

Effect of retinoic acid on cell proliferation kinetics and retinoic acid receptor expression of colorectal mucosa

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Abstract

AIM: To investigate the effect of retinoic acid (RA) on cell proliferation kinetics and retinoic acid receptor (RAR) expression of colorectal mucosa.

METHODS: One hundred sixty healthy male Wistar rats were randomly divided into 4 groups. Rats in groups I and II were subcutaneously injected with dimethylhydrazine (DMH) (20 mg/kg, once a week,) for 7 to 13 weeks, while groups III and IV were injected with normal saline. Rats in groups II and III were also treated with RA (50 mg/kg, every day, orally) from 7th to 15th week, thus group IV was used as a control. The rats were killed in different batches. The expressions of proliferating cell nuclear antigen (PCNA), nucleolar organizer region-associated protein (AgNOR) and RAR were detected.

RESULTS: The incidence of colorectal carcinoma was different between groups I (100 %) and II (15 %) ($P<0.01$). The PCNA indices and mean AgNOR count in group II were significantly lower than those in group I ($F=5.418$ and 4.243 , $P<0.01$). The PCNA indices and mean AgNOR count in groups I and II were significantly higher than those in the groups III and IV (in which carcinogen was not used) ($F=5.927$ and 4.348 , $P<0.01$). There was a tendency in group I that the longer the induction with DMH the higher PCNA index and AgNOR count expressed ($F=7.634$ and 6.826 , $P<0.05$). However, there was no such tendency in groups II, III and IV ($F=1.662$ and 1.984 , $P>0.05$). The levels of RAR in normal and cancerous tissues in groups treated with RA were significantly higher than those in groups not treated with RA ($F=6.343$ and 6.024 , $P<0.05$).

CONCLUSION: RA decreases the incidence of colorectal carcinoma induced by DMH. Colorectal cancer tissue is associated with abnormal expression of PCNA, AgNOR and RAR. RA inhibits the expression of PCNA and AgNOR, and increases RAR concentration in colorectal tissues.

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INTRODUCTION

The occurrence and development of colorectal carcinoma usually need a long and multistep process. Intervention treatment to block the canceration course from precancerous lesion of colorectal carcinoma is an important step to decrease the incidence of colorectal carcinoma. Some results obtained from *in vitro* experiments have shown that retinoic acid (RA) plays a role in blocking canceration induced by carcinogen and promotes normal differentiation of leucocythemia cells^[1-3]. However, the effect of RA on colorectal carcinoma, especially on cell proliferation kinetics and the expression of retinoic acid receptor (RAR) of colorectal mucosa, has not been reported. To provide theoretic data on prevention and treatment of colorectal carcinoma, we investigated the effect of RA on cell proliferation kinetics and expression of RAR of colorectal mucosa.

MATERIALS AND METHODS

Animals and groups

One hundred sixty healthy male Wistar rats (body weight 134 ± 12 g) were randomly divided into 4 groups. There were 40 rats in each group. Rats in group I and II were subcutaneously injected with dimethylhydrazine (DMH) (20 mg/kg, once a week,) for 7 to 13 weeks, while groups III and IV were injected with normal saline. Rats in groups II and III were treated with RA (50 mg/kg, every day, orally) from 7th to 15th week, group IV was used as a control. Eight rats in each group were killed randomly at 7th, 14th and 21st week in each group. The other rats were killed at 28th week. The number of colorectal carcinoma lesions was examined, and the normal colorectal tissues were also collected. The colorectal samples were fixed with 10 % formalin and embedded in paraffin. The expression of proliferating cell nuclear antigen (PCNA) and nucleolar organizer region-associated protein (AgNOR) was studied.

Detection of PCNA and AgNOR

Normal colorectal tissues ($n=8$) and the colorectal tissues ($n=8$) free of cancer induced by DMH after 7, 14, 21 and 28 weeks, were included. The samples including well-differentiated adenocarcinoma ($n=8$) and poorly differentiated adenocarcinoma ($n=8$) were also collected.

The immunohistochemical staining method was used to detect PCNA indices^[4-7]. Representative regions with a double blind method were selected, and at least 1 000 cells were counted. The rates of positive cells over total cells counted were defined as the PCNA indices. Ploton one-step method was used for the detection of AgNOR count^[8-11].

Detection of retinoic acid receptor (RAR)

Specimen disposal The mesentery tissues were removed and

part of the colorectal tissues was cut to pieces and placed in DMEM buffer. A tissue was homogenated by a high speed disperser, 4 000 r.min⁻¹ for 10 min and a homogenizer 4 000 r.min⁻¹ for 30 min and then by centrifugation 1 000 r. min⁻¹ for 30 min. The buffer was added to the deposit, and the suspension was centrifugated, 750 r. min⁻¹ for 15 min. Finally, the deposit was made to nucleus fluid. DNA concentration was determined by the dimethylamine method. The rest part of tissues was treated with liquid nitrogen and preserved in an ultra cold storage freezer.

