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Development of Aberrant Crypt Foci Involves a Fission Mechanism as Revealed by Isolation of Aberrant Crypts

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Morphological analysis of isolated colonic crypts in rats, postnatally, indicated that the crypts reproduce themselves by a fission mechanism, the division beginning at the crypt base and proceeding upwards until there are two separate crypts. Occasionally, before the separation is complete, a second fission process starts on one or both sides of a bifurcating crypt and a triple-branched or quadruple-branched crypt results. Analysis of isolated aberrant crypt foci (ACF) in rats treated with 1,2-dimethylhydrazine revealed that the development of ACF consisting of multiple crypts is also due to a fission mechanism. Initially, an indentation appears at the base of a single ACF crypt, with subsequent formation of a bifurcation and eventual crypt division.

Key words: Aberrant crypt foci — Fission mechanism — Crypt isolation technique

Aberrant crypt foci (ACF) in methylene blue-stained whole mount preparations of colons[1] have been proposed as putative preneoplastic lesions in both rodents[2,3] and man.[4,5] ACF may be histologically normal or display varying degrees of dysplasia,[5,6] altered enzyme activity[7] and molecular abnormalities.[8–10] They consist of 1, 2, 3, or 4 or more crypts, and it has been suggested that the number of crypts increases with time due to crypt multiplication and/or branching.[11] However, the actual process involved is unclear.

Postnatally in rats, there is a rapid increase in the number of colonic crypts during the first 6 weeks of life.[12] The crypts appear to reproduce themselves by a fission mechanism.[12,13] By applying a crypt isolation technique,[14] the three-dimensional development of ACF was investigated in comparison with that of normal crypts in the postnatal phase.

MATERIALS AND METHODS

Fischer rats (Charles River Japan Inc., Kanagawa) were maintained on basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and water *ad libitum* and housed in plastic cages in an air-conditioned room at 24°C with a 12 h light-12 h dark cycle. Newborn rats were nursed by their mothers until they began taking solid food 18 days after birth.

Group 1: From three litters of rats, 5 males at 5 days old, five 10-day-old males, five 15-day-old males and five 16-week-old male rats were killed. Group 2: Seven 6-week-old males were given weekly s.c.-injections of 20 mg/kg body weight 1,2-dimethylhydrazine (DMH) (Wako Chemical Co., Ltd., Tokyo) for up to 5 weeks. Group 3: Seven 6-week-old males were given weekly s.c.-injections of 20 mg/kg body weight DMH for up to 10 weeks. At ten weeks after the start of the experimental period the animals (16 weeks old) in groups 2 and 3 were killed and autopsied.

The colons were removed, opened longitudinally, and rinsed in Hanks' balanced salt solution. In the DMH-treated cases they were stained with 0.2% methylene blue in phosphate-buffered saline (pH 7.4) for 5 min,[1] and then assessed for ACF development by stereoscopic microscopic examination. The numbers of ACF per colon and the numbers of crypts per ACF were recorded.

A modification of the method of Bjerknes and Cheng[14] was used for the isolation of intestinal epithelium. Colon samples cut into 1–2 cm longitudinal segments were incubated three times at 37°C in calcium-magnesium-free Hanks' balanced salt solution containing 30 m*M* EDTA for 10 min. At the end of the incubation, the tissue was stirred and crypts were isolated from the stroma. The supernatant fluid was aspirated, and the isolated crypts were fixed with 2% paraformaldehyde in 0.1*M* phosphate buffer (pH 7.4). In these crypts ACF were identified on the basis of their distinctive features under a stereoscopic microscope.

For scanning electron microscopy (SEM), the isolated and fixed ACF were washed for 30 min in distilled water, dehydrated through a graded series of ethyl alcohol (50–100%), and immersed in isoamyl acetate. The specimens were then processed in a critical point drier (HCP-1, Hitachi, Ltd., Tokyo) using dry CO_2 gas, thinly coated with gold in an ion coater (IB-2, Eiko Engineering Co., Ltd., Ibaragi), and examined at 20 kV (S-2250N, Hitachi, Ltd.).

