Treatment of Ethylnitrosourea Induced Lymphocyte Hyperproliferation by DNA Hypomethylation in the Rat Colon

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Summary N-ethyl-N-nitrosourea (ENU) is a potential carcinogenic agent commonly used in industry, and it may cause an uncontrollable cell proliferation and eventually tumourgenesis. On the other hand, the hypomethylation of DNA by 5-aza-2'-deoxycytidine is the best known anti-tumoural mechanism used for the treatment of leukemia. Therefore the present study aimed to find out the possible healing effects of 5-aza-2'-deoxycytidine on lymphocyte hyperproliferation in the rat colon through the above mentioned DNA hypomethylation mechanism. Rats were injected with 300 mg/kg body weight ENU (i.p.) in order to induce tumour development. Following 45 weeks when the tumourgenesis was proved visually, animals were treated with 5-aza-2'-deoxycytidine 100 µg/100 g body weight twice a week intraperitoneally for 15 weeks. After the experimental procedure, all animals were sacrificed and colonal tissues were obtained. Tissues were processed for light and electron microscopy. While no colonal tumour development was observed in the control group, an extensive tumour development was seen in the subcutaneous region in the high dose ENU treated group. The light and electron microscopical examination of the rat colonal tissue revealed a lymphocyte hyperproliferation and invasion in the submucosal region, an increased number of polymorphonuclear leukocytes (PMNLs) and occasional epithelial lesions. On the other hand, the evaluation of the 5-aza-2'-deoxycytidine treatment group rat colon demonstrated features similar to those seen in the control and PEG treated groups indicating a possible anti-neoplastic effect of 5-aza-2'-deoxycytidine via DNA hypomethylation.

Key words 5-aza-2'-deoxycytidine, Antineoplastic effect, Colon, Rat.

It has been shown that ENU induces tumour development in various organs of mammalian species (Henderson *et al.* 1998, Sasaki *et al.* 1997, Özdemir *et al.* 2001). This monofunctional alkylating agent is a potent inducer of cellular stress leading to chromosomal aberrations such as point mutations, translocations, deletions, insertions and cell killing (Nikolova *et al.* 1996, Wilhelm *et al.* 1997). This agent is also known as a potent cell mutagen due to its alkylating function and induced DNA damage in the cell (Op-het-Veld *et al.* 1997, Suzuki *et al.* 1997, Tong *et al.* 1997).

5-aza-2'-deoxycytidine (5-azadCR, DAC, Decitabine) and its ribose congener 5-azacytidine are pyrimidine analogs and specific inhibitors of DNA methyltransferase enzyme. Both drugs are able to reduce the biochemical activity of DNA methyltransferase enzyme in cells. A great deal of preclinical studies have shown that 5-azadCR is able to induce maturation of human leukemic cells and inhibit clonogenic potential of cells *in vitro* in the absence of acute cell killing with a mechanism involving DNA hypomethylation (Mandelli 1993). These cytidine analogs, modified in position 5, were originally developed as antitumour drugs, and have been used in the treatment of both childhood and adult leukemias (Taylor 1993).

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The DNA methyl inhibitor, 5-azadCR, also causes chromosome decondensation in the human lymphocyte cell culture (Özdemir *et al.* 1998). 5-azadCR is the most potent specific inhibitor of DNA methylation (Jones and Taylor 1980). Agent was demonstrated to be an active antileukemic drug (Momparker *et al.* 1985, Rivard *et al.* 1981). Pinto *et al.* (1990) presented evidence for *in vivo* induction of leukemic cell differentiation by cytidine analogs.

The present study aimed to investigate the effects of 5-azadCR treatment on hyperproliferated lymphocyte cells of rat colon that induced by a potent carcinogen and an alkylating agent, ENU.

Materials and methods

Animals

Animals used in the present study were non-transgenic and were bred and fed in optimal laboratory conditions. Forty male 7–8-week old Wistar albino rats (*Rattus norvegicus*), obtained from “The Experimental Animal Laboratory of Cumhuriyet University, Sivas-Turkey”, were used in the present study. Three group rats were used in the present study, control, ENU treatment and 5-aza-2'-deoxycytidine treatment groups.

Experimental Design

N'-ethyl-N'-nitrosourea (ENU) (CASRN: 759-73-9, Sigma Chemical Company, MO, U.S.A.) and polyethylene glycol (PEG) were obtained from Sigma Chemical Company, U.S.A. ENU is dissolved in PEG and stored at -70°C . While the experimental group rats (15 male and 15 female in a total of 30) were injected once a week with 300 mg/kg body weight with ENU (i.p.), and the control group animals received no agent administration. Following 45 weeks of experimental period, 10 rats per treatment group were sacrificed and colonic tissues were obtained. The rest of the animals (20 rats) from ENU treated group were used for 5-azadCR administration.