Receptor radio-ligand binding test^[16-20] All the procedures of the test were carried out at 4 °C. 0.1 ml of nucleus fluid and 0.05 ml of 3H-atRA (2×10⁻⁶ mol/L) and 0.05 ml of buffer were mixed at different concentrations (the end concentration was 0.1-10 nM, with 6 concentration points). At the same time, the control test tube of non-specific binding was 200-time of unlabelled 9-cis-atRA. After 20 h, the reaction mixture was filtered with a multi-head collecting device and the free RA was removed, and examined by the filter membrane method. Saturation binding curve, Scatchard diagram and receptor maximum dissociation constant KD were analyzed by a receptor radio-ligand binding analyzing software.

Statistical analysis

Experimental results were analyzed by variance analysis and chi-square test with SPSS software. Statistical significance was determined at $P < 0.05$.

RESULTS

Incidence of colorectal carcinoma

At the 14th week after induction, 12.5 % of rats in group I developed colorectal carcinoma, but colorectal carcinoma was not found in group II. At the 21st and 28th weeks, the incidence of colorectal carcinoma reached 60 % and 100 % respectively in group I, compared with 12 % and 20 % respectively in group II. There were significant differences between the two groups ($P < 0.05$ and $P < 0.01$). All the carcinomas were adenocarcinomas. In group I, 12 cases of adenocarcinoma were well-differentiated and 9 cases were poorly-differentiated. All the 4 cases in group II were well-differentiated (Table 1).

Table 1 Incidence (%) of colorectal carcinoma in the groups

Weeks	n	Number of cancer			
		I	II	III	IV
7	8	0	0	0	0
14	8	1(12.5)	0	0	0
21	8	5(60.5)	1(12.5)	0	0
28	15	15(100.0)	3(20.0)	0	0

Expression of PCNA indices

At the 7th week, PCNA indices reached 96.75±6.88 and 95.50±14.01, respectively, in group I and group II, which were significantly higher than those in group III and group IV (34.38±6.30 and 33.63±4.75, respectively, $P < 0.01$). In metaphase and late-phase, PCNA indices in group I and group II were continuously increased, especially in group I. PCNA indices in group I reached 168.13±14.34 at the 28th week and approached the level of well-differentiated adenocarcinoma (169.13±11.68), but were still significantly lower than that of poorly-differentiated adenocarcinoma (181.63±23.38, $P < 0.05$). Analysis of variance showed that there was a tendency in group I that the longer the induction with DMH the higher the PCNA index ($F = 7.634$, $P < 0.05$). However, there was no such tendency in groups II, III and IV ($F = 1.662$, $P > 0.05$).

In comparison between the groups, the results showed that PCNA indices in group I and group II were significantly higher than those in groups III and IV at all stages of carcinoma induction ($F = 5.927$, $P < 0.01$). Moreover, PCNA index in group I was significantly higher than that in groups II at all stages ($F = 5.418$, $P < 0.01$), except at the 7th week (Table 2).

Table 2 Expression of PCNA indices in groups

Weeks	n	I	II	III	IV
7	8	96.75±6.88	95.50±14.01	34.38±6.30	33.63±4.75
14	8	110.88±15.51	97.88±8.90	35.13±3.91	35.88±2.17
21	8	149.50±15.15	98.25±25.09	36.00±3.46	34.13±4.39
28	8	168.13±14.34	98.88±25.30	35.88±4.29	33.13±4.32
Well-differentiated adenocarcinoma	8	169.13±11.68	-	-	-
Poorly differentiated adenocarcinoma	8	181.63±23.38	-	-	-

Expression of AgNOR count

At the 7th week, AgNOR count reached 3.78±0.88 and 3.71±0.95, respectively, in group I and group II, which was significantly higher than that in group III and group IV ($P < 0.05$). As the time of induction with DMH prolonged, the AgNOR count in group I was continuously increased. Analysis of variance showed that there was a tendency in group I that the longer the induction with DMH the higher the AgNOR count ($F = 6.826$, $P < 0.05$). However, there was no such tendency in groups II, III and IV ($F = 1.984$, $P > 0.05$). At the 28th week, the AgNOR count already approached the level of well-differentiated adenocarcinoma, but was still significantly lower than that of poorly-differentiated adenocarcinoma ($P < 0.05$).

In comparison between the groups, the results showed that the AgNOR counts in group I and group II were significantly higher than those in groups III and IV at all stages of carcinoma induction ($F = 4.348$, $P < 0.05$). The AgNOR count was significantly higher in group I than that in group II at all stages ($F = 4.243$, $P < 0.05$), except at the 7th week (Table 3).

