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Transretinoic acid inhibits rats gastric epithelial dysplasia induced by N-methyl-N-nitro-N-nitrosoguanidine: influences on cell apoptosis and expression of its regulatory genes

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INTRODUCTION

Gastric epithelial dysplasia (GED) hypothetically is a straight-forward concept: dysplastic epithelium replacing the normal gastric epithelium of the stomach[1]. In the stomach, like any other segment of the gut, it is defined as an unequivocal non-invasive epithelial change[2,3]. The observation of gastric dysplasia as a cancerous lesion was recognized over a century ago, but it is only after the advent of gastroscopy that its clinical significance has been stressed[4-7].

All-trans retinoic acid can effectively reverse dysplasia in gastric epithelial cells, thereby inhibiting its progression to gastric cancer[8-10]. However, its mechanism is not yet clear. We used N-methyl-N-nitro-N-nitrosoguanidine (MNNG) to establish a rat model of gastric epithelial cell dysplasia, and to study the influence of all-trans retinoic acid. Changes in apoptosis and expression of Bcl-2, Fas and ICE were observed to investigate into insight of its mechanism.

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MATERIALS AND METHODS

Induction of gastric epithelial cell dysplasia and treatment with all-trans retinoic acid

Forty-five 8-week-old male Wistar rats weighing 120 g-140 g were housed in individual cages at a controlled temperature of 22℃, and a relative humidity of 50%. These animals were randomly divided into three groups each 15 rats. Group 1 served as blank control. Groups 2 and 3 were fed with MNNG to induce gastric epithelial cell dysplasia. Two grams MNNG (Fluka Co.) was dissolved in 2000 mL distilled water, placed in a brown bottle and kept at 4℃. The preserved MNNG solution was further diluted to the concentration of 1 g/mL for use as drinking water ad lib[11,12]. In addition, at one, three, five and seven weeks, 2 mL absolute alcohol was infused into the stomach of each animal. After 24 weeks, when gastric epithelial cell dysplasia had been induced, animals in group 2 were given 40 μg/kg all-trans retinoic acid (Shanghai No.6 Pharmaceutical Co.) through infusion into the stomach every day. Group 3 served as treatment controls and a placebo (distilled water) was given instead of all-trans retinoic acid. The animals were killed at 36 weeks.

The stomachs of the rats were cut along the greater curvature. Specimens were taken from the pyloric area and five paraffin sections were made for each rat following the conventional method. These sections were used for routine pathological examinations, apoptosis determination and measurements for the expression of Bcl-2, Fas and ICE. Pathological diagnoses and gradings were carried out according to the criteria set by the National Gastric Cancer Research Group[13,14].

Determination of apoptosis by TUNEL method

After dewaxing a 4 μm thick section, the fundamental steps of the procedure were: addition of proteinase K (20 μL/mL); incubated at 37℃; addition of Triton x-100 in 0.1% sodium citrate on ice, stood up; addition of 50 g TUNEL (Boehringer Co., Cat. no. 1684817); incubated at 37℃ in a humidified chamber; staining with diaminobenzidine (DAB); sealing the slide; and

examined under a light microscope. Apoptotic cells presented as brownish staining in the nucleus, although part of the cytoplasm also can be stained because of leakage of some nuclear DNA fragments. At least 500 cells were examined and the number of apoptotic cells per 100 cells calculated to arrive at the apoptosis index (AI)[15,16].

Expression of Bcl-2, Fas and ICE measured by immunohistochemical staining

Bcl-2 After dewaxing, the 4 μm thick section was stained using the Avidin-Biotion Complex (ABC) method[16]. The primary antibody used was 2 mg/L rabbit antirat Bcl-2 polyclonal antibody (Santa Cruz Co. USA, Cat. No. Sc-578) diluted 1:50.

Fas To detect Fas activity, sections were stained by ABC method. The primary antibody used was 2 mg/L rabbit antirat Fas polyclonal antibody (Santa Cruz Co. USA, Cat. No. sc-716G) diluted 1:50.

ICE To detect ICE activity, sections were stained by the ABC method. The primary antibody used was 2 mg/L rabbit antirat ICE polyclonal antibody (Santa Cruz Co. USA, Cat. No. sc-514) diluted 1:50.

Positivity control Slides of multiple gene-protein-positive gastric cancer specimens were used as positivite controls. If brown granules appeared on the nuclear membrane and in the cytoplasm of gastric epithelial cells, the specimen was considered to be Bcl-2 positive. If brown granules appeared on the cell membrane, the specimen was considered to be Fas positive; and if brown granules appeared in the cytoplasm, the specimen was considered to be ICE positive. The expression was graded according to the degree of staining: strongly positive (+++), moderately positive (++) and weakly positive (+). If more than 30% of cells in a slide were also considered positive. Those with strongly and moderately positive responses were considered to be overexposing the protein in question[17-20].

