

Inhibition of Large Intestinal Cancers by Celecoxib Using a Serial Sacrifice Technique*

BELA TOTH and MELISSA COLES

*The Eppley Institute for Research in Cancer and Allied Diseases,
University of Nebraska Medical Center, Omaha, Nebraska, U.S.A.*

Abstract. *In this serial sacrifice experiment, celecoxib (C) was administered at a 0.1% dose level, in the diet of female Swiss Webster CFW outbred mice. The animals also received either 1,2-dimethylhydrazine dihydrochloride (1,2-DMH) as ten weekly subcutaneous (s.c.) injections at 20 µg/g body weight or physiological saline (PS) as ten weekly s.c. injections at 0.01 ml/g body weight. Subsequently, the mice were sacrificed at 26 weeks or 35 weeks after the first injection of 1,2-DMH or PS. The number of mice with large intestinal tumors and the total number of these tumors were: Group 1 (1,2-DMH), 29 and 438; Group 2 (C + 1,2-DMH), 18 and 64; and Group 3 (PS), 1 and 1, in the mice sacrificed at 26 weeks. The corresponding tumor incidences in the mice sacrificed at 35 weeks were: Group 1 (1,2-DMH), 30 and 323; Group 2 (C + 1,2-DMH), 23 and 134; and Group 3 (PS), 0 and 0. Histopathologically, the tumors were diagnosed as polypoid adenomas and adenocarcinomas of the cecum, colon and rectum. Celecoxib treatment inhibited the development of large intestinal cancers in mice sacrificed at 26 or 35 weeks after the first injection of the carcinogen.*

In a recent study, we administered celecoxib (C), at a 0.1% dose level, in the diet of female Swiss Webster CFW outbred mice for life. The animals also received 1,2-dimethylhydrazine dihydrochloride (1,2-DMH) as ten weekly subcutaneous (s.c.) injections at 20 µg/g body weight. The administration of C reduced, in a statistically significant manner, the number of mice with large intestinal cancer and the total number of tumors (1). In the present series of

experiments, essentially identical treatments were given to the same type of mice, except that the animals were sacrificed at either 26 or 35 weeks after the first injection of 1,2-DMH or physiological saline (PS).

Serial sacrifice investigations are frequently employed to disclose the time sequence of cancer induction and progression in chemical carcinogenesis and prevention studies. Additionally, the current studies were part of some immunological experimentation in which the immune phenotype, cytokine expression and the reversal of tumor-associated immune suppression were altered. The induction of large intestinal tumors was associated with a significant increase in immature myeloid suppressor cells and a significant decrease in CD⁺T cells in the spleen, both of which were reversed by C administration (2).

Thus, celecoxib, a non-steroidal anti-inflammatory agent selective for the cyclooxygenase-2 (COX-2) enzyme, which is essential to the synthesis of prostaglandins, was proven to prevent large intestinal carcinogenesis in experimental animals.

Materials and Methods

Female Swiss albino Webster, CFW, outbred mice (Charles River Laboratories, Wilmington, MA, USA) were used. The mice were housed, in a modified barrier facility, in groups of five in plastic micro-isolator cages on ventilated racks and provided with granular cellulose bedding. They were given a Harlan Teklad Rodent powdered diet and tap water *ad libitum*. The temperature was kept between 18 and 26°C, the humidity 30% and 70%, while the lighting was rotated in a 12-hour on and 12-hour off cycle.

The carcinogen used was 1,2-dimethylhydrazine dihydrochloride, symmetrical (1,2-DMH), (molecular weight, 133.02, melting point, 168°C), which was obtained from Aldrich Chemical Company, Inc., (Milwaukee, WI, USA). The 1,2-DMH was dissolved in sterile physiological saline (PS). The mice were s.c. injected in the interscapular region using a tuberculin syringe with 24-gauge needle.

Celecoxib (celebrex, C) (molecular weight, 381.37, melting point, 156-158°C), was obtained from LKT Laboratories, Inc., (St. Paul, MN, USA) in powdered form mixed with the diet and given orally.