Table 3 Expression of AgNOR count in groups

w	n	AgNOR count ($\bar{x} \pm s$)			
		I	II	III	IV
7	8	3.78±0.88	3.71±0.95	2.17±0.53	2.45±1.06
14	8	5.15±1.87	4.30±0.84	2.20±0.86	2.16±0.80
21	8	7.54±0.73	4.39±0.62	2.20±0.77	2.49±0.90
28	8	9.37±0.71	4.75±0.98	2.35±1.01	2.38±1.04
Well-differentiated adenocarcinoma	8	9.93±1.47	-	-	-
Poorly-differentiated adenocarcinoma	8	11.14±1.86	-	-	-

Table 4 RAR count (fmol/μg DNA) and KD (nmol) in cells. ($\bar{x} \pm s$)

Group	n	Bmax	KD
I	6	1.16±0.34	1.99±0.25
II	6	1.78±0.36	2.16±0.18
III	6	2.61±0.55	2.39±0.43
IV	6	2.64±0.22	2.45±0.23
Cancer tissue	6	1.02±0.21	1.74±0.16

Expression of RAR

Six samples of colorectal and cancer tissues were collected randomly from groups I, II, III and IV respectively. Expressions

of RAR were detected, Bmax and KD were calculated. The Bmax and KD in group I approached the level of cancer tissues (1.02 ± 0.21 and 1.74 ± 0.16 , $P > 0.05$). The Bmax and KD in group II were significantly higher than those in group I, but significantly lower than those in groups III and IV, ($F = 6.343$ and 6.024 , $P < 0.05$).

DISCUSSION

Recently, the mechanism of preventing carcinoma by RA has been studied by scholars all over the world. Some researchers reported that leukaemia cells could respond to the effect of differentiation induced by RA to put up the potential of diphasic differentiation^[21-23]. Some reported that RA could result in reversion of liver cancer cells^[24,25]. In our research, we found that the incidence of carcinoma developed in RA treatment group (group II) was significantly lower than that in group I during induction. The results showed that retinoic acid (RA) had an effect on blocking canceration induced by carcinogen and decreased the incidence of colorectal cancer.

PCNA is the 36 KD polypeptide which is synthesized and expressed just in proliferating cells. It has been proved that PCNA expression is related to cell generation cycle^[11,12]. Expression of PCNA increases in G₁ phase gradually, reaches pinnacle in S phase, and decreases in G₂/M phase. It plays an important role in understanding cell generation state to detect PCNA indices. The higher the PCNA expression, the higher the cell malignancy trend^[12-15]. Our experimental results showed that there was a tendency in group I that the longer the interval induced by DMH, the higher the PCNA index would be ($P < 0.05$). At the 28th week, the PCNA indices already approached the level of well-differentiated adenocarcinoma, but were still significantly lower than that of poorly-differentiated adenocarcinoma ($P < 0.05$). The PCNA indices in group II were higher than those in groups III and IV, but still lower than those in group I. RA may have an effect on blocking canceration induced by carcinogen and decreasing the incidence of colorectal carcinoma. The mechanism is not clear, maybe it is related to blocking the transition of cancer cells from G₀/G₁ phase to S₁, G₂+M phase. Our results also showed that RA could not block canceration entirely.

AgNOR is the biochemical symbol of rDNA and transcription. AgNOR count can reflect the cell active state and cell malignant trend of carcinoma^[8-10]. We found that AgNOR count of colorectal mucosa cells in group II was significantly lower than that in group I, but significantly higher than that in groups III and IV during the period of inducement. The reasonable explanation was that RA could inhibit the process of canceration induced by carcinogen but could not block canceration entirely.

Our results showed that there were plenty of RARs in colorectal tissues. The normal RAR contents in colorectal cells were $2.64 \text{ f mol}/\mu\text{g DNA}$, and KD was 2.45 nmol . However, RAR contents in colorectal cancer cells decreased significantly ($1.02 \text{ f mol}/\mu\text{g DNA}$, and 1.74 nmol). It is possible that the development of colorectal carcinoma is related to abnormal expression of RAR, and especially decrease of RAR content. After interference treatment with RA, the expression of RAR increased. The carcinogenic course induced by DMH was slowed down distinctly. The results revealed that RA had an effect on inhibiting cellular proliferation and RA could regulate the expression of RAR^[24-30].

There are plenty of similarities between human colorectal cancer and experimental colorectal cancer. However, it is possible that colorectal cancer occurs in total colorectal mucosa under the action of carcinogenic factor. It is possible that clinical application of RA can inhibit the precancerous lesion of colorectal carcinoma, block the canceration course, and

decrease the incidence of colorectal cancer^[31-35]. It is expected that clinical application of RA after colorectal operation would prevent and decrease the recurrence of carcinoma.

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