Strain Differences of Macrophage Distribution in the Experimental Colorectal Cancer Model

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実験的大腸癌におけるマクロファージ分布の系統差

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ABSTRACT

Colorectal cancer (CRC) is one of the most common forms of malignancy in humans. Patients with long-standing ulcerative colitis (UC) have an increased risk for developing CRC compared to the general population. For investigation of the mechanisms and prevention of UC, and UC-related CRC, we first induced UC by DSS and then induced CRC by DMH. It took only 3 months to found the CRC model in Wister rats. The important similarities of the model may be seen in the clinical and pathological findings in animals and patients with CRC developing from UC. It is especially suited to the study of UC and CRC. In this study the mean percent of ED1, and ED3 positive macrophages in Wistar rats was higher than that in DA and Lewis rats ($p < 0.05$). The mean percent of Mar1, and Mar3 positive macrophages in DA and Lewis rats was higher than that in Wistar rats ($p < 0.05$). These results suggested that the existence of four types of macrophages may play an important role in inducing colitis and rectal cancer. Furthermore, the mechanisms of strain difference in the experimental models are discussed.

Key Words : experimental colorectal cancer, rats, macrophages

INTRODUCTION

Colorectal cancer (CRC) is one of the most common forms of malignancy in humans, and its incidence is steadily increasing. Patients with long-standing ulcerative colitis (UC) have an increased risk for developing CRC compared to the general population[1, 2, 3]. Despite the risk of CRC in patients with UC being recognized for years, there is considerable controversy over the exact magnitude of the risk. Choi[4] reviewed published date and calculated the relative risk in UC to very between 1 and 20 when compared to the general population. The increased risk of cancer in UC is related to the anatomical extent and the duration of the disease [2, 4, 5].

For investigation of the mechanisms and prevention of UC, and UC-related CRC, an appropriate animal model for such disease should be established. The experimental model systems are important to test the various hypotheses concerning etiology and pathogenesis of UC and lead to understand the mechanisms involved in CRC. Finding in patients with chronic UC are paralleled in animal models, that spontaneously development an idiopathic colitis with histopathological similarity in human UC [3].

The cause of the increased incidence of colorectal cancer in chronic UC is unknown but may be associated with repeated episodes of chronic inflammation and repair of the colonic epithelium. Macrophages play a key role in the process of inflammation and cancer in many different tissues. Macrophages represent one of the largest compartments of the mononuclear phagocyte system in the body. They are localized preferentially in the subepithelial region and constitute in the intestinal lamina propria[6].

In the present study, we attempted to investigate the Induction of CRC in dextran sulfate sodium (DSS)-induced experimental ulcerative colitis in specific pathogen-free (SPF) rats, and. We employed immunohistochemical staining and flow cytometric analysis methods using monoclonal antibodies for detection macrophage-surface antigens in the CRC of three kinds of strains in rats. Furthermore, the mechanisms of strain difference in the experimental models are discussed.

MATERIALS AND METHODS

Animals and chemicals

7 weeks old specific pathogen-free (SPF) male rats of inbred strains were purchased from Japan SLC Inc. (Hamamastu, Japan) and maintained in a specific pathogen-free animal facility at the Hamamatsu University School of Medicine. The rats of inbred strains Wistar ($RT1^{u}$), DA ($RT1^{a}$) and Lewis ($RT1^{l}$) were used throughout the study.

Dextran sulfate sodium (DSS, molecular weight 5,000) and 1, 2-Dimethlhydrazine (DMH) was obtained from Sigma Chemical Co. USA.

Carcinogenesis

Induction of UC in group A. The UC was induced by drinking water containing 3 % DSS, as described by Kimura I et al.[7] for 11 days of DSS administration.

Induction of CRC in groups A and B. The rats of group A after 11 days of DSS administration and group B received DMH subcutaneously once a week on the same day and the same time (10pm) in a dosage of 30 mg/kg body weight for 12 weeks. DMH was prepared in 0.9% NaCl and adjusted to pH 6.5 with sodium bicarbonate immediately before injection[8].

Control in group C. The rats of group C received 0.9% NaCl subcutaneously only once a week at the same day and same time as groups A and B (Table 1).

Table 1. Group separating and treatment

Group	Stain (n=6)	Treatment	
	Wister	DSS	DMH
A	DA	DSS	DMH
	Lewis	DSS	DMH
	Wister		DMH
B	DA		DMH
	Lewis		DMH
	Wister		NaCl
C	DA		NaCl
	Lewis		NaCl

Tissue preparation

Rats were anesthetized with diethyl ether and perfused through the aorta with a physiological saline, followed by periodate-lysine-paraformaldehyde (PLP). The colon and rectum were removed and immersed in the same fixative for overnight at 4°C and then placed in 30% sucrose in 0.01 M phosphate buffered saline (PBS) for 6 hours at 4°C. The specimens were embedded in OCT compound. (Miles Inc., IN, USA), then frozen in –80°C until sectioned. Serial tissue sections (5 μm) sections were cut perpendicular to the mucosal surface by a cryostat, and stored at –80°C until use. All experiments were approved by the Ethical Committee for Animal Experiments of Hamamatsu University School of Medicine.

