Pharmacological Reports 66 (2014) 114–120

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

Antitumoral effect of a selective Rho-kinase inhibitor Y-27632 against Ehrlich ascites carcinoma in mice

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ARTICLE INFO

Article history:
Received 5 December 2012
Received in revised form 21 May 2013
Accepted 13 June 2013
Available online 1 February 2014

Keywords:
Rho-kinase
Y-27632
Ehrlich ascites carcinoma
Experimental tumor

ABSTRACT

Background: The Rho proteins and Rho-kinase (ROCK) enzymes are responsible for signal transduction, and cause cell permeability, contractility, differentiation, migration, proliferation or apoptosis depending on cell types. All of these functions are vital for cancer initiation and progression. In this study, the preventive and protective effects of a selective ROCK inhibitor Y-27632 against Ehrlich ascites carcinoma in *Swiss* albino mice were investigated.
Methods: Adult male albino mice were divided into five equal groups, and Y-27632 (0.1, 1, and 10 mg/kg) was given to groups as two steps; before (pre-carcinoma) and after inoculation of carcinoma cell suspensions (post-carcinoma). At the end of the experiments (at day 15), cardiac blood samples, the ascitic fluid, and intestinal specimens were collected for histopathology and biochemical investigation.
Results: Significant decreases in the body weight and immunostaining scores in small and large intestine for ROCK2, preservation of serum glutathione (GSH) levels, and an increase in tumor level of nitric oxide were recorded in groups pretreated with Y-27632. However, treatment with Y-27632 after tumor inoculation did not affect body weight and ROCK2 immunostaining scores, increased serum MDA levels, and decreased GSH levels.
Conclusions: This is the first study on the effectiveness of Y-27632 in this experimental tumor model. Our findings provided direct evidence for ROCK involvement in tumor development. These data suggest that pretreatment with Y-27632 has a protective effect against tumor formation.

Introduction

The Rho/Rho-kinase pathway plays an essential role in reorganization of the actin cytoskeleton, leading to the biological processes such as cell movement, migration, chemotaxis, cell adhesion, cell cycle, gene expression, tumor progression and apoptosis [18,28,30]. Rho-kinase (ROCK) is a serine–threonine kinase and has two isoforms: ROCK1 and ROCK2. Currently available pharmacological inhibitors cannot discriminate between ROCK isoforms [18].

Several lines of evidence indicate that the Rho/ROCK pathway may be important for cancer invasion, growth and metastasis. Increased expression of ROCK has been shown to contribute to the metastatic tumors [26]. Activation of ROCK increases myosin light chain phosphorylation, and induces myosin contraction and subsequently the assembly of actin stress fibers and focal adhesions [2]. Activation of Rho/ROCK signaling pathway is also known to stimulate the assembly of actin stress fibers and enhance the motility and invasion of cancer cells [14,20]. Y-27632 is a highly potent, cell-permeable, and a well known specific synthetic inhibitor of ROCK. Y-27632 binds to the Rho-kinase ATP binding pocket in a ATP competitive manner [8]. Y-27632 also inhibits cellular migration and morphological change of rat ascites hepatoma cells [13]. Because of these functions, inhibition of ROCK is promising target for the development of novel anticancer drugs. Numerous investigations utilizing *in vitro* models have reported that ROCK inhibition blocks cell invasion, proliferation, cell migration and chemotaxis, angiogenesis, metastasis [28,30,35], but only a few studies have evaluated the effectiveness of ROCK inhibition in *in vivo* models [26,37]. However, the effect of ROCK inhibition in experimental tumors is not entirely known. In this study, we showed that the efficacy of Y-27632 against Ehrlich ascites carcinoma (EAC) *in vivo*. This was the first study investigating the effects of ROCK inhibition by Y-27632 against to a transplantable spontaneous experimental tumor EAC.

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1734-1140/$ – see front matter
http://dx.doi.org/10.1016/j.pharep.2013.06.006

Materials and methods

Animals

Adult Swiss male albino mice (26–33 g) were used in the study. Mice were housed in polycarbonate cages in a controlled environment (temperature 25 ± 2 °C and 12 h dark and light cycle) with standard diet and water *ad libitum*. The animal experiments were carried out in accordance with the Institutional Protocols of Animal Care. The study was conducted after obtaining institutional Animal Ethics Committee approval (ethical number: 06/2009-4).

