

Distribution of 1,2 DMH-induced colonic aberrant crypt foci after administration of a gastrin receptor antagonist (CR2945), in the murine model



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Introduction

A possible role of gastrin in the development of colorectal cancer has been proposed years ago, when some reports showed that gastrin stimulates the growth of epithelial cultures derived from gastric and intestinal mucosa and enhances cancer growth in rodents as well as in culture (13, 14, 15, 7, 32, 25, 37, 12, 33).

Gastrin receptors have been located on normal and neoplastic gastric and colonic mucosa (25, 28, 35, 19) and intracellular gastrin has been found in human gastric and colorectal cancer cells (30). These tumours can produce gastrin-like peptides able to react with antagastin antibodies (22, 36, 29, 21).

The hypothesis that explains these reports is an autocrine or paracrine loop in which gastrin or gastrin-like substances, produced by the tumour itself, bind to tumour receptors and stimulate tumour growth (5, 23). This hypothesis suggests that gastrin receptor antagonists could be useful in the adjuvant treatment of colorectal cancer.

Previous findings have proposed aberrant crypt foci (ACF) as "putative preneoplastic lesions" in colon carcinogenesis: ACF in fact are observed in the colons of rodents treated with colon carcinogens (3), they are absent in control groups (3), they increase in a dose dependent manner in response to chemical carcinogens (18), and some of them show atypia and dysplasia after histological examination (18).

The purpose of this study is to investigate whether the administration of a new selective CCK-B gastrin receptor antagonist (CR2945) can affect the appearance and the distribution of ACF in dimethylhydrazine (DMH) treated mice.

Abstract

Our previous experimental data demonstrated that a new gastrin receptor antagonist (CR2945) has a chemopreventive effect on dimethylhydrazine-induced colon cancer in mice. The aim of this study is to test the effect of CR2945 on the appearance and distribution of aberrant crypt foci (ACF), proposed as early "preneoplastic" lesions in colon carcinogenesis, in the murine model. 176 CD1 male mice were randomly divided into 4 groups: group 1, sham group received 2 daily intra-peritoneal injections of saline solution; group 2 received 1 weekly intra-peritoneal injection of DMH 20 mg/kg, for 5 weeks, and 2 daily intra-peritoneal injections of equal volume of NaCl 0.9%; group 3 and 4 received the same weekly dose of DMH and 2 daily injections of CR2945 at the respective doses of 2.5 and 7.5 mg/Kg for 5 weeks. The rodents were sacrificed 15, 20, 25, and 38 weeks after receiving the first injection. The number of ACF per area (ACF frequency), their multiplicity (number of crypts per focus), ACF frequency according to each colonic site were recorded. No ACF were found in the sham group. No substantial differences were observed in ACF distribution between the remaining groups. Our hypothesis is that CR2945 does not alter the final number of ACF but might induce a regression of some dysplastic ACF.

Key words: Aberrant crypt foci (ACF);, colon cancer.

Riassunto

DISTRIBUZIONE DEI FOCI DI CRIPTE ABERRANTI DMH-INDOTTI NEL COLON, DOPO SOMMINISTRAZIONE DI UN ANTAGONISTA RECETTORIALE DELLA GASTRINA (CR2945) NEL MODELLO SPERIMENTALE SUL TOPO

Nostri precedenti dati sperimentali hanno dimostrato che un nuovo antagonista recettoriale della gastrina (CR2945) ha un effetto chemiopreventivo sul carcinoma del colon DMH-indotto nei topi. Lo scopo di questo studio è quello di saggiare l'effetto del CR2945 sulla comparsa e la distribuzione dei foci di cripte aberranti (ACF), proposti quali lesioni "preneoplastiche" nel modello sperimentale di cance -

rogenesi del colon.

176 topi maschi tipo CD1 sono stati randomizzati in 4 gruppi: il gruppo 1, di controllo, ha ricevuto due somministrazioni intraperitoneali giornaliere di soluzione salina; il secondo gruppo ha ricevuto una somministrazione intraperitoneale settimanale di 20mg/Kg di DMH per 5 settimane e due iniezioni intraperitoneali giornaliere di uguale volume di NaCl 0.9%; i gruppi 3 e 4 hanno ricevuto la stessa dose settimanale di DMH e due iniezioni giornaliere di CR2945 alle dosi, rispettivamente, di 2.5 e 7.5 mg/Kg per 5 settimane. I roditori sono stati sacrificati dopo 15, 20, 25 e 38 settimane dalla prima iniezione. È stata valutata la frequenza degli ACF, la loro molteplicità (numero di cripte per ciascun focolo), la frequenza di ACF in relazione ai vari segmenti del colon.