Antibodies

Two different monoclonal antibodies (MAbs), Mar1 (anti macrophage specific), and Mar3 (anti phagocytosis specific) were produced in Second Department of Anatomy University School of Medicine[9]. ED1 (anti macrophage and monocyte), and ED3 (anti macrophage subset) were purchased from Daiichi Pharma. Co. (Tokyo, Japan). A second antibody, alkaline phosphatase-conjugated anti-mouse IgG, and fluorescein isothiocyanate-conjugated rabbit anti mouse IgG antibody was purchased from Sigma Chemical Co. (MO. USA).

Immunohistochemical staining

5 μm sections made by a cryostat were stained by an indirect immunoalkaline phosphatase method and the Fast red technique[10]. To minimize non-specific reactions, the sections were preincubated with diluted normal serum from the same species as the source of the secondary antibody. The sections were incubated with the primary antibodies for 60 min at room temperature. After washing with 0.01 M cold PBS (pH 7.4), the slides were incubated with secondary antibodies for 45 min at room temperature. The slides were developed, following additional washes with PBS, using ABC alkaline phosphatase substrate kit 1 (Vector red) (Funakoshi Co., Tokyo, Japan). Finally, the slides were slightly counterstained with hemotoxylin solution, dehydrated, mounted and observed by light microscope. The specificity of histochemical staining was confirmed by omitting the first antibody and by using PBS.

Isolation of rectum macrophages

The macrophages infiltrated in rectal cancer were isolated according to a modification of the method of Bull & Bookman[11] and Gu[12]. The CRC tissue were washed three time in calcium-magnesium-free HBSS with 1mM EDTA for 10 min at 37°C under gentle shaking to remove intestine epithelial cells and was cut into smaller pieces. Specimens were washed with HBSS and incubated for 20–30 min in 2 ml RPMI 1640 with 1 mg/ml collagenase type 1 (=336 U/ml),

0.1 mg/ml DNAse and 1 mg/ml hyaluronidase without FCS at 37°C. Cells were washed twice with PBS and finally submitted to Ficoll density gradient centrifugation for 20 min at 2000 rev/min (~ 690g. without brake) for isolation of mononuclear cells. The interphase was carefully removed and washed with PBS. The cells were resuspended in medium, and used within 3 – 6 h.

Flow cytometric analysis

The macrophages (1 x 10^6) were treated with MAbs for 30 min at 4°C. After twice washes with PBS, the fluorescein isothiocyanate-conjugated rabbit anti mouse IgG antibody at 1:50 dilution was added and incubated for a further 30 min at 4°C After twice washes with PBS, the cells were analyzed on an EPICS Profile (Colt Co. Fla., USA).

Statistical analysis

Statistical analysis was performed using the two-tailed Student's t-test.

RESULTS

Carcinogenesis

In order to induce the experimental ulcerative colitis model, 7 weeks old rats were administrated by the water containing 3% DSS. During the period of drinking the DSS water, the order of colitis occurred is Wistar (day-3), DA (day-11) and Lewis (day-11) rats according to the bloody stools. After11days of being administrated 3% DSS, group A rats were drunk the water instead of DSS water. After 12 weeks of receiving DMH subcutaneously all group A rats had development of colorectal cancer. The localization of the tumors was from proximal colon to rectum. Distribution, size, and histopathology of the CRC in group A are shown in Table 2.

In group A the most different of carcinogenic morbidity in Wister rats was the 1.7-fold increase compared with DA rats ($p < 0.05$) and the 2.6-fold increase compared with Lewis rats in the rectum (the last 5 cm of the large intestine). Whereas 71% of large intestinal tumors were located in the rectum of the Wister rats, only 43% were found there in DA rats and 33% were found there in Lewis rats (n = 1). However, No significant difference in the CRC neither size nor tumor histology was observed in the three of strains (Table 2). It was difficult to say what difference between the tumors sizes localized in colon and rectum because the CRC rats were not enough to do the statistics. No any CRC was found in group B, and group C either by eyes or by microscopy.

Table 2. Frequency, size and histological types of colorectal cancer in group A

Strain	Localization Colon	Localization Rectum	Mean tumor size (mm) ± S.E.	Histology Polyplid neoplasia	Histology Adeno-carcinoma
Wister	2(28)	5(71)	3.9 ± 1.2	1	6
DA	4(57)	3(43)	3.3 ± 0.7	2	5
Lewis	2(67)	1(33)	4.2 ± 1.8	1	2

Immunohistochemical analysis of rectal cancer tissues with DHM-induced CRCs

As shown in Table 3, the ED1 and ED3 positive macrophages infiltrated in rectal cancer tissue of Wistar rats were more stronger than those in that of DA and Lewis rats,. Otherwise, Mar1 and Mar3 positive macrophages in tumor tissue of DA and Lewis rats were much higher than in that of Wistar rats (Table 3). The macrophages containing immuno-reactive ED1, ED3 Mar1 and Mar3 were distributed throughout the tumor tissue and densely distributed around

the tumor tissue (date not shown).