EAC modeling

Ascites carcinoma cells were counted in a cell counter (Cedex, Roche) using the trypan blue dye exclusion method. Cell viability was >95%. Carcinoma cell suspensions were prepared in phosphate balanced salt solution (PBS) at pH 7.4 to final concentrations of 1×10^6 viable cells/ml. Mice were inoculated intraperitoneally (*i.p.*) with 1×10^6 viable carcinoma cells/mouse in a volume of 0.2 ml as previously described [23].

Antitumoral activity

Animals were divided into 5 groups (n = 10 for each group) for both of the pre- and post-carcinoma experiments. All experimental groups were injected with EAC cells except healthy control group. This was taken as day zero for post-carcinoma groups. Two days after inoculation, animals started receiving daily *i.p.* administration of different concentration of Y-27632 (Tocris Cookson Ltd., Bristol, UK, dissolved in 0.9% NaCl). In pre-carcinoma groups, Y-27632 was given *i.p.* daily starting from two days before EAC cells inoculation. EAC cells inoculation was taken as day zero for pre-carcinoma groups.

Control groups were treated with the same volume of 0.9% NaCl solution. All the treatments were given for 14 days.

Experimental groups:

Group 1 – Normal control.
Group 2 – Tumor control, EAC cells (1×10^6 cells/mouse).
Group 3 – EAC cells (1×10^6 cells/mouse) plus 0.1 mg/kg/day Y-27632.
Group 4 – EAC cells (1×10^6 cells/mouse) plus 1 mg/kg/day Y-27632.
Group 5 – EAC cells (1×10^6 cells/mouse) plus 10 mg/kg/day Y-27632.

These groups were designed separately for both pre- and post-carcinoma treatment. After 14 days of treatment, all the animals were fasted for 12 h, but still allowed to access to water. Then, the animals from each group were euthanized on 15th day by ether anesthesia. Cardiac blood samples were collected for biochemical parameters. The ascitic fluid from the peritoneal cavity of carcinoma bearing mice was quantitatively isolated by peritoneal lavage after death. The total number of EAC cells was counted by the trypan blue exclusion method [23] using by the Cedex counter [15]. The antitumoral effect of Y-27632 was assessed by observing the changes with respect to body weight, viable and nonviable cell count, biochemical parameters, tissue pathology and immunohistochemistry.

Biochemical parameters

All blood samples were drawn into a tube (BD Vacutainer, Canada, USA) containing ethylenediamine tetra-acetic acid (EDTA) to prevent coagulation. The blood was centrifuged and the upper layer of plasma was transferred into a conical tube and stored at −80 °C. Plasma malondialdehyde (MDA) [38] and reduced glutathione (GSH) levels [5] were measured by spectrophotometrically. MDA level was measured by using the trichloroacetic acid (TCA) method, which is based on the fact that MDA reacts with TCA to form a compound with a maximum absorbance at 535 nm [30]. GSH level in plasma was measured by using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), which reacts with GSH to form a product with a maximum absorbance at 412 nm [5]. Plasma and tissue NO levels were measured by a NO/ozone chemiluminescence technique published previously [1,11].

Histopathological procedure

Small and large intestine tissues were excised and fixed in 10% tamponized formaldehyde solution. The preserved tissues were dehydrated, cleared, and processed for routine paraffin-block preparation. Sections of about 4 μm thickness were cut, stained with hematoxylin–eosin [23]. The slides were examined for histopathological changes by light microscopy.

Immunohistochemistry

Five-micrometer sections from paraffin embedded tissues were cut from paraffin-embedded tissue blocks onto silane-coated slides. Sections were dewaxed (xylene 2 min × 3 min) and rehydrated by passing through graded alcohols and rinsed in water. Immunohistochemical staining of 1/40 diluted monoclonal anti-ROCK2 primary mouse antibody (Tocris Bioscience, USA) was performed following by boiling procedure for 60 min in Ventana Bencmark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The sections were assessed in light microscopy. Immunoreactivity score was used to evaluate the intensity of immunohistochemistry staining. ROCK2 expression was evaluated in entire slides in the area of the tumor due to cytoplasmic staining pattern, as follows: 0 – no reactivity (staining less than 1%); +1 – weak (staining in up to 10% of positive cells); +2 – moderate cytoplasmic staining (in 11–50% of the cells); +3 – marked cytoplasmic staining (in 51–80% of positive cells); +4 – high expression (more than 80% of positive cells). Staining severity was scored as follows; 0 – no staining, 1 – weak, 2 – moderate and 3 – strong. And then the formulas "(1 + staining severity/3) × staining rate" was calculated to evaluate ROCK2 positive cells (in objective magnification × 100) [9].