Non è stata rilevata alcuna ACF nel gruppo di controllo. Non è stata rilevata alcuna sostanziale differenza nella distribuzione di ACF nei rimanenti gruppi.

La nostra ipotesi è che il CR2945 non modifichi il numero finale delle ACF ma possa indurre la regressione di alcuni ACF displastici.

Parole chiave: Foci di cripte aberranti (ACF), cancro del colon.

Materials and methods

Animals: 176 CD1 male mice, 42 days old, weighing about 38 g, (Charles River Italy Laboratories) were housed following Interdisciplinary Principles and guide-lines for the Use of Animals in Research, Marketing and Education. They received food and water ad libitum prior to and through the study.

Reagents: The following substances were used: 1,2-dimethylhydrazine (DMH) and CR2945 (Rotta Research Laboratorium), (chemical name: (R)-1-naphthalenepropanoic acid, beta-[2-[(8 - azaspiro [4.5] dec - 8 - ylcarbonyl) -4,6 - dimethylphenyl] amino]-2-oxoethyl]).

DMH was dissolved in 0.9% NaCl solution, neutralized to pH 6.5 with 1M NaHCO₃ and then injected into each animal, at a weekly dose of 20 mg/Kg intraperitoneally (i.p.) for five weeks (20). CR2945 required a specific preparation: 25 mg of CR2945 (0.0475 mM) were suspended in 15 ml of sterile distilled water. After stirring, 0.6 ml of NaOH (0.1N) was added to achieve pH= 11 - 12. Then mannitol was used to obtain an isotonic solution. pH was corrected to a value of 8-8.1 with HCl 0.1N. This solution was filtered with a sterile filter of cellulose acetate PRO-X (0.8m LIDA) and then administered to the mice. Both substances (DMH and CR2945) had a pH very similar to physiological pH, they were isotonic and they did not cause irritation for the animals.

Experimental protocol: After 4 weeks of acclimatisation, mice were randomly divided into 4 groups:

Group 1: sham group. 40 animals received 2 daily

intra-peritoneal injections of equal volume of NaCl 0.9%, for 5 weeks.

Group 2: control group. 46 animals received 1 weekly intra-peritoneal injection of DMH 20 mg/kg and 2 daily intra-peritoneal injections of equal volume of NaCl 0.9%, for 5 weeks.

Group 3: treated group. 36 animals received 1 weekly intra-peritoneal injection of DMH 20 mg/kg and 2 daily intra-peritoneal injections of CR 2945, 2.5 mg/kg, for 5 weeks

Group 4: treated group. 54 animals received 1 weekly intra-peritoneal injection of DMH 20 mg/kg and 2 daily intra-peritoneal injections of CR 2945, 7.5 mg/kg, for 5 weeks.

The rodents were sacrificed 15,20,25 and 38 weeks after receiving the first i.p. injection. Colons were removed, flushed with Ringer solution, opened longitudinally from caecum to anus, fixed in 10% buffered formalin.

The colons were conventionally subdivided as follows: rectum (the most distal two cm of colon), caecum (as anatomically defined); distal and proximal colon (subdividing the remaining into two equal segments).

Visualization and quantification of ACF: The colons were placed on microscope slides with the mucosa side up and ACF were scored under the light microscope at a magnification of x 40 or x 100, by two different examiners. As described by Bird (3), ACF were identified by their increased size, thicker epithelial lining and increased pericryptal zone. Total mucosal area, seat and frequency of ACF (n./cm²) were scored. Multiplicity (number of crypts/focus) was evaluated.

Tumours: Frequency and localization of neoplasms were always scored, by two different examiners. All tumours underwent histological evaluation based on Morson's criteria (20).

Statistical methods: Data on ACF were log-transformed for better normal approximation or for variance-stabilizing (1). One way analysis of variance (ANOVA) with Dunnett's test for multiple comparison between the controls and treated groups were performed. Non parametric Mann-Whitney test for unpaired data was performed for comparison between two groups. Cancer frequency in each group was considered as a proportion and Fisher's exact test was performed for comparison between two proportions after combining the low-dose and the high-dose groups together. All the statistical tests were performed at the 0.05 p-value at two tails using the BMDP/Dynamic computer programmes (8).

Results

A total of 1352,85 cm² of colonic mucosa was analyzed. The mean areas examined for each animal were similar between the groups at each date of sacrifice ($p>0.05$ for each comparison) and ranged from 7.9 to 12.6 cm². Tab. I shows the frequency of ACF at the time of sacrifice in each group.

Neither ACF nor tumours were observed in the sham group. No substantial differences were observed between the control and treated groups at week 15 and 20.

