Effects of Imbalance of Apoptosis and Proliferation on Large Bowel Carcinogenesis in Mice

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OBJECTIVE To observe the pattern of changes in the proliferation and apoptosis at different stages of large bowel carcinoma in mice, and to explore the effects of the imbalance of apoptosis and proliferation at different stages of large-intestine carcinogenesis.

METHODS An experimental animal model for large intestine carcinogenesis of KUNMING-strain mice was used. The carcinomas were induced by subcuteneous injection of dimethylhydrazine (DMH) and the distribution and density changes of proliferating and apoptotic cells observed through multistages toward cancer formation. The animals were killed in groups at the 12th, 18th, 24th,and 32nd weeks of carcinoma induction. The apoptotic and proliferating cells were labeled separately using TUNEL and PCNA immunohistochemical staining methods

RESULTS In the normal mouse mucosa, all the apoptotic cells were situated in the superficial layers, however, the proliferating cells were situated in the basement layers, and the amount of both were small. In the early stage of carcinoma induction, the proliferation and the apoptotic cells slightly increased in amount, but there were no obvious changes in their ratio. In the medium stage, the densities of both distinctly increased, but there were no obvious changes in the ratio. In the late stage, the densities of the proliferating and the apoptotic cells in the non-carcinoma mucosa were higher than those at other stages. The proliferating cells in the dysplastic mucosa increased progressively with the increasing degree of the lesions. Although the apoptotic cells increased, their changes did not occur with the degree of the lesions. Their ratio showed a decreasing tendency with the degree of the lesions.

CONCLUSIONS ①The presence of an imbalance between cell proliferation and apoptosis was confirmed in the course of large intestine carcinogenesis in a mouse model. ②In the early stage of carcinoma induction both proliferation and apoptosis were at a low level; in the medium stage, they were both at a high level; and in the late stage (that is in carcinoma), proliferation was at a very high level, while apoptosis was at a low level. ③The proliferating cells increased progressively with the degree of dysplasia. There were no obvious changes in the apoptotic cells and their ratio to the proliferating cells showed a progressively increasing tendency. ④In the stage of cancer formation, the most essential change was the excessive decrease in the ratio of apoptosis to proliferation. These results support the hypothesis of "Cell Selective Proliferation", which was raised by authors previously in a study on human large bowel carcinoma.

KEYWORS: mouse, large bowel carcinoma, apoptosis.

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The dynamic balance of cell proliferation and apoptosis is not only physiologically significant in maintaining a stable number of cells in a tissue and controlling the organogenesis during fetal development, but also of importance in eliminating transformed cells to avoid cancer development. The problem of an imbalance of cell proliferation with apoptosis has become one of concern in recent years. Studies concerned with large bowel cancer, breast cancer, leukaemia and their precancerous lesions have shown that the dynamic imbalance of cell proliferation and apoptosis is uniformly present in malignant tumors and their precancerous lesions. However, most investigations have been confined to observations in a static state of some pathological changes and were deficient in studying a continuous multistage –dynamic process of cancer formation. In this study, using an experimental animal model for the formation of large intestine carcinoma of mice induced by dimethylhydrazine(DMH), the dynamics of cell proliferation and apoptosis were observed in the lesioned tissue in multistages in order to clarity the function of cell proliferation and apoptosis during different stages in large intestine carcinogenesis.

MATERIALS AND METHODS

Experimental objective

Seventy two KUNMING strain mice, male, five weeks of age, 24–30g, were divided randomly into the experimental group (48) and control group (24) and fed for one week prior to initiating the study.

Animal treatment

DMH was dissolved in physiological saline and injected subcutaneously into the experimental group of mice at a dosage of 20mg per kilogram. Ten mice were sacrificed at each of these weeks after injection: the 12th, 18th , 24th and 32nd. In the control group, the same quantity of physiological saline was administrated and 6 mice were killed at the same week as the experimental group.