Statistical analysis

All data are expressed as mean ± SD or the percentage incidence. Unpaired Student t test was used for comparisons of the differences between mean values of two groups. Statistical comparison of more than two groups was performed by analysis of variance followed by Student–Newman–Keuls multiple comparisons test. The Kruskal–Wallis test plus Dunn's test were used to detect significant differences between immunostaining scores. All statistical analyses were carried out with SPSS 13.0 software program. A p value <0.05 was considered indicative of statistical significance.

Results

Viability of EAC cells

EAC cells stained with trypan blue and counted in Cedex cell counter (Roche, Mannheim Germany). The viable EAC cells in both pre- and post-carcinoma groups were decreased when compared to the pre-inoculation stage (Group 1) (Fig. 1). Mortality rate was 90% in Group 5 of post-carcinoma group, so the result of this group

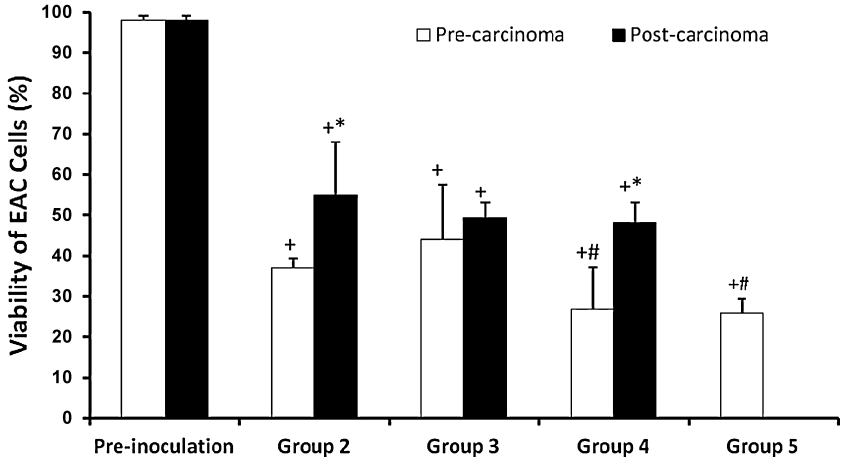

Fig. 1. Viability of Ehrlich ascites carcinoma (EAC) cells in experimental groups. $^{+}p < 0.05$ when compared to pre-inoculation to inoculation, $^{*}p < 0.05$ when compared to pre-carcinoma group, $^{\#}p < 0.05$ when compared to Group 2.

was not included. In all the other groups, mortality was not seen during the experimental period. There were markedly high viable cell numbers in Groups 2 and 4 in post-carcinoma groups when compared to the pre-carcinoma groups (Fig. 1).

Mean body weight changes

Body weight was markedly increased with the EAC tumor in both pre-carcinoma and post-carcinoma groups. In pre-carcinoma groups, there were reductions in increased body weight at all doses of Y-27632 (Groups 3–5) as compared to EAC tumor-bearing mice (Group 2) (Fig. 2). These decreases were significant in Groups 3 and 5 when compared to Group 2. Administration of Y-27632 did not markedly affect the body weight in post-carcinoma groups ($p > 0.05$). The result of Group 5 in post-carcinoma group was not included due to high mortality rate (90%).

