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Combination of aging and dimethylhydrazine treatment causes an increase in cancer–stem cell population of rat colonic crypts

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ABSTRACT

Aging is associated with increased incidence of colon cancers. It is also becoming evident that cancer stem cells (CSC) play a vital role in the pathogenesis and prognosis of colon cancer. Recently, we reported the presence of colon cancer stem-like cells in macroscopically normal mucosa in patients with adenomatous polyps and that they increase with aging, suggesting that aging may predispose the colon to carcinogenesis. In the current study we have examined the combined effects of aging and carcinogen exposure on the status of colon CSCs in an experimental model. We used young (4–6 months) and aged (22–24 months) rats and exposed them to the carcinogen, dimethylhydroxide (DMH). We investigated the expression of colon cancer stem cell markers, CD44, CD166, EpCam, and ALDH1 as well as EGFR expression in normal colonic crypt epithelium following carcinogen treatment. Our results demonstrate that aging per se or carcinogen treatment alone causes an increase in the number of colon cancer stems cells, as evidenced by increased immunoreactive-CSC-markers positive cells in the colonic mucosa. In aged rats, carcinogen exposure results in a more pronounced increase in colon cancer stem cells. Our study shows that in aging colon the effects of carcinogens are more pronounced, and an increase in colon CSCs is one of the earliest changes preceding tumor development. Moreover, the current investigation of the use of a panel of immunohistochemical markers of colon CSC can potentially serve as a prognostic marker during screening for colon cancer.

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Introduction

A growing body of evidence supports the contention that epithelial cancers including the colorectal cancer are diseases driven by a small set of self renewing cells, termed cancer stem cells (CSC) or cancer-initiating cells, that are distinct from the bulk of the cells in the tumor [1]. CSCs are widely believed to arise from the normal stem cells or progenitor cells upon mutation(s) [1–3]. Two recent transgenic animal studies have demonstrated that crypt stem cells are the cell of origin of intestinal cancers [4,5]. CSC possess not only the ability to self-renew but also differentiate and can give rise to heterogeneous tumors [1–5]. Currently, CSCs are identified by specific surface epitopes. Several stem cell markers have been proposed that highlight the stem cell or stem cell-like populations in several tissues including breast, colon and pancreas [6,7]. Most commonly used markers for detection of colonic stem cells are CD44, EpCAM, CD133, CD166, Lgr5, etc. Dalerba et al. demonstrated that only a subpopulation of CD44 high/EpCAM high cells were able to form tumors in xenografts [6]. More recently aldehyde dehydrogenase 1 (ALDH-1) has also been shown to be a stem cell marker for colon [8].

Aging is associated with an increased risk for colon cancer [9–11]. The occurrence of benign and malignant tumors increases with age in the colon [12]. It has also been demonstrated that during aging, colonic crypt proliferative activity increases and apoptosis decreases [13]. The mechanisms related to the increased incidence of colorectal cancer with advancing age have been a subject of intense investigations. Currently there is no definitive explanation for the changes observed in the colonic crypts, such as increased proliferation and decreased apoptosis, as well as increased expression of EGFR, preceding overt neoplasia. Since the stem cells are considered to be the origin of neoplastic transformation in solid tumors as well as intestinal neoplasias, we decided to investigate the status of stem cells in the normal appearing colonic crypts in aging. Recently, we reported the presence of colon cancer stem-like cells in macroscopically normal mucosa in patients with adenomatous polyps and that they increase with aging [14]. These changes are paralleled by increases in the expression of EGFR [11,15].

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In the current study we examined the combined effects of aging and carcinogen exposure on the stem cell population of colonic crypts of rats. We focused on the expression of stem cell markers in normal appearing colonic mucosa of rats exposed to the carcinogen dimethylhydrazine (DMH) in young and aged rats.

Materials and methods

Animals

Male Fischer-344 rats, aged 4–6 (young) or 22–24 month (old) were purchased from the National Institute on Aging (Bethesda, MD). All procedures were performed according to the standards for use of laboratory animals established by the Institute of Laboratory Animal Resources, National academy of Sciences, and were approved by the Animal Investigation Committee at Wayne State University School of Medicine. The details of animal handling have been previously published [11].

Carcinogen treatment

Groups ($n = 6$) of 4–6 and 22–24 month old male Fischer rats were injected i.p. once per week for 4 weeks with either 1,2-dimethylhydrazine (DMH), in 30 mg/kg dose, dissolved in 300 μl neutral buffered 10 mM $NaHCO_3$ or an equivalent volume of buffer only (controls). All animals were sacrificed by CO_2 asphyxiation 1 week after the last carcinogen injection. The abdominal cavity was opened and the colons were removed, rinsed with cold saline, opened and cut in half longitudinally. One half of the colon from each rat was fixed overnight in 10% buffered formalin. These tissues were processed, embedded in paraffin.

