0	4.0	0	2.8	0	1.9	0	1.4
Corn oil	4.5	0.5	3.3	0.5	2.4	0.5	1.9	0.5
Total	100	100	100	100	100	100	100	100