Oxidant status

In pre-carcinoma groups, plasma MDA levels were not markedly changed among the groups (Fig. 3). However, there was a significant elevation in MDA level in Group 2 in post-carcinoma group. This elevation was further augmented with Y-27632 treatment in Groups 3 and 4 (Fig. 3). Marked increases in plasma GSH levels were noted in Group 2 when compared to control (Group 1) in both pre-carcinoma and post-carcinoma groups (Fig. 4). In pre-carcinoma groups, ROCK inhibition did not markedly modify the GSH levels. However, GSH levels were significantly decreased in Groups 3 and 4 with Y-27632 treatment in post-carcinoma groups (Fig. 4). No marked differences were observed in plasma NO concentrations in both pre- and post-carcinoma groups (Fig. 5A). NO levels in tumor ascitic fluid were also similar to the control values except for Group 5 of pre-carcinoma animals, where a marked increase was recorded (Fig. 5B).

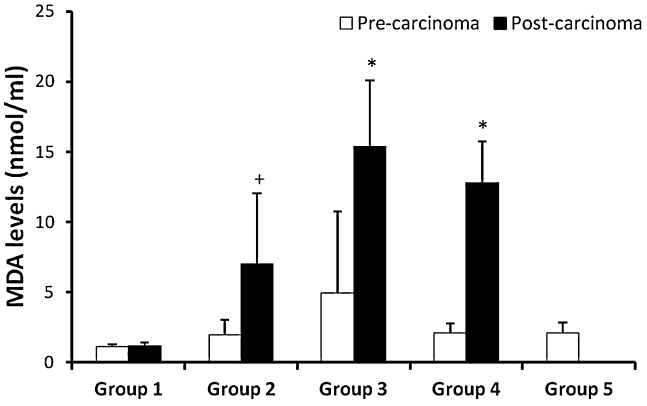

Fig. 3. Plasma MDA levels in experimental groups. $^{+}p < 0.05$ when compared to Group 1, $^{*}p < 0.05$ when compared to Group 2.

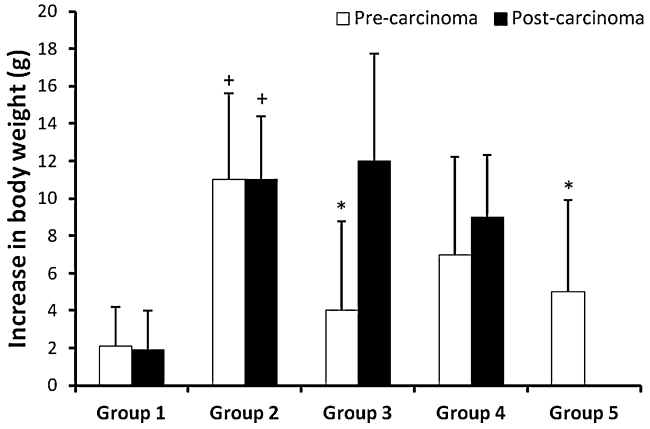

Fig. 2. The body weight changes of mice in experimental groups. $^{+}p < 0.05$ when compared to Group 1, $^{*}p < 0.05$ when compared to Group 2.

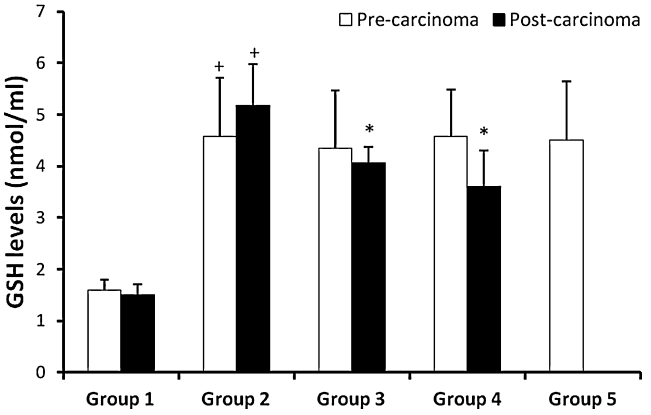

Fig. 4. Plasma GSH levels in experimental groups. $^{+}p < 0.05$ when compared to Group 1, $^{*}p < 0.05$ when compared to Group 2.