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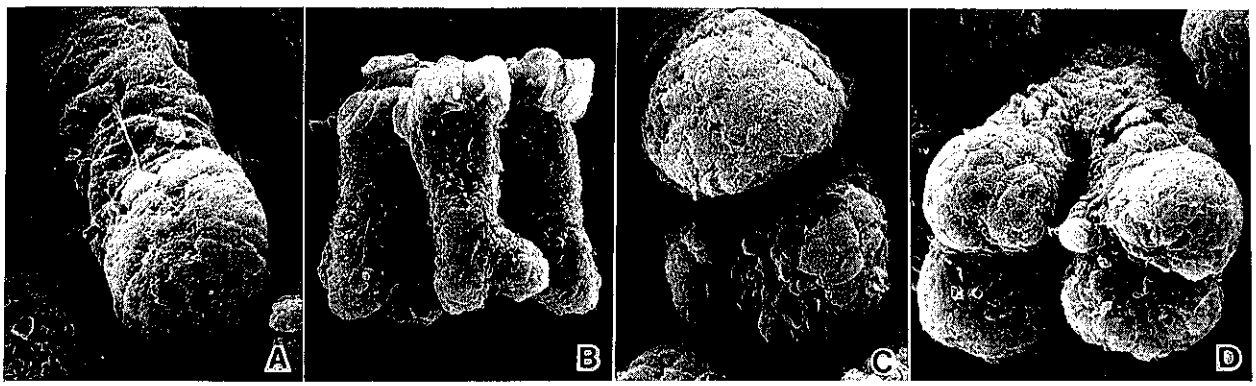

Fig. 1. Postnatal development of large intestinal crypts as revealed by SEM of isolated crypts. A, A non-branched crypt. ×2.0 k. B, Bifurcating crypts. ×600. C, A triple-branched crypt. ×2.0 k. D, A quadruple-branched crypt. ×1.5 k.

Table I. Incidences of Single, Bifurcating, Triple-branched and Quadruple-branched Isolated Crypts

Time after birth	No. of animals	Crypt (%)[a]			
		Single	Bifurcating	Triple	Quadruple
5 days	5	40.2±2.3	45.2±2.6	12.8±1.3	1.8±0.9
10 days	5	22.8±1.5	45.4±2.5	27.0±2.2	4.8±1.5
15 days	5	32.0±3.5	51.2±3.2	14.0±4.0	2.8±1.2
16 weeks	5	92.8±1.5	7.2±1.5	0	0

a) More than 1000 crypts/rat were examined.

RESULTS

The results demonstrated that, in postnatal development, large intestinal crypts reproduce themselves by a fission mechanism. The fission process begins at the basal region and proceeds upwards until there are two separate crypts. Occasionally before the separation is complete a second fission process starts on one or both sides of a bifurcating crypt and a triple-branched or quadruple-branched crypt then results. In adult rats, almost all crypts were single and no triple-branched or quadruple-branched crypts were found, although in post neonatal rats the latter were frequent. The SEM appearance of non-branched, bifurcating, triple-branched and quadruple-branched crypts is shown in Fig. 1. Over one thousand isolated crypts from each rat were examined and the incidences of single, bifurcating, triple-branched and quadruple-branched types at 5, 10 and 15 days old and in adult (16 weeks old) rats are summarized in Table I.

Data for ACF found by stereoscopic microscopic examination in groups 2 and 3 are summarized in Table II. After isolation of intestinal epithelium, ACF were easily detected under the stereoscopic microscope and SEM due to their larger diameter and longer length than normal crypts (Figs. 2 and 3). Increase in crypt number was again found to be due to a fission mechanism with the initial appearance of an indentation at the base of a single crypt, subsequent bifurcation and eventual formation of an ACF consisting of 2 crypts (Fig. 2). As each crypt independently showed bifurcating activity, ACF consisting of two crypts were further classified into 1) ACF consisting of two single crypts, 2) ACF consisting of one single crypt and one bifurcating crypt and 3) ACF

Table II. Numbers of ACF in Rats Treated with DMH

No. of crypts/ ACF	Group 2 ($n=7$)	Group 3 ($n=7$)
Single crypt	142.1±18.0[a] (45.2%[b])	127.1±33.6 (37.3%)
2 crypts	120.4±14.8 (38.3%)	120.6±36.1 (35.3%)
3 crypts	38.1±11.5 (12.1%)	58.9±19.0 (17.3%)
4 crypts	9.4±6.2 (3.0%)	22.9±9.1 (6.7%)
5 crypts and over	4.3±2.7 (1.4%)	11.7±6.6 (3.4%)

a) Mean±SD of number of ACF/rat.
b) Percentage of the mean number of ACF/rat.

consisting of 2 bifurcating crypts. ACF consisting of 3, 4 or more crypts also comprised single crypts and bifurcating crypts. The incidences of each subtype of ACF consisting of 1, 2, 3, 4 and over 4 crypts are summarized in Table III.

Almost all indentations of crypts occurred centrally at their bases with clear demarcation into 2 morphologically similar crypts. However, occasionally, irregular indentations and divisions were found (Fig. 3).