On the contrary, at the 25th week a deflection in ACF frequency was seen without significant difference between the groups.

At the last sacrifice, higher ACF frequency was found in the control group with respect to treated animals, without significant difference though.

In every group the highest frequency of ACF was observed in the left colon, with significant difference with respect to the right colon at every time point of sacrifice (Tab. II).

No significant differences between groups were found in ACF multiplicity at each week (Tab. III).

Discussion

Experimental and human data indicate that colon carci-

Tab. I - ACF FREQUENCY (ACF/cm²) MEAN VALUES ± STANDARD DEVIATIONS, AT DIFFERENT WEEKS OF SACRIFICE.

Group	15 th week	20 th week	25 th week	38 th week
1	0	0	0	0
2	0.76 ± 0.37*	0.88 ± 0.53*	0.063 ± 0.81*	1.29 ± 1.17*
3	1.17 ± 1.13	1.11 ± 0.66	0.55 ± 0.25	1.05 ± 0.32
4	0.93 ± 0.91	0.66 ± 0.22	0.68 ± 0.50	0.96 ± 0.54
Anova	P>0.1	P>0.1	P>0.1	P>0.1

*Anova: $p=0.09$ using the Kruskal-Wallis test.

Tab. II – ACF FREQUENCY (ACF/cm²) MEAN VALUES FOR EACH COLONIC SEGMENT ± STANDARD DEVIATIONS, AT THE 38th WEEK OF SACRIFICE.

Group mice	whole colon	proximal	distal	rectum	
1	10	0	0	0	
2	18	1.40 ± 1.11	0.49 ± 0.64	2.32 ± 2.57	1.86 ± 2.31*
3	11	1.05 ± 0.32	0.23 ± 0.75	1.47 ± 1.16	5.00 ± 4.02*
4	14	0.96 ± 0.54	0.13 ± 0.20	1.70 ± 1.54	2.53 ± 3.83
Anova	P>0.1	P>0.1	P>0.1	P=0.04	

*Scheffé's test for multiple comparisons: $p<0.05$ when comparing the controls and mice treated with 2.5 mg of CR2945.

Table. III – ACF MULTIPLICITY (CRYPTS/FOCUS) MEAN VALUES ± STANDARD DEVIATIONS, AT DIFFERENT WEEKS OF SACRIFICE.

Group	15 th week	20 th week	25 th week	38 th week
1	0	0	0	0
2	1.98 ± 0.43	2.09 ± 0.40	2.40 ± 0.57	2.22 ± 0.40
3	1.26 ± 0.57	2.65 ± 1.44	1.26 ± 0.27	1.56 ± 0.40
4	1.57 ± 0.54	1.83 ± 0.67	1.55 ± 0.66	1.92 ± 0.62
Anova	P>0.1	P>0.1	P>0.1	P>0.1

nogenesis is a multistep process in which subsequent pre-neoplastic lesions accumulate in mucosa cells leading finally to neoplastic transformation (16).

Previous studies demonstrated that administration of a chemical colon carcinogen in rodents induces an increase of ACF, followed by the growth of colonic tumours, that exhibit pathological features similar to sporadic human colon cancer (4). ACF have been proposed as preneoplastic lesions in both rodent (3, 18) and human (34, 31, 27, 6) colon carcinogenesis and they are thought to be useful biological markers of the effects of carcinogens and eventually agents preventing carcinogenesis in the large bowel. After the first description of ACF (3), some researchers have tried to identify dysplastic characteristics or molecular abnormalities in ACF (17, 24, 26). The results of these studies indicate that only some ACF are dysplastic lesions.

However, several controversies persist, since recent reports did not find association between ACF characteristics and colonic tumour incidence (10, 11).

Preliminary results of this study showed (9) that CR2945 has a chemopreventive effect on DMH-induced colon cancer in mice: in fact while 4% of mice that received the gastrin receptor antagonist had cancer, 37.4% of controls developed colon cancer, the difference being highly significant.

But while we found a significant effect of CR2945 on tumours in our previous study (9), we did not observed a significant effect on ACF distribution. An hypothesis is that CR2945 does not alter the final number of ACF but induce a regression or remodelling of some dysplastic ACF and alter the rate of transformation of ACF to adenocarcinoma.

ACF are heterogeneous lesions with different multiplicity, types of luminal patterns, proliferation rate and types of mucins expressed: only some of ACF eventually represent true premalignant lesions progressing to colon cancer.

On the other hand, ACF growing features, their increase in frequency and multiplicity as a function of time after carcinogen administration, could represent an unspecific indicator of exposure to a colon carcinogen. The total number of ACF alone could not be considered as a single valid biomarker of colorectal cancer, but other indicators must be seeked.

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