*Presented, in part, at the Annual Meeting of the American Association for Cancer Research, April 1-5, 2006, Washington, D.C., U.S.A.

Correspondence to: Dr. Bela Toth, Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198, U.S.A. Tel: 402, 559-4229, Fax: 402, 559-4651, e-mail: btoth@unmc.edu

Key Words: Intestine, cancer, prevention, celecoxib, COX-2.

Table I. Treatments and survival rates in 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), celecoxib (C) and physiological saline (PS)-treated Swiss mice sacrificed at 26 weeks after the start of 1,2-DMH or PS treatment.

Group	Treatment	Initial no. + sex of mice	No. of survivors (age in weeks)							
			5	10	15	20	25	30	35	40
1	1,2-DMH, 10 weekly s.c. injections at 20 µg/g	30 ♀	30	30	30	29	29	27	21	-
2	C, 0.1% in diet + 1,2-DMH as in group 1	30 ♀	30	30	30	30	29	28	28	-
3	PS, 10 weekly s.c. injections at 0.01 ml/g	30 ♀	30	30	30	30	30	29	27	-

The mice were divided into six experimental groups:

Group 1: Thirty mice, nine weeks old, given ten weekly injections of 1,2-DMH at 20 µg/g body weight in 0.01 ml PS.

Group 2: Thirty mice, seven weeks old, given C at 0.1% w/w basis in the powdered diet. The C treatment was followed by 1,2-DMH, as described for Group 1.

Group 3: Thirty mice, nine weeks old, given PS as 10 weekly injections of 0.01 ml/g body weight.

Group 4: Identical treatment as Group 1.

Group 5: Identical treatment as Group 2.

Group 6: Identical treatment as Group 3.

The animals in Groups 1-3 were sacrificed at 26 weeks, while those in Groups 4-6 were sacrificed at 35 weeks after the first injection of 1,2-DMH or PS.

During the study, the animals were allowed to die or were killed with CO₂ when found to be in poor condition and complete necropsies were performed. All organs were examined macroscopically and were fixed in 10% buffered formalin. Histological examination was routine for the intestines (large and small) as well as any organs showing gross pathological changes. Sections from these tissues were stained with hematoxylin and eosin and studied by light microscopy.

Results

Experiments in which the animals were sacrificed at 26 weeks after the first injection of 1,2-DMH or PS. Table I summarizes the survival rates of the treated mice in five-week intervals. Of the animals treated with 1,2-DMH, 29 mice (96%) developed 438 tumors of the large intestine. Their average age at death was 33.8 weeks. The first tumor was observed at the 28th week and the last at the 35th week of age. Of these, seven mice had seven adenocarcinomas of the cecum, 27 mice developed 270 adenocarcinomas of the colon, 28 mice had 160 adenocarcinomas of the rectum and one mouse developed a squamous cell carcinoma of the anus.

Of the animals treated with C plus 1,2-DMH, 18 mice (60%, $p < 0.001$) developed 64 tumors of the large intestine.

Their average age at death was 35.0 weeks. The first tumor observed was at the 35th week of age, as was the last. Three mice had three adenocarcinomas of the cecum, 16 mice developed 39 adenocarcinomas of the colon, 11 mice had 21 adenocarcinomas of the rectum and one mouse developed a squamous cell carcinoma of the anus.

Among the PS-treated mice, only one (3%) developed a single adenocarcinoma of the cecum at the 35th week of age.

Table II presents the number and percentage of mice with tumors and their ages at death.

Experiments in which the animals were sacrificed at 35 weeks after the first injection of 1,2-DMH or PS. Table III summarizes the survival rates of the treated animals in five-week intervals. Of the animals treated with 1,2-DMH, 30 mice (100%) developed 323 tumors of the large intestine. Their average age at death was 38.6 weeks. The first tumor observed was at the 27th week and the last at the 44th week of age. Of these, four mice had four adenocarcinomas of the cecum, 26 mice developed 217 adenocarcinomas of the colon, 24 mice had 95 adenocarcinomas of the rectum, one mouse had a polypoid adenoma and four adenocarcinomas of the rectum and two mice developed two squamous cell carcinomas of the anus.