5-Aza-2'-deoxycytidine (5-azadCR) administration

5-azadCR was purchased from Sigma Chemical Co. Stock solutions of drug were prepared in phosphate-buffered saline (PBS) at a concentration of 25 mg/2 ml (55 mM), aliquoted and stored at -40°C . Each aliquot was used once only, thawed immediately before use, serially diluted with distilled water, and treatment group rats were injected intraperitoneally in 100 $\mu\text{g}/100\text{ g}$ body weight or 0.5 μM of final concentration for 2 times a week (approximately 72 h). Following 15 weeks of drug administration, all animals were killed and colonic tissues were obtained.

Microscopy

For light microscopy, colonic tissue samples were fixed in 3% glutaraldehyde, dehydrated through the increasing concentrations of ethanol and embedded in JB4 glycol metacrylate. 2–2.5 μm thick sections were stained with toluidine blue acid fuchsin. They were evaluated under a Jenamed 2 (Carl Zeiss Jena, Germany) light microscope, and appropriate field of views were photographed.

Tissue samples for electron microscopy were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2–4 h. Tissues were rinsed with buffer, post-fixed in 1% aqueous osmium tetroxide for 2 h, dehydrated in ethanol, and embedded in Epon resin. Semithin tissue sections were stained with toluidine blue and evaluated at the light microscopical level whereas the ultra-thin sections were double stained with uranyl acetate-lead citrate, and observations were done at the ultra-structural level using a Zeiss (Germany) electron microscope.

Results

All ENU treated rats exhibited large subcutaneous tumours (approximately 5–9 cm in size) at

Figs. 1–4. 1) Control group rat colon epithelial and stromal features are in their regular appearances. Epithelium (ep), stroma (str). $\times 100$, acid fuchsin-toluidine blue. 2) The ENU treated group rat colon has lymphocyte accumulation (arrows) in the lymphatic vessels of the lamina propria. $\times 100$, acid fuchsin-toluidine blue. 3) The submucosal region of the rat colon contains dense lymphocytic cell invasion (arrows) due to the result of hyperproliferation following ENU administration. $\times 100$, acid fuchsin-toluidine blue. 4) Shows the increased number of lymphocytes (arrow) in the lamina propria of ENU treated rat colon at the ultrastructural level. $\times 7000$, uranyl acetate-lead citrate.

different body regions when evaluated visually. These tumours were also investigated histopathologically and showed distinct characteristics of subcutaneous gliosarcoma (data not shown). Control group rat colonic tissues demonstrated mucosal crypts that contained simple columnar epithelium and underlying lamina propria in their regular appearances (Fig. 1).

The ENU treated group rat colonic features revealed distinct histopathological changes such as mucosal and submucosal hyperproliferation of lymphocyte cells and lymphocytic invasion into the mucosal layer from the underlying submucosa (Figs. 2, 3). These histopathological alterations must be the result of ENU treatment since the PEG only administration caused no such changes (data not shown). Electron microscopical examination of the ENU treated group submucosal region of the rat colon supported the hyperproliferation of lymphocytes (Fig. 4). After the 5-azadCR administration of the ENU treated rats, it was seen that the drug reduced the number of lymphocytes in colonic tissues (Fig. 5a, b). The electron microscopical examination of 5-azadCR treated rat colon revealed ultrastructural features similar to the control group (Fig. 6). However, an increased collagen fiber content was present in the 5-azadCR treated group stromal tissue possibly due to DNA hypomethylation and distinct gene reactivation (Fig. 7).

Discussion

A consistent amount of data has been accumulated over the last 15 years about the clinical activity and biological properties of 5-azadCR in leukemic diseases. The results of *in vitro* and *in vivo* studies showed that the drug is a powerful antileukemic agent and displays healing activity in acute

Figs. 5–7. 5) The 5-azadCR treated group rat colonic features are similar to those seen in the control group. Epithelium (ep), submucosa (sm). a) $\times 50$, b) $\times 100$, acid fuschin-toluidine blue. 6) The electron microscopical examination of the 5-azadCR treated rat submucosal region has fibroblastic-like cells which is in consistence with the light microscopical evaluation of the same group that there are mostly regular connective tissue cells. $\times 4400$, uranyl acetate-lead citrate. 7) Demonstrates the increased amount of callogen fibers synthesis (arrows) in the 5-azadCR treated group rat colonic submucosa. $\times 3000$, uranyl acetate-lead citrate.