Probes

The following oligonucleotide probes were used in this study. Bcl-2, TGATACCAGCACTGGAGCAG, was synthesized by Shanghai Shenggong Company. Fas, CAGCCAGGAAAGATCAAACAGAGAGC, was bought from Fuzuo Company.

Northern-blot analysis

Total RNA was isolated from the gastric epithelial tissue by extraction of guanidine isothiocyanate and centrifugation in cesium chloride[21,22]. Poly(A) +RNA was selected by oligo(dt)-cellular chromatography[23]. Six micograms of poly(A) +RNA from each sample was electophoresed in 1% agarose gel containing 0.66 mol/L formaldehyde and ethidium bromide (0.66 mg/L).

After electrophoresis, the gels were photographed under UV light to confirm that approximately equal amounts of RNA were loaded. The gels were pretreated with 0.05N NaOH for 30 minutes at room temperature[24,25] and RNA was transferred onto nitrocellulose. Then, appropriate probes were labled with 32P deoxycytidine triphosphate using a Random Prime DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The blots were hybridized overnight at 62℃ (bcl-2) or 52℃ (Fas) in 59% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate, 1 mol/L NaCl, and 100 g/L of sonicated salmon sperm. Then the blots were washed in 2 × standard saline citrate (1×SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.4), and 0.5% sodium dodecyl sulfate four times at room temperature for 5 minutes and also washed (3 × 10 minutes) in 0.2 × SSC and 0.5% sodium dodecyl sulfate at 60℃. The blots were then exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70℃. The nitrocellulose filter was boiled in 0.1% SSC and 0.1% SDS for 30 minutes to strip off the radioactivity probes and rehybridized with another ^{32}P labelled Cdna prob in a similar manner[26]. The quantity of specificy transcripts in different lanes was determined by densitometric analysis of autoradiographs.

RESULTS

Histopathological changes of the gastric mucosa

The incidence of dysplasia in group 3 was significantly higher than that in group 2 (73.3% *vs* 26.7%, $P = 0.05$, Table 1).

Table 1 Histopathological changes and apoptosis of the gastric mucosa

Group	*n*	Modest dysplasia (*n*)	Modest and severe dysplasia (*n*)	Percentage (%)	Apoptotic index
Normal	15	0	0	0	8.3±3.1
DIM	15	3	11	73.3%	2.2±0.4[a]
DIMTR	15	4	4	26.7%[ab]	7.8±2.6[bc]

[a]$P<0.05$ *vs* normal; [b]$P>0.05$ *vs* normal; [c]$P>0.05$ *vs* DIM group.

Apoptosis

The apoptosis index was not significantly different in groups 1 and 2 ($P>0.05$), but there was a significant difference between groups 1 and 3 ($P<0.05$) and also between groups 2 and 3 ($P<0.05$).

Expression of apoptosis-associated proteins

Bcl-2 In group 1, two rats (13.3%) expressed Bcl-2 and one rat (6.7%) overexpressed Bcl-2. Expression of Bcl-2 was found in 10 rats (66.7%) and overexpression of Bcl-2 in five rats (33.3%) in group 3. Both the expression and overexpression

were significantly higher than that in group 1 ($P<0.05$). Group 2 had five rats (33.3%) expressing Bcl-2 and one rat (6.7%) overexpressing Bcl-2. Neither expression nor overexpression was significantly different from that of group 1 rats ($P>0.05$) but was significantly different from that of group 3 ($P<0.05$).

Fas In group 1, seven rats (46.7%) expressed Fas and two rats (13.3%) overexpressed of Fas. Group 3 had two rats (13.3%) with expression of Fas and two rats (13.3%) with overexpression of Fas. Expression of Fas was significantly different from that of group 1 ($P<0.05$), but overexpression was not ($P>0.05$). In group 2, six rats (40%) expressed Fas and four rats (26.7%) overexpressed Fas. Neither expression nor overexpression were significantly different from that of group 1 ($P>0.05$), but when compared with group 3, a significant difference was seen in the expression of Fas ($P<0.05$), yet not in overexpression ($P>0.05$).

ICE In group 1, three rats (20%) expressed ICE and no rats were found to be overexpressing this protein. Group 3 had two rats (13.3%) with expressing ICE and one rat (6.7%) with overexpressing ICE. Neither expression nor overexpression were significantly different from that in group 1 ($P>0.05$). In group 2, expression of ICE was seen in nine rats (60%) and overexpression was found in two rats (13.3%). Expression of ICE was significantly different from that in group 1 ($P<0.05$) but overexpression was not ($P>0.05$), and when compared with that of group 3, expression of ICE was significantly different ($P<0.05$) while overexpression was not ($P>0.05$).

Bcl-2 and Fas mRNA expression

The expression of bcl-2 mRNA increased and Fas mRNA was decreased in comparison of group 2 with group 1. The expression of bcl-2 mRNA was decreased and Fas mRNA was increased in comparison of group 2 with group 3 (Figure 1).