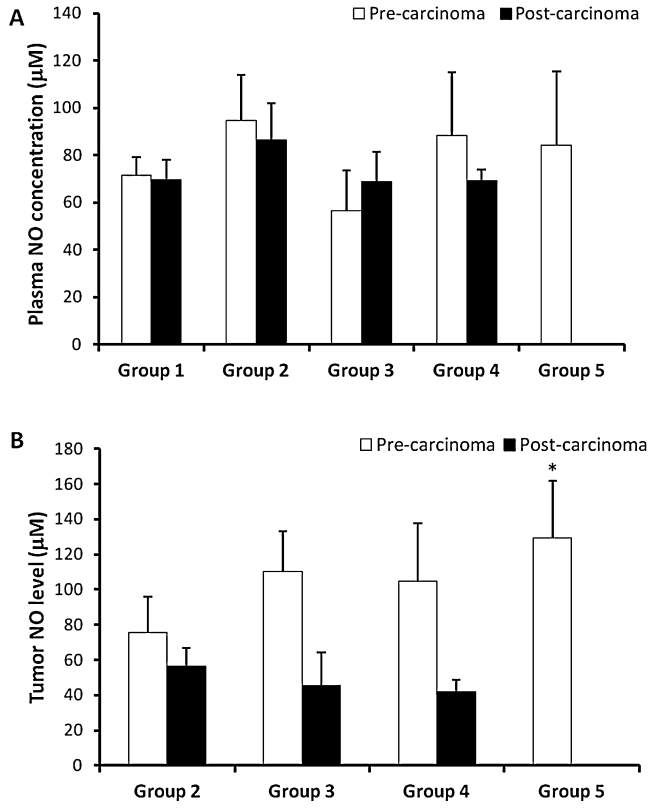

Fig. 5. Nitric oxide (NO) levels in plasma (A) and tumor ascitic fluid (B) in experimental groups. $^{*}p < 0.05$ when compared to Group 2.

Histopathology

Small and large intestine of healthy control groups showed normal intact histological structure. In pre-carcinoma inoculated groups, both of small and large intestine revealed edema inflammatory and pigmented cells infiltration in only serosal layer, but inflammatory cells infiltration with mild edema in focal manner at the muscularis propria layers from 40% of small intestine and 100% of large intestine. Y-27632 pretreatment has diminished 60% of pathological changes with a great degree on tumor spreading and progression in Group 3, 65–70% in Group 4 and 50% in Group 5 (Fig. 6). In post-carcinoma inoculated groups, edema inflammatory cells infiltrated to serosal layer of small and large intestine in all groups. Although 60% small intestine and full of large intestine were invaded, the treatment with Y-27632 had no marked effects on tumor spreading and progression (Fig. 7).

Immunostaining for ROCK2

ROCK2 positive cells were seen gradually in small intestine and also in large intestine from pre- and post-carcinoma groups. ROCK2 stainings were markedly increased in Group 2 in both pre-carcinoma and post-carcinoma groups. The immunostaining scores in both small and large intestine were significantly decreased in pre-carcinoma, but not in post-carcinoma groups ($p < 0.05$) (Fig. 8).

Discussion

Rho/ROCK pathway was reported that it may be important for cancer invasion, growth and metastasis [11,12,17,21]. In this regard, inhibition of ROCK is promising target for the development of novel anticancer drugs. There were limited investigations related with ROCK inhibition by well-known and selective inhibitors (Y-27632 and fasudil) in cancer therapy [21,23,25,29]. In addition, the effect of ROCK inhibition in experimental tumors is not entirely known. This is the first study showing that pretreatment with a ROCK inhibitor suppresses tumor formation in EAC-bearing mice. Our findings indicate that ROCK is involved in this experimental tumor development. The EAC implantation induces a local inflammatory reaction, with increasing vascular permeability (due to rise in the VEGF), which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation [10]. It is known that inhibition of ROCK reduces VEGF-induced endothelial migration and angiogenesis *in vitro* [34]. Ascitic fluid has severally been shown to be the direct source of nutrition for tumor cells. Since ascitic fluid has an inhibitor factor which suppresses cell proliferation [6], EAC cells decrease with increasing concentration of ascitic fluid [17]. These results of this study showed that pre-carcinoma administration of Y-27632 reduced the ascitic burden as depicted by the body weights of mice.