myelocytic leukemia (AML) and acute lymphoblastic leukemia (ALL) patients. As a reversible epigenetic modification which can affect gene expression and DNA hypomethylation has been attractive candidate for the biochemical mechanism of genomic imprinting (Tycko 1997). Transcriptional blocks in p16INK4A and p15INK4B genes in gastric carcinomas were reversed by 5-azadCR treatment (Lee *et al.* 1997). Frequent aberrant methylation patterns of p16INK4A gene was reported in primary rat lung tumours (Swafford *et al.* 1997).

Differential repair of structurally distinct mutagenic lesions in particular genes may influence the cellular risk of malignant conversion. Complete carcinogens must possess both initiating and promoting properties in tumour development (Brustle *et al.* 1993). While most N-nitroso compounds are potential mutagens and considered to be tumour initiating agents, some are not mutagenic and yet are complete carcinogens. The present study investigated the ethylnitrosourea-induced rat colonic structural changes at the light and electron microscopical levels and these structural changes were treated with 5-azadCR which causes gene reactivation/DNA hypomethylation possibly in the tumour suppressor p53 gene or in the related genes. The DNA methyl inhibitor, 5-azadCR, took place significant role in the treatment of hyperproliferative lymphocytes of rat colon in the present study. There were neither tumours nor colonic tissue alterations in the untreated group whereas distinct colonic lymphocyte hyperproliferation, invasion and lymphoepithelial lesions were evident in the ENU treated rats. Sequential intra-peritoneal injections of ENU (300 mg/kg) strongly induced subcutaneous sarcomas (data not shown) and colonic tissue changes 45 weeks after treatment. In colon, however, ENU at its highest dose caused adverse alterations in rat colonic tissue. Favor suggested the threshold model for explaining the ENU mutagenicity in germ

cells claiming that the doses below the threshold dose result in induced DNA adducts that are repaired (Favor 1998). ENU may possibly cause some changes in A/T to G/C or G/C to A/T sequences (Favor 1998, Cerutti *et al.* 1994) indicating that the high dose of ENU was effective in inducing mutations in colonic tissue changes. Similarly the present study used a high dose of ENU and found reversible alterations in the colon. Loss of the wild type allele results in a mutator phenotype, accelerating tumorigenesis which specifically occurs in the gastrointestinal and genitourinary tracts (de-Wind *et al.* 1998, Shoemaker *et al.* 1997). In addition, the long term ENU exposure to rats may cause different type of tumours at different organs, one of which was a very large subcutaneous sarcoma that observed visually in rats 45 weeks after ENU treatment in the present work. There is wide variation of AGTC transversion/transitional mutation levels between the organ and cell types, which appears to correlate with cell and tissue type sensitivity to the mutagenic and carcinogenic effects of alkylating agents. Findings of the present study supported the idea that the lymphocyte hyperproliferation caused by ENU may possibly lead to the development of lymphoma in the rat colon. There is however, a possibility that the increased number of lymphocytes could be activated and proliferated by inflammation of the subcutaneous tumours induced by ENU. It could be postulated that ENU presumably initiates the triggering signal for colonic carcinogenesis by alkylating the bases of A/T-G/C, A/T-C/G, A/T-T/A, G/C-C/G and G/C-T/A base substitutions as suggested by Shibuya (Pourand and Cerutti 1993, Shibuya and Morimoto 1993). The injection of 5-azadCR reversed the rat colonic tissue changes caused by ENU due to DNA hypomethylation. Therefore the 5-azadCR administration produced an antineoplastic effect on the colonic lymphocyte hyperproliferation. In addition, 5-azadCR treatment caused increased protein synthesis. In conclusion, it could be postulated that 5-azadCR has the potential of tumour suppressor gene activation which have been spontaneously silenced by DNA hypermethylation in the hyperproliferated lymphocytes.

It could be suggested that a distinct subcutaneous tumour development and a lymphocyte hyperproliferation which might be the triggering signal for carcinogenesis were observed in rat colon due to the alkylating characteristics of ENU. On the other hand, 5-azadCR treatment reversed those colonic alterations through the DNA hypomethylation mechanism. Although the molecular basis of this mechanism has not been completely explained, the altered methylation patterns and reactivation of some distinct genes could be an important step in tumour therapy.

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