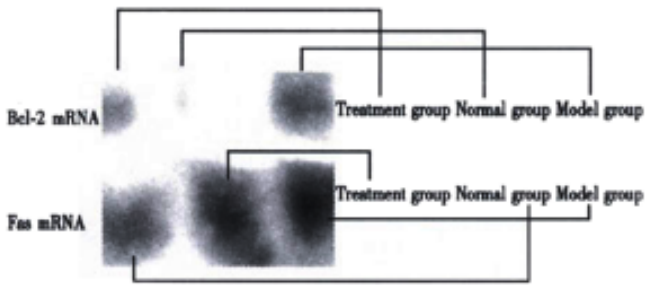

Figure 1 Bcl-2 and Fas mRNA expression.

DISCUSSION

With the introduction of fiberoptic endoscopy in the late 1960s and early 1970s, Nakamura and Nagayo in Japan were among the first to identify possible precancerous lesion on biopsical material and develop several categories for dysplasia[27,28]. In the West, Grundmann in 1975 quote for the first time the word dysplasia to describe exclusively precancerous gastric lesions[29]. Shortly after the WHO committee sanctioned this usage and detailed general diagnostic principles based on cellular atypia, abnormal differentiation and disorganised architecture[30,31]. There are differences between the Japanese and the Western criteria. A multicenter study is now under way to unify the diagnostic criteria[32-34]. So far there has been no unifed criteria of dysplasia among Chinese. We used the Japanese criteria in this study.

Is dysplasia reversible Several investigators have addressed the issue, but the cumulative results remain inconclusive. There are still coutroversies in the interpretation of atrophic changes and intestinal metaplasia resulting in wide discrepancy in the conclusions reached by different authors. Controlled, long-term prospective studies conducted in different ethnic and geographic settings are needed to provide sound evidence-based answers to the question of reversibility of atrophy, intestinal metaplasia, and epithelial dysplasia[6,35], trying to look for new drugs to reverse the dysplasia of great importance.

Normal gastric mucosal epithelial cells undergo apoptosis to clear up the senile cells and maintain the physiological balance of mucosal epithelial cells[36]. The homeostasis of gastric epithelial cells is maintained by the balance between cell proliferation and apoptosis. Alterations of these physiological cellular events in chronic pathological conditions of the stomach, as far as the proliferative pattern is concerned, an increase in the total number of epithelial proliferating cells and an abnormal distribution of the latter are frequently observed in chronic gastritis, gastric atrophy, intestinal metaplasia, gastric dysplasia and gastric cancer. Conversely, apoptosis has been found to be impaired in intestinal metaplasia, gastric dysplasia and cancer[37,38]. We consider the development of gastric cancer as a simple problem of balance, which is explained in the following formula: cumulative rate of epithelial cells' proliferation rate of epithelial cells' apoptotic rate of epithelial cells. When overaccumulation of gastric mucosal epithelial cells due to any reason, there is the possibility of development of gastric cancer. Thus, abnormal apoptosis might be one of the causes of gastric cancer development.

Apoptosis is modulated by regulatory genes. Bcl-2, Fas and ICE are the regulatory genes that have been predominantly studied. The statement of Bcl-2 can inhibit apoptosis, allowing the

proliferating cells to accumulate and inhibiting the removal of the malignant potential cells, thereby facilitating the development of cancerous change. Several scientists have studied the relation among bcl-2, gastric epithelial dysplasia and apoptosis and found that the expression abnormality leads to the apoptosis changed, the result in dysplasia and carcinoma[19,39-42]. The statement of Fas promotes apoptosis and his abnormality has relation with dysplasia too[43]. Interleukin-1-coverzyme can induce apoptosis in certain types of cells[44-46].

A few investigation of bcl-2 expression and dysplasia found that bcl-2 expression did not correlate with the presence or degree of dysplasia in either benign gastric mucosa or gastric carcinoma (GC) patients. bcl-2 protein is frequently expressed in GC[39,47]. The reason perhaps is the difference of rat's model and human gastric epithelial dysplasia, model induced by MNNG caused mainly by chemistrial carcinogenese, is not all the same with human.

Retinoic acid and its analog retinoid has a reverse effect on experimentally induced gastric mucosal precancerous lesions in rats[8-10,48]. The results of the present study further confirmed this. The results of the present study also revealed that retinoic acid can inhibit the overstatement of the Bcl-2 protein, promote the normal statement of the Fas protein and enhance the overstatement of the ICE protein, thereby promoting the apoptosis of gastric mucosal dysplastic epithelial cells. This may be one of the mechanisms by which retinoic acid reverses gastric mucosal precancerous lesions and the high statement of ICE may partly explain the side effects of retinoic acid.

The results of the present study reveal that in moderate and severe gastric mucosal dysplasia precancerous lesions, there is already abnormality in apoptosis and changes in associated genes. The increase in Bcl-2 statement, decrease in Fas statement and inhibition of apoptosis may be an important mechanisms in the progression of dysplasia to cancer.

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