Of the animals treated with C plus 1,2-DMH, 23 mice (76%, $p < 0.01$) developed 134 tumors of the large intestine. Their average age at death was 43.3 weeks. The first tumor was observed at the 39th week and the last at the 44th week of age. Of these, one mouse developed an adenocarcinoma of the cecum, 20 mice had 78 adenocarcinomas of the colon, 20 mice developed 52 adenocarcinomas of the rectum, two mice had two squamous cell carcinomas of the anus and one mouse had a sebaceous gland adenoma of the anal gland.

None of the PS-treated mice developed tumors of the large intestine.

Table II. *Treatments and tumor incidences in 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), celecoxib (C) and physiological saline (PS)-treated Swiss mice sacrificed at 26 weeks after the start of 1,2-DMH or PS treatment.*

Group	Treatment	No. and sex of mice	Animals with tumors of:									Other tumors**
			Large intestine			Malignant lymphomas			Lungs			
			No.	%	Latent period*	No.	%	Latent period*	No.	%	Latent period*	
1	1,2-DMH, 10 weekly s.c. injections at 20 µg/g	30 ♀	29	96	33 (28-35)	3	10	34 (34-35)	3	10	35 (35-35)	1 Adeno-carcinoma of duodenum (32)
2	C, 0.1% in diet + 1,2-DMH as in group 1	30 ♀	18	60	35 (35-35)	5	16	31 (21-35)	10	33	35 (35-35)	1 Adeno-carcinoma of ileum (35)
3	PS, 10 weekly s.c. injections at 0.01 ml/g	30 ♀	1	3	35	4	13	32 (28-35)	-	-	-	-

*: Average and range in weeks.

**: Age at death given in weeks in parentheses.

Table III. *Treatments and survival rates in 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), celecoxib (C) and physiological saline (PS)-treated Swiss mice sacrificed at 35 weeks after the start of 1,2-DMH or PS treatment.*

Group	Treatment	Initial no. + sex of mice	No. of survivors (age in weeks)								
			5	10	15	20	25	30	35	40	45
1	1,2-DMH, 10 weekly s.c. injections at 20 µg/g	30 ♀	30	30	30	30	30	29	25	16	-
2	C, 0.1% in diet + 1,2-DMH as in group 1	30 ♀	30	30	30	29	28	26	25	24	-
3	PS, 10 weekly s.c. injections at 0.01 ml/g	30 ♀	30	30	30	29	28	26	25	25	-

The location and distribution, gross appearance and histological descriptions of the large intestinal tumors were similar to those described in our previous publications (3). Table IV presents the number and percentage of animals with tumors and their ages at death.

Statistical analysis. The number of intestinal tumors per mouse was compared between the 1,2-DMH and C-treated and 1,2-DMH alone groups using the Wilcoxon rank sum test (4). The differences were statistically significant in mice sacrificed at 26 and 35 weeks after the first injection of the carcinogen.

In addition to the incidence of large intestinal tumors, the animals developed several types of tumors in other organs, which are listed in Tables II and IV. Because they occurred in low incidences the incidence of these other tumors were not related to the treatments.

Discussion

The aim of the current study was to further determine the possible anticarcinogenic effect of C in a serial sacrifice investigation. In an earlier study, the oral administration of C for life at a 0.1% dose level reduced the incidence of large intestinal cancer induced by 1,2-DMH at 20 µg/g body weight (1). In the present group of experiments, identical treatments were administered to Swiss mice, but they were sacrificed at 26 or 35 weeks after the first injection of the carcinogen or PS. As anticipated, the incidence of large intestinal cancer was substantially reduced in the mice sacrificed at 26 and 35 weeks after the first injection of 1,2-DMH. In both of our investigations, the administration of C reduced the induced large intestinal cancer incidence.

Table IV. Treatments and tumor incidences in 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), celecoxib (C) and physiological saline (PS)-treated Swiss mice sacrificed at 35 weeks after the start of 1,2-DMH or PS treatment.

