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Combination Of Aging And Dimethylhydrazine Treatment Causes An Increase In The 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 stem 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.

Keywords

CD166; ALDH1; Aging; Cancer stem cells; Colon Cancer; Dimethylhydrazine

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

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(1). CSCs are widely believed to arise from the normal stem cells or progenitor cells upon mutation(s) (1,2,3). 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).

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 NaHCO₃ or an equivalent volume of buffer only (controls). All animals were sacrificed CO₂ 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 mm sections. The slides were deparaffinized. For antigen retrieval, tissues were microwaved for 15 minutes 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 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 Students 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, Figure 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 Figure 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 Figures 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 Figures 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 Figure 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. However, the changes in expression of colon cancer stem-like cell markers were more striking when stratified by age (14).

In the current study we not only demonstrate the effect of aging on the expression of colon cancer stem-like cell markers, but we also show that in response to carcinogen exposure, one of the earliest changes that occur in the normal appearing colonic crypts is the increase in cancer stem-like cell population. The model we have utilized in this study is representative of the course of events in normal aging population where there is a time dependent accumulation of mutagenic agents and an increased vulnerability due to aging associated changes. The finding of an early increase in the number of cancer stem-like cells in colonic crypts is very significant for a better understanding of the pathobiology of colorectal cancer, and supports the crypt overpopulation by cancer stem cells hypothesis proposed by Boman et al (3,8). Boman et. al. first demonstrated in a mathematical modeling study that the best explanation for the occurrence of aberrant crypt foci and crypt fission which precedes carcinogenesis is an initial increase in the cancer stem cell population (3). Recently they have also shown that in normal appearing FAP crypts the earliest abnormality is the expansion of the cancer stem cell fraction in the crypt with a concomitant shift of cancer stem cells to crypt lumina (8).

We have also demonstrated in our animal model that the expression of EGFR is significantly increased only when aging is also combined with carcinogen exposure. Changes in EGFR expression probably contributes to the increased proliferative activity in colonic crypts associated with aging and carcinogen exposure.

Since the increase of cancer stem cell population is an early event preceding overt neoplastic changes such as adenoma formation and eventually leading to high grade dysplasia and cancer, we propose that cancer stem cell markers can be utilized as prognostic indicators of colonic neoplasia in normal population. These markers can be tested on biopsies obtained from normal appearing mucosa during screening endoscopy procedures, or may even be tested on fecal material as a molecular screening test.

Before the demonstration of CD166 and CD44 as colon cancer stem cell markers, these markers were known as cell adhesion molecules and several studies exist which demonstrate their value as prognostic markers in several cancers including colonic adenocarcinoma. Weichert et al (16) have demonstrated that CD166 is overexpressed in colorectal carcinoma and membranous expression is an independent prognostic variable in invasive colonic adenocarcinomas in a multivariate model. In their study, they also demonstrated that in normal colonic mucosa, CD166 expression is limited to few cells in the crypt base (16). However, in adenomas and carcinomas, the staining pattern is more widespread. Another interesting finding they demonstrated was the pronounced expression of CD166 in the invasive fronts of cancers which has been associated with stem cell like properties (16).

The markers utilized in this study as cancer stem cell markers have been tested in previous studies by selectively xenografting the tumor population with or without expression of the markers. CD44 and ALCAM (CD166) have consistently been associated with tumor growth in xenografts. However, it can be argued that these markers are not very specific for cancer stem cells per se, but they highlight a population that has stemness to a certain extent. The recent study by Huang et al. claims that Aldehyde Dehydrogenase 1 is a more accurate marker for stem cells, while CD44 and CD133 highlight a wider population including stem cells (8). In our study the results of ALDH1 expression were in concordance with the other markers and demonstrated highest level of expression in the aged, DMH treated rats. We believe that even though the markers we have used in the study, are not exclusively highlighting the stem cells per se, they are still very useful in demonstrating the increase in stem cell population and they do have potential as prognostic markers for screening colorectal cancer. Our results do not favor one marker in expense of others and we believe that using a panel of markers is a safer approach in analyzing cancer stem cell populations, until a definitive marker is found.

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Fig 1. Photomicrograph showing immunoreactive CD166 (ALCAM) or Aldehyde Dehydrogenase 1 (ALDH1) cells in the colonic mucosa of young and aged rats treated with DMH or vehicle (control). 600 $\times$ magnification. The arrows highlight the staining cells.

Fig 2.
Relative levels of CSC markers (CD44, EpCAM, CD166(ALCAM), ALDH1) and EGFR as determined by immunohistochemistry in colonic mucosa of young and aged rats treated with DMH or vehicle (control).

Table 1

Expression levels of 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