DISCUSSION

In this study, examination of isolated colonic crypts clearly indicated that the crypts reproduce themselves by a fission mechanism, in line with earlier descriptions.[12, 13] Postnatally, there is a rapid increase in the number of colonic crypts over the first 6 weeks of life,[12] and before

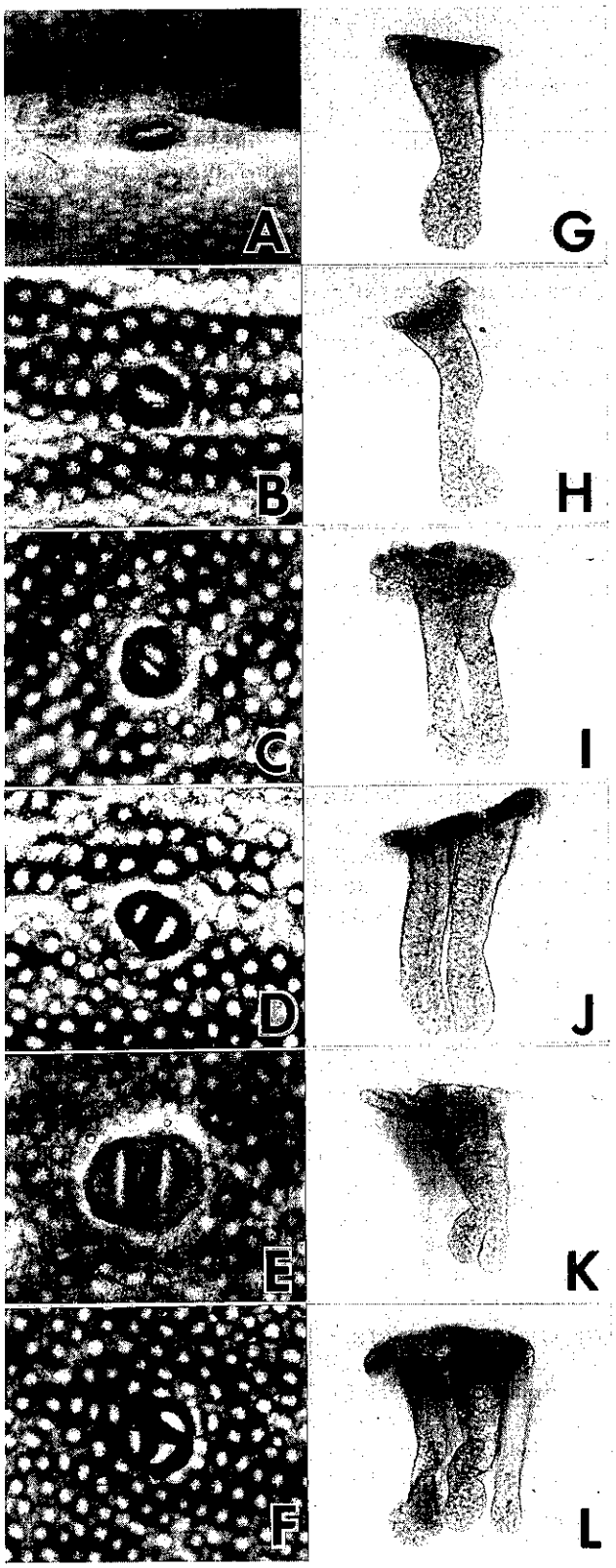

Fig. 2. Development of ACF followed by stereoscopic microscopy of methylene blue-stained whole mount preparations of colons (A–F) and isolated crypts (G–L). Initially, an indentation appears at the base of a single crypt (A, B, G–H) with subsequent development of a bifurcation (C, I) and then an ACF consisting of 2 crypts appears (D, J). In this case, a second indentation then appears at the base of the crypt on one side of the ACF consisting of 2 crypts (E, K) with development into an ACF consisting of 3 crypts (F, L). × 200.

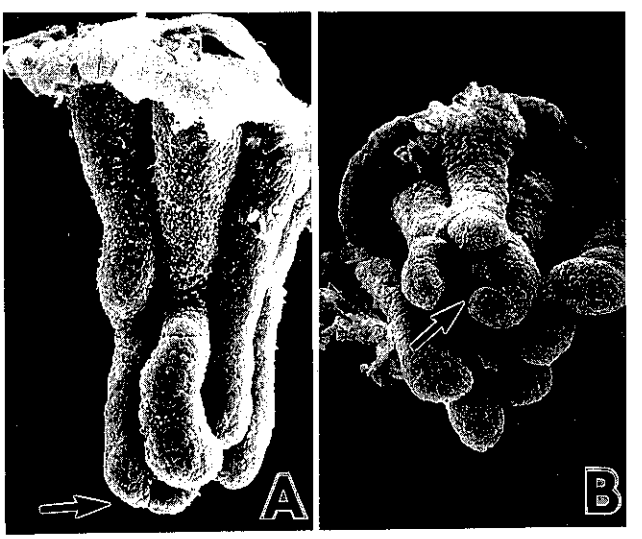

Fig. 3. Appearance of isolated ACF in SEM: Almost all indentations of crypts occur at the center of their bases (A); however, occasionally, irregular indentations are found. The illustrated example has an irregular indentation(arrow) and shows irregular division (B). ×400.