Immunohistochemistry. The antibodies utilized were as follows: CD44 (156-3c11, Cell Signaling Technologies, Beverly MA), CD166 (ALCAM, R&D systems MN), Ep-CAM(VU1D9 Cell Signaling Technologies, Beverly MA), EGFR (Cell Signaling Technologies, Beverly MA), and ALDH1 (BD Biosciences, San Jose CA).

Immunohistochemistry was performed according to our standard protocol [11,14]. Briefly, the paraffin blocks of the fixed colon tissues were cut into 5 μm sections. The slides were deparaffinized. For antigen retrieval, tissues were microwaved for 15 min in Citrate pH:6.0 buffer, then allowed to cool to room temperature. Endogenous peroxide was quenched by incubation of the sections with 3% hydrogen peroxide. Non specific binding was blocked by application of 5% horse serum. Primary antibodies were applied overnight at 4 °C and antibody detection was completed utilizing the secondary antibody detection kit from Vector. AEC was used as chromogen.

Results

For each tissue sample, the colonic crypts with longitudinal orientation and complete section from base to lumen, were analyzed. The expression of markers in the colonic crypts was quantitated by counting the stained cells in the crypt base under high power field of the microscope. Twenty colonic crypts were counted and an average number of stained cells per crypt was recorded. The same procedure was applied to all the markers tested. The differences in expression levels were tested by Student's *t* test.

CD44

CD44 staining was present in the lymphocytes residing in the lamina propria as well as in the colonic crypts. There was minimal expression of CD44 in the crypt of normal 5-month-old rats. But, its expression was increased in the crypt base of DMH treated animals. Aging by itself (data from the control rats) was associated with an increase in CD44 staining. However, the combination of DMH and aging was additive, since the highest number of cancer stem-like cells were detected in aged and DMH treated rats (see Table 1, Fig. 2).

EpCam

EpCam expression was very low in normal young rat colons. However, DMH exposure caused an increase in the EpCam expression in both young and old rats. Although the magnitude of this increase was higher in aged than in young rats following DMH treatment, this increase was not found to be additive as noted for CD44 expression (see Table 1 and Fig. 2).

CD166 (ALCAM)

CD166 expression was low in young control rats, but DMH treatment caused a modest increase in its expression. Aging alone caused an increase in expression of CD166. Again, as observed for CD44, the effect of DMH treatment was found to be additive (Table 1 and Figs. 1 and 2).

ALDH1

Staining of ALDH1 in normal crypts in young control animals was minimal. In aged and DMH treated animals there was a significant increase in ALDH1-immunoreactive cells, however, the increase was more pronounced in DMH treated aged animal (Table 1 and Figs. 1 and 2).

EGFR

Expression of EGFR was also increased in aging animals. The expression of EGFR was most pronounced in the aged rats, treated with DMH.

The differences in expression are summarized in Table 1 and demonstrated in Fig. 2.

Discussion

In this study we demonstrate that the number of cancer stem-like cells in colonic crypts of rats increase significantly due to aging and in response to carcinogen treatment. Moreover, these effects are additive. The increase in stem cell population is maximal in aged and carcinogen treated animals.

These results are very similar to our recent observation in normal appearing colonic mucosa from patients with adenomatous polyps, which are considered to be precursors of colorectal cancer [14]. In our previous study, we had demonstrated the increased colon cancer stem-like cell population in normal appearing colonic mucosa between groups younger than 55 years of age vs. older than 55. The expression of CD166 and EpCam were also higher in patients with more than 2 polyps on surveillance colonoscopy.

Table 1
Expression levels of cancer stem cell markers and EGFR in normal appearing colonic crypts.

	5 m Control	5 m DMH	22 m Control	22 m DMH
CD44	0 ± 0.1	2 ± 0.2*	8 ± 0.3*	18 ± 0.6**
ALDH1	1 ± 0.1	3 ± 0.2*	3 ± 0.2*	9 ± 0.5**
CD166	0 ± 0.1	1 ± 0.1*	3 ± 0.2*	8 ± 0.4**
Ep-CAM	2 ± 0.2	10 ± 0.5*	13 ± 0.5*	14 ± 0.6*
EGFR	1 ± 0.1	2 ± 0.2*	2 ± 0.3*	12 ± 0.5**

* $p < 0.05$ compared to 5 month control.
** $p < 0.05$ compared to 22 month control.