Index to be investigated and method of treatment

The mice were sacrificed by decapitation and the whole large intestine and important organs were taken out to be observed with the naked eye. Then the tissue was fixed in 10% formalin followed by dehydration and paraffin–embedding. Then the tissue was sectioned at 5μm thickness. The investigation included four aspects.

To observe the dysplasia and type of tumor tissue in the section with HE stain.

To demonstrate the distribution and degree of apoptosis by use of the labeling TUNEL technique.

To observe the distribution and degree of proliferation by noting cells found in G1/S phase detected by PCNA immunohistochemical stain.

Counting methods and statistics: The number of positive cells in the sections stained with TUNEL and PCNA were counted under microscopy at 400× magnification with 16D grids. Ten gridding per section were counted and the average was considered as the positive cell density. Analysis of variance and t –test were used to analyze the data.

RESULTS

Examination of routine pathology

Macroscopic examination: Single or multiple nodes whose diameters ranged from 0.1cm to 0.3cm situated on the surface of the large intestinal mucosa were found in 22.2 percent of the experimental group at the 24th week (Fig.1). Multi nodes with the maximum diameter of o.8cm were demonstrated in 86.7% of the mice at the 32nd week with necrosis and ulcer formation in some nodes. The nodes usually were seen at the distal end of the large intestine. Thickening of the large intestinal wall, rigidity, and adhesion were observed in some mice. One mouse of the experimental group 1 died of lung infection and three mice of experimental group 4 died of intestinal obstruction from a tumor. Table 1 and 2 show the number and size of the tumors at the 24th and 32nd week.

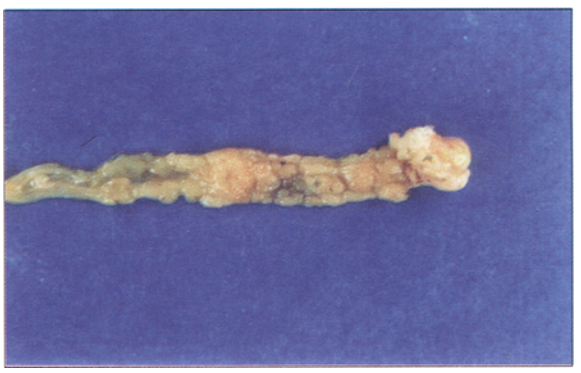

Fig. 1. Experimental group at 32nd weeks of inducing carcinoma. Single and multiple nodes appeared on the surface of the mucosa of the large intestine with the intestinal wall becoming thicker and rigid.

Table 1. Number and incidence of the tumors at the 24th and 32nd week in the tumor-induced experiment

Week	n	o	≤5	≤10	≤15	>15	Incidence
24th	9	7	1	1	0	0	22.2
32nd	15	2	2	6	3	2	86.7
Control	12	0	0	0	0	0	0

Examination under microscopy: Mild, moderate and severe dysplasia were observed in the large intestinal mucosa of the mice during the process of tumor induction (Fig.2). Cancer development in the large intestine appeared by the 24th week. The incidence of cancer formation was 66.7% by the 24th week and 93.3% by the 32nd week. The histological type of these tumors was mainly adenocarcinoma with only few adeno – squamous cell carcinomas. Three mice had liver metastastic foci. The pathological changes in the large intestinal mucosa at various times shown in Table 3.

Table 2. The average diameter (mm) of tumors at the 24th and 32nd week in the tumor-induced experiment

Week	n	0	≤1	≤2	≤3	≤4	≤5	>5
24th	9	7	0	1	1	0	0	0
32nd	15	2	0	5	2	1	3	2

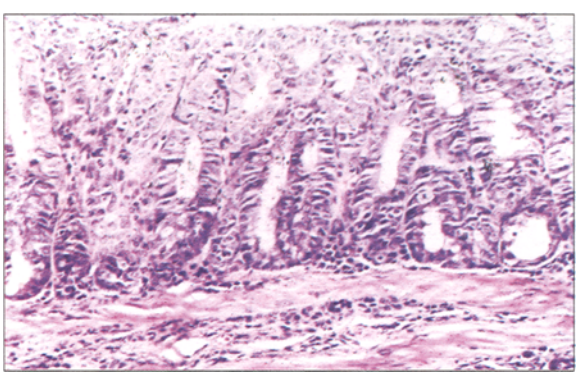

Fig. 2. Model group at 24th weeks of inducing carcinoma. Dysplasia of the mucosa epithelium, HE staining ×200.