Group	Treatment	No. and sex of mice	Animals with tumors of:									Other tumors**
			Large intestine			Malignant lymphomas			Lungs			
			No.	%	Latent period*	No.	%	Latent period*	No.	%	Latent period*	
1	1,2-DMH, 10 weekly s.c. injections at 20 µg/g	30 ♀	30	100	38 (27-44)	4	13	38 (37-43)	3	10	40 (38-44)	1 Adeno-carcinoma of ileum (35) 1 Granulosa cell tumor (41)
2	C, 0.1% in diet + 1,2-DMH as in group 1	30 ♀	23	76	43 (39-44)	5	16	33 (17-44)	4	13	41 (32-44)	2 Hepatomas (44, 44)
3	PS, 10 weekly s.c. injections at 0.01 ml/g	30 ♀	-	-	-	4	13	32 (17-44)	-	-	-	-

*: Average and range in weeks.

**: Age at death given in weeks in parentheses.

Our findings corroborate those of other investigators who used C to inhibit the development of cancers in the intestine, stomach, urinary bladder, skin, breast and prostate (5-15), while similar protective results were obtained in the duodenum, colon and rectum of humans (16-18).

After the anti-inflammatory painkiller rofecoxib (Vioxx) was withdrawn from the market, similar COX-2 inhibitors, including celecoxib, came under suspicion. The National Institutes of Health recently halted a large scale colorectal cancer prevention trial involving more than 2,000 people, since there was evidence of a 2.5-fold increased risk of major fatal and non-fatal cardiovascular events in those taking C versus the placebo patients (19). In addition, Pfizer, the manufacturer of the COX-2 inhibitor celecoxib, is involved in an international trial with approximately 20,000 patients, focusing on those with heart disease, including patients who had undergone bypass surgery and those at risk of cardiac problems (20). Even though the trial is still in progress, a number of criticisms have been leveled against the protocol in terms of the variables involved in the experimental set-up.

One advantage of the Swiss mouse model is that it develops few, if any, spontaneous large intestinal cancers. Whether the molecular genetics of chemically-induced rodent models are significantly different from those observed in human colorectal carcinogenesis remains to be seen. The Apc transgenic mouse strain, on the other hand, is certainly different since, in both humans and mice, over

expression of the Apc gene is responsible for the appearance of intestinal tumors. The Apc transgenic murine model is characterized by the spontaneous development of small and large intestinal tumors, and our on-going studies are designed to clarify this field of interest.

Our preliminary study nevertheless indicated that the survival rate was substantially prolonged and the intestinal tumor incidence considerably reduced by the life-long administration of C to Apc mice.

Acknowledgements

This work was supported by grant 1R21 AT001739 from the National Institutes of Health, USA. The authors wish to thank Dr. James C. Lynch for his excellent statistical analysis.

References

- Toth B and Coles M: Prevention of tumors of the large intestine by celecoxib in mice. *In Vivo* 19: 661-664, 2005.
- Talmadge JE, Hood KC, Zobel LC, Shafer LR, Coles M and Toth B: Chemoprevention by cyclooxygenase-2 inhibition prevents immature myeloid suppressor cell expansion. Carcinogenesis, submitted for publication.
- Toth B, Malick L and Shimizu H: Production of intestinal and other tumors by 1,2-dimethylhydrazine dihydrochloride in mice. I. A light and transmission electron microscopic study of colonic neoplasms. *Am J Pathol* 84: 69-86, 1976.
- Altman DG: Practical Statistics for Medical Research. Chapter 9: Comparing groups-continuous data. Chapman & Hall, New York, pp. 194-215, 1991.