Table III. Numbers of ACF Isolated from Rats Treated with DMH

	Group 2 (%) $n=5$	Group 3 (%) $n=5$
Single crypt/ACF	397 (54.7)	465 (49.8)
S	319 (80.4)	375 (80.6)
B	78 (19.6)	90 (19.4)
2 crypts/ACF	242 (33.3)	312 (33.4)
S+S	205 (84.7)	246 (78.8)
S+B	35 (14.5)	57 (18.3)
B+B	2 (0.8)	9 (2.9)
3 crypts/ACF	71 (9.8)	120 (12.9)
S+S+S	59 (83.1)	95 (79.1)
S+S+B	12 (16.9)	23 (19.2)
S+B+B	0	2 (1.7)
B+B+B	0	0
4 crypts/ACF	13 (1.8)	26 (2.8)
S+S+S+S	12 (92.3)	21 (80.8)
S+S+S+B	1 (7.7)	3 (11.5)
S+S+B+B	0	2 (7.7)
S+B+B+B	0	0
B+B+B+B	0	0
5 crypts and over/ACF	3 (0.4)	10 (1.1)
Total[a)]	726	933

a) Total number of ACF selected from all crypts isolated from the distal colon.
S, single crypt; B, bifurcating crypt.

separation a second fission process often starts so that multiply branched crypts are observed. The presence of triple- or quadruple-branched crypts thus suggests a rapid increase in the number of colonic crypts.

Analysis of isolated ACF revealed their crypts to be significantly longer and larger than isolated normal crypts. The presence of a notch at their bases and bifurcating crypts at various stages was interpreted as indicating fission of crypts within ACF, as indicated in Fig. 2. The lack of triple- or quadruple-branched crypts in ACF suggested that their rate of crypt division is slower than that of postnatal normal crypts in the first 2 to 3 weeks of life.[12, 13] On the other hand, the percentage of bifurcating crypts in normal adult rat colonic mucosa was found to be approximately 7%, while bifurcating crypt values of over 15% were noted for ACF in many cases. Therefore the increased rate in ACF might be intermediate between those in the adult and in the postnatal colonic mucosa. One study of cell proliferation in azoxymethane-induced ACF of rat colon, using immunohistochemical markers, revealed labeling indices for ACF which were significantly higher than that of normal crypts.[15] This provides support for the conclusions drawn from the present results.

The accuracy of quantitative assessment of isolated ACF crypt multiplicity is limited by their fragility and loss during processing. However, the incidence of fractures in ACF consisting of 2 or more crypts was relatively low and differences in the ratios for ACF crypt multiplicity as counted in methylene blue-stained whole mount preparations of colons and in isolated ACF were not statistically significant.

As each ACF crypt has its own proliferative zone of cells at the base, the cell turnover in each crypt may be specific to the individual crypt itself, as in the normal crypt case. However, crypt cells in mouse chimeras are all derived from one partner or the other, which strongly suggests a clonal origin.[16, 17] ACF consisting of multiple crypts might have a clonal origin because they could be expected to be derived from single crypts via the fission mechanism.[12, 13]

The relationship between ACFs and carcinogenesis appears to be rather complicated. It is known that colon carcinogens induce ACFs dose-dependently and that the number of ACFs is correlated with the potency of the carcinogens.[3, 18] However, some authors[19, 20] have reported poor correlations between the incidence of cancer and ACF number or size. A decrease in numbers of ACF consisting of 1, 2 or 3 crypts was also reported during DMH-induced rat colon carcinogenesis, suggesting that some ACF are transient in nature[11] and that they are eliminated or undergo phenotypic reversion, analogous to the well-documented observations with respect to putative preneoplastic lesions in the rat liver carcinogenesis model.[21] In this study, almost all non-bifurcating and bifurcating crypts in ACF exhibited no significant morphological difference, except in size, from those in normal colonic epithelium. However a few irregular indentations and divisions were found. The crypt base is the area where the functional stem cells are found[22] and it is probable that each fission event begins with a localized proliferation of stem cells in both normal crypts and in those in ACF. The level of initiation of stem cells in ACF not demonstrating irregularity might be insufficient to ensure persistence so that these ACF are reversible in nature.[11] Only a few ACF crypts demonstrated irregular bifurcation in the present study but those that were observed might be regarded as more atypical, with a higher risk of developing into tumors. This possibility clearly warrants further investigation.

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