The distribution and density of proliferation and apoptosis in the large intestinal mucosa at tumor-inducing multistages

The number of TUNEL-positive cells in the normal mucosa from the control group were few and were located on the surface of the epithelium as a cluster distribution. The number of PCNA positive cells located at the mucosa bottom was also small and the distribution was the same as with TUNEL-positive cells (Fig.3,4). As to the dysplasia of the experimental group, the number of TUNEL-positive cells ranging from the surface layer to the bottom increased as did the PCNA-positive cells. In the carcinoma tissue, the number of apoptotic cells was few and the distribution appeared sprinkled, but the distribution of PCNA-positive cells was clustered.

Analysis of the density of proliferating and apoptotic cells in different tumor-induced stages showed that the proliferating and apoptotic cells slightly increased at the 12th week but there wasn't a statisticly significant difference between the experimental group and control group. At the 18th and 24th week, the density of proliferating and apoptotic cells was higher in the experimental group than in the control group and had markedly increased by the 32nd week compared to other stages in the mucous layer, especially in moderate and severe dysplasia. In the large bowel cancer tissue, the density of proliferating cells was the highest and the density of apoptotic cells was the lowest. The ratio of proliferating cells to apoptotic cells dropped gradually (Fig.5,6). In a word, the density of proliferating cells significantly increased and the changes in density of apoptotic cells were unconspicuous in the dysplasia stage. The ratio of apoptosis and proliferation took on a descending trend, but the differences were not significant. In the tumor tissue, the ratio of apoptosis to proliferation dropped to the lower end, that is to say, the density of proliferating cells was the highest and that of apoptotic cells the lowest in cancer tissue(Table 4, 5).

Table 3. The pathological changes of large intestinal mucosa at different tumor-inducing stages

Week	n	Normal	Dysplasia			Adenocarcinoma	
			Mild	Moderate	Severe	Intramucous carcinoma	Infiltrative carcinoma
12th	10	2	3	4	1	0	0
18th	10	0	2	4	4	0	0
24th	9	0	0	1	2	5	1
32nd	15	0	0	0	1	2	12

Outcome of rank test: $H=33.35$, $P<0.01$.

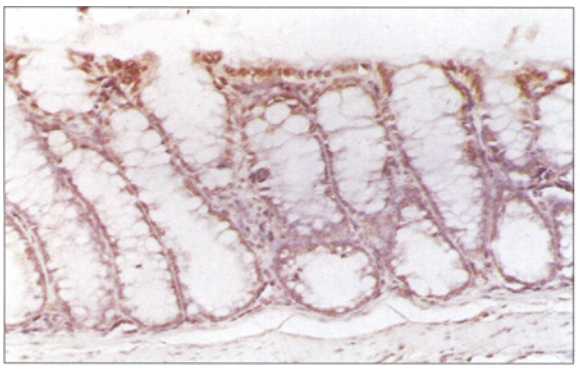

Fig. 3. Control group. The apoptotic cells located on the surface layer of the normal mucosa forming an apoptotic cell strip. TUNELstaining ×200.

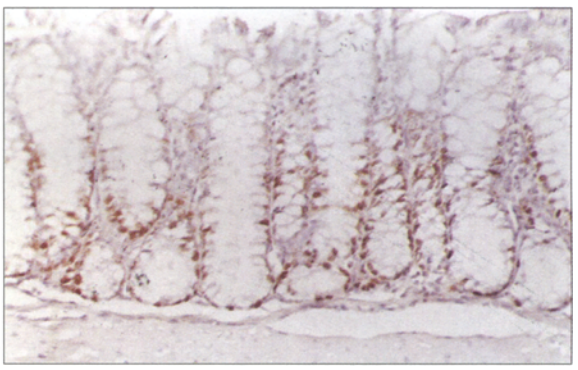

Fig. 4. Control group. The proliferating cells of the normal mucosa distribute on the bottom of the mucous layer forming a proliferating cell strip. PCNA staining ×200.