- 5 Kawamori T, Rao CV, Seibert K and Reddy BS: Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res* 58: 409-412, 1998.
- 6 Jacoby RF, Seibert K, Cole CE, Kelloff G and Lubet RA: The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. *Cancer Res* 60: 5040-5044, 2000.
- 7 Reddy BS, Hirose Y, Lubet R, Steele V, Kelloff G, Paulson S, Seibert K and Rao CV: Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res* 60: 293-297, 2000.
- 8 Brown WA, Skinner SA, Malcontenti-Wilson C, Misajon A, Dejong T, Vogliagis D and O'Brien PE: Non-steroidal anti-inflammatory drugs with different cyclooxygenase inhibitory profiles that prevent aberrant crypt foci formation but vary in acute gastrotoxicity in a rat model. *J Gastroenterol Hepatol* 15: 1386-1392, 2000.
- 9 Brown WA, Skinner SA, Malcontenti-Wilson C, Vogliagis D and O'Brien PE: Non-steroidal anti-inflammatory drugs with activity against either cyclooxygenase 1 or cyclooxygenase 2 inhibit colorectal cancer in a DMH rodent model by inducing apoptosis and inhibiting cell proliferation. *Gut* 48: 660-666, 2001.
- 10 Rao CV, Indranie C, Simi B, Manning PT, Connor JR and Reddy BS: Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. *Cancer Res* 62: 165-170, 2002.
- 11 Fischer SM, Lo H-H, Gordon GB, Seibert K, Kelloff G, Lubet RA and Conti CJ: Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, and indomethacin against ultraviolet light-induced skin carcinogenesis. *Mol Carcinog* 25: 231-240, 1999.
- 12 Grubbs CJ, Lubet RA, Koki AT, Leahy KM, Masferrer JL, Steele VE, Kelloff GJ, Hill DL and Seibert K: Celecoxib inhibits n-butyl-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-334 rats. *Cancer Res* 60: 5599-5602, 2000.
- 13 Howe LR, Subbaramaiah K, Patel J, Masferrer J L, Deora A, Hudis C, Thaler HT, Muller WJ, Du B, Brown AMC and Dannenberg AJ: Celecoxib, a selective cyclooxygenase 2 inhibitor, protects against human epidermal growth factor receptor 2 (HER-2)/neu-induced breast cancer. *Cancer Res* 62: 5405-5407, 2002.
- 14 Fischer SM, Conti CJ, Viner J, Aldaz CM and Lubet RA: Celecoxib and difluoromethylornithine in combination have strong therapeutic activity against UV-induced skin tumors in mice. *Carcinogenesis* 24: 945-952, 2003.
- 15 Hu PJ, Yu J, Zeng ZR, Leung WK, Lin HL, Tang BD, Bai HAC and Sung JY: Chemoprevention of gastric cancer by celecoxib in rats. *Gut* 53: 195-200, 2004.
- 16 Steinbach G, Lynch PM, Phillips RKS, Wallace MH, Hawk E, Gordon G, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su L-K, Levin B, Godio L, Patterson S, Rodriguez-Bigas MA, Jester SL, King KL, Schumacher M, Abbruzzese J, DuBois RN, Hittelman WN, Zimmerman S, Sherman JW and Kelloff G: The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Eng J Med* 342: 1946-1952, 2000.
- 17 Phillips RKS, Wallace MH, Lynch PM, Hawk E, Gordon GB, Saunders BP, Wakabayashi N, Shen Y, Zimmerman S, Godio L, Rodrigues-Bigas M, Su L-K, Sherman J, Kelloff G, Levin B, Steinbach G and FAP Study Group: A randomized, double blind, placebo controlled study of celecoxib, a selective cyclooxygenase 2 inhibitor, on duodenal polyposis in familial adenomatous polyposis. *Gut* 50: 857-860, 2002.
- 18 Rahme E, Barkun AN, Toubouti Y and Bardou M: The cyclooxygenase-2 selective inhibitors rofecoxib and celecoxib prevent colorectal neoplasia occurrence and recurrence. *Gastroenterology* 125: 404-412, 2003.
- 19 National Cancer Institute. News: NIH halts use of COX-2 inhibitors in large cancer prevention trial. <http://www.nih.gov/news/pr/dec2004/od17Q&A.htm>.
- 20 Couzin J: Massive trial of celebrex seeks to settle safety concerns. *Science* 310: 1890-1891, 2005.

Received April 4, 2006

Revised May 3, 2006

Accepted May 8, 2006