Table 3. Expression of macrophage phenotypes on colorectal cancer in group A

CRC rats	MAbs used			
	Mar 1	Mar 3	ED 1	ED 3
Wister	+	+	++	++
DA	++	++	+	+
Lewis	++	++	+	+

+, stained moderately; ++, stained strongly for the particular antigen listed

Phenotypic characterization of the macrophages infiltrated in rectal cancer tissue

After administrating DMH subcutaneously for 12 weeks, expression of the surface marker phenotypes of the CRC macrophages isolated from Wistar, DA , and Lewis rats was analyzed on an EPCS flow cytometer to quantify the degree of cell staining. Figure 1 showed the expression of Mar1, Mar3, ED1 and ED3 in Wistar, DA and Lewis rats. The pattern of surface marker expression on the isolated macrophages was the same as the results of Immunohistochemical analysis. The mean percent of ED1, and ED3 positive cells in Wistar rats was higher than that in DA and Lewis rats ($p < 0.05$). The mean percent of Mar1, and Mar3 positive cells in DA and Lewis rats was higher than that in Wistar rats ($p < 0.05$).

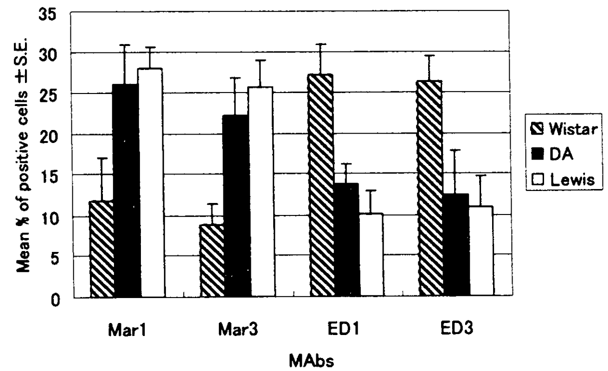

Fig 1. The cell surface phenotype of CRC macrophages isolated from Wistar, DA, and Lewis rats after administrating DMH subcutaneously for 12 weeks. MAbs binding was visualized with a FITC-conjugated secondary antibody, and analyzed by fluorescence flow cytometry.

DISCUSSION

The organ-specific approach to unlocking the etiology of CRC has been spurred on in the past several decades by the knowledge that several types of cancer occurring readily in humans have been reproduced in the laboratory using either specific carcinogenic agents or by combining carcinogenic agents with specific inbred strains of animal populations[13, 14]. These reproducible models provide the opportunity to study the CRC and to allow the investigator to manipulate certain variables such as diet, colon anatomy, microbiological factors and immunological parameters that may prove important in etiology of CRC. On drawback using this particular tumor model is the latency period of least 6 months needed to produce tumors when DMH administered. In this study, we first induced UC by DSS and then induce CRC by DMH. It took only 3 months to found the CRC in Wister rats. The important similarities of the model may be seen in the clinical and pathological findings in animals and patients with CRC developing from UC. It is especially suited to the study of UC and CRC. Clinical study of large patient populations and the introduction of specialized instrumentation such as colonoscopy have helped to answer questions regarding epidemiology and anatomical changes, but little has been gained working with human model relating to the underlying cause of this disease.

Our previous study[15, 16] revealed that after drinking the DSS water, the order of positive macrophages distributed is also Wistar (day-0), DA (day-7) and

Lewis (day-7) rats. Mar 1 and Mar 3 positive macrophages were more numerous than those in control, throughout the follicle as well as in the dome and interfollicular regions were. Degree of the distribution of ED1, ED3 positive macrophages in DA and Lewis rats (day-3) were much higher than in Wistar rats (day-11). The macrophages containing immunoreactive ED1 were densely distributed throughout the lamina propria and in the submucusa and ED3 positive macrophages were densely distributed in the lamina propria and around the lymphoid follicles in DA and Lewis rats.

In this study the mean percent of ED1, and ED3 positive macrophages in Wistar rats was higher than that in DA and Lewis rats ($p < 0.05$). The mean percent of Mar1, and Mar3 positive macrophages in DA and Lewis rats was higher than that in Wistar rats ($p < 0.05$). It was suggested that the existence of four types of macrophages play an important role in inducing of colitis and rectal cancer. However, the relationship Mar positive and ED positive macrophages remains unclear, and their respective relevance to a protective immune response, if any, within the UC and CRC are unknown. The high density of the macrophage types, of itself, dose not confirm the presence of an active, ongoing macrophage-mediated immune response to tumor antigens; this high density may simply reflect a tumor-mediated modulation of cellular influx and efflux mechanisms[17, 18]. The functional nature of this impressive tumor infiltration by macrophages must await the further development of specific markers of macrophage subsets and also in vitro analysis of the cytotoxic and antigen- presenting capabilities of isolated tumor- infiltrating macrophages[19, 20]. The expression of major histocompatibility complex (MHC) class II molecules on the macrophages and the relationship between the experiment colitis and colorectal cancer are studying in the strain different rats.

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