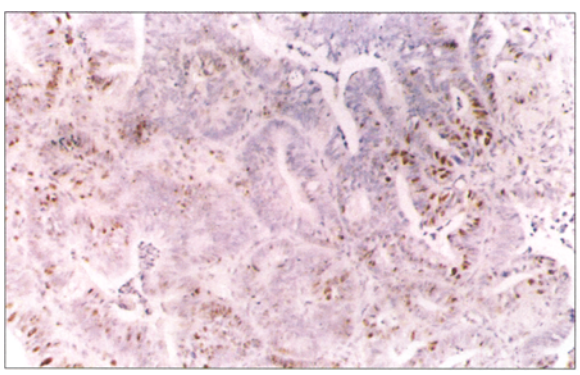

Fig. 5. Experimental group. There are a few apoptotic cells in the large intestinal carcinoma disseminately distributed. TUNEL staining ×200.

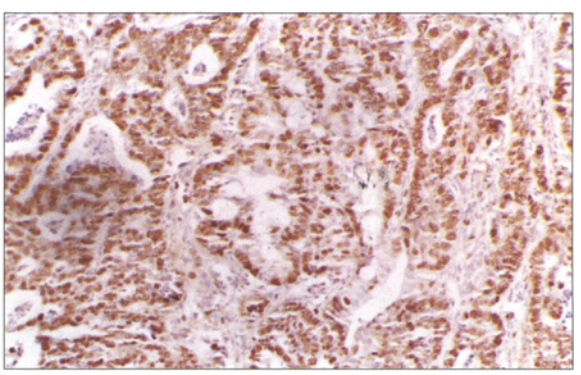

Fig. 6. Experimental group. There are a large number of proliferating cells in the large intestinal carcinoma. PCNA staining × 200.

Table 4. The density of apoptotic and proliferating cells during different stage of carcinoma induction (number of positive/area)

Stages	n	Proliferating cells	Apoptotic cells	Ratio
12th	10	25.3±9.2	12.5±5.7	0.58
18th	10	34.5±11.6$^{\triangle}$	16.7±8.4$^{\triangle}$	0.48
24th	9	63.6±18.5$^{\triangle}$*	37.4±17.3$^{\triangle}$	0.43
32nd	15	108.3±26.5$^{\triangle}$#	37.5±18.2$^{\triangle}$*	0.35
control group	23	17.2±6.7	9.8±3.40.49	0.56

$^{\triangle}$ compared to control group $P<0.01$,

* compared to the 12th and 18th week $P<0.01$,

compared to the other group $P<0.01$.

DISCUSSION

The dynamic balance of cell proliferation and apoptosis can maintain a steady state in normal tissue [1-3]. Apoptotic cells are situated on the surface layer of the mucosa forming an apoptotic cell zone. The proliferating cells are located at the bottom of the mucosa forming a proliferating cell zone. This distributive trait shows that the epithelial cells of the large intestine undergo a progressive procession of differentiation, maturation, senility and death. This balance has broken down in the large bowel cancer and precancerous lesions with the number and distribution of cell apoptosis and proliferation appearing abnormal. The result of this study showed that the number of apoptotic cells decreased and the degree of descent was parallel with the degree of cancer development[4-6]. A study on familial adenoma disease, single adenoma and adeno carcinoma by Bedi using the TUNEL labeling technique showed that there

Table 5. The density of apoptotic and proliferating cells during different lesions of induced cancer(number of positive/area)

Type of lesion(dysplasia)	n	Proliferating cells	Apoptotic cells	Apoptotic cells/proliferating cells
Mild	5	8.88±25.7	46.6±19.1	0.52
Moderate	9	114.7±31.7$^{\triangle}$	47.1±17.3	0.50
Severe	8	159.0±45.9$^{\triangle *}$	61.0±24.6	0.38
Cancer	20	214.7±24.1$^{\triangle *\#}$	26.7±17.0$^{\#}$	0.12

$^{\triangle}$compared to mild dysplasia P<0.01, *compared to moderate dysplasia P<0.01, #compared to the other group P<0.01.

were few apoptotic cells in the single adenoma and familial adenoma disease, which were located on the surface of the mucosa, and no apoptotic cells in the adenocarcinoma[2,8]. In 27 cases of colonic polypoid adenoma and 8 cases of villous adenoma studied by Arai, the apoptotic index (AI) was reported to be 3.5%~8.8% in polypoid adenoma and 1.8% in villous adenoma. Therefore, an excessive accumulation of cells from restrained apoptosis might be the cancerous basis in the large intestine[4].

The imbalance between proliferation and apoptosis in mice was verified by our study using a tumor-induced experimental model. Furthermore, an abnormal distribution, with the loss of the characteristic proliferating and apoptotic cell zones and the changes of density and ratio between proliferating and apoptotic cells were verified in the study. In fact, the changes of increasing density of proliferating and apoptotic cells in the mucosa emerged from the 12th week. Severe dysplasia and partial cancer development appeared from the 18th and 24th week. The number of proliferating and apoptotic cells increased but the ratio between them had only a slight decrease. In the 32nd week, cancerous colonic tissue appeared in most animals. The increasing density of proliferating cells with a decreasing change in apoptotic cells led to the distinct fall in the ratio between apoptosis and proliferation[9,10]. Statistical analysis of the large intestinal lesions in the process of cancer induction showed that the density of proliferating and apoptosis cells was higher in dysplastic than normal tissue. With the progress of the lesions, the density of proliferation definitely increased with the density of apoptotic cells remaining in a stationary state. All this led to a descending ratio between apoptosis and proliferation. As to the cancer tissue, the degree of proliferating cells increased but the degree of apoptotic cell decreased. In a word, the trend of density changes of proliferation and apoptosis at different cancer-inducing stages slightly increased in the early stage, both occured increased in the middle stage and a high-proliferation and low-apoptosis state occurred in the late stage [11]. The density changes of proliferating and apoptotic cells in the pre-cancer stage differed from those in the cancer stage. During the pre-cancer stage, the epithelial cells showed a high-proliferative and high-apoptotic state changing to a high-proliferative and low-apoptotic state in the cancer stage which led to the decreased ratio of apoptosis to proliferation. Once the cumulative speed of cellular proliferation accelerated it may mean that a tumor will emerge[12-14].

This result in which high-proliferation and high-apoptosis appeared in the stage of precancerous lesions followed by high-proliferation and low-apoptosis in the stage of cancer development maybe different from the findings of Bedi and Arai, they concluded that the process of apoptotic restraint appeared during the whole stage of cancer formation without variation in the different stages of tumor induction, that is to say, the number of apoptotic cells decreased in the stage of precancerous lesions as well as in the stage of carcinogenesis [2,15]. The result of the animal experimentation was consistent with the study of large intestinal villous adenoma (precancerous lesion stage) and papillary adenoma (cancerous stage). Hence the idea of Cell Selective Proliferation was suggested according to the unbalanced law of proliferation and apoptosis during the process of large intestinal epithelium cancerization [3,16]. In the early and middle stage of high-proliferation and high-apoptosis, some cells kept on proliferating but some other cells were removed because of apoptosis. During the cancerous stage, high-proliferative activity cells remained and escaped from apoptosis by selection. All this led to the descent of the apoptotic cell number. The highly-proliferative cells then gradually progressed showing a behavior of invasion and metastasis.

The mechanism of Cell Selective Proliferation maybe related to genetic, influence P53, Bcl-2, Bax, Fas and Fas-L regulation may have an effect on cell proliferation and apoptosis. The detailed mechanism awaits further studies for clarification.

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