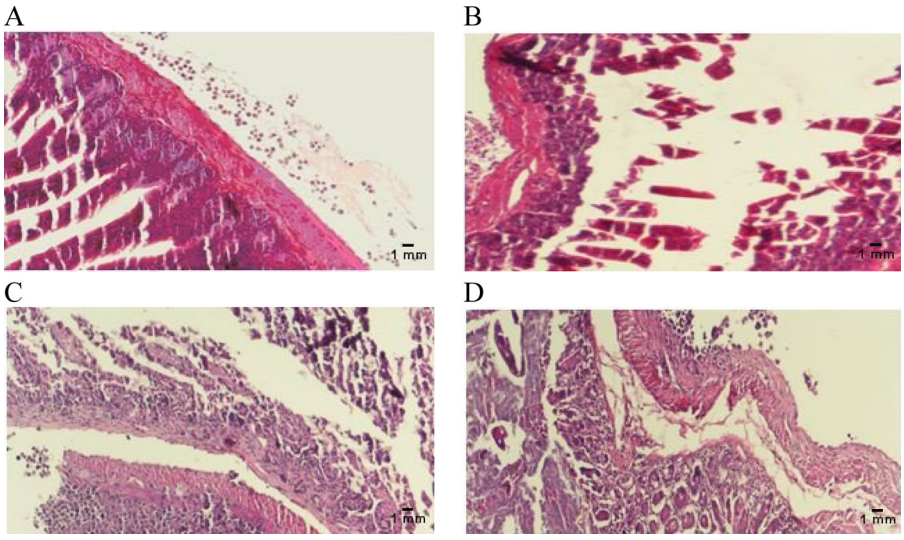

Fig. 6. Small and large intestinal tissue stained with hematoxylin–eosin in pre-carcinoma groups. Large intestine with serosal invasion in Group 2 (A), small intestine with invasion in serosa and muscularis propria layers in Group 2 (B), large (C) and small (D) intestine with serosal invasion in Group 4 (100×).

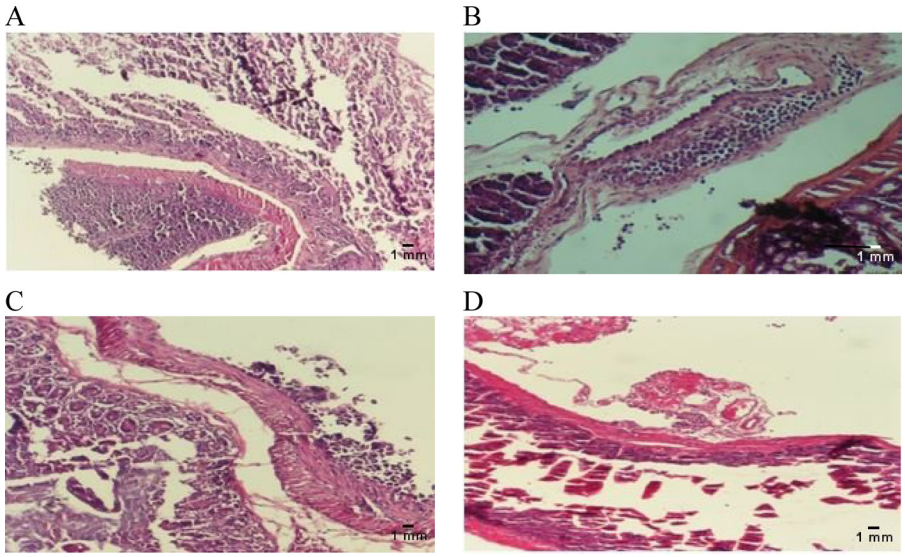

Fig. 7. Small and large intestinal tissue stained with hematoxylin–eosin in post-carcinoma groups. Large intestine with serosal invasion in Group 2 (A), small intestine with invasion in serosa and muscularis propria layers in Group 2 (B), large (C) and small (D) intestine with serosal invasion in Group 4 (100×).

In this experimental tumor model, host defense cells with prevalence of neutrophils were observed infiltrating the solid tumor or adjacent to disseminated tumor cells [27]. The number of peritoneal leukocytes has been reported to be increased as a consequence of tumor growth [10]. The activation process of leukocytes is accompanied by the intensive production of reactive oxygen species [3]. There is evidence that treatment with Y-27632 result in a significant reduction in the accumulation of neutrophils [4]. Polymorphonuclear leukocytes aggregation can also be decreased by Y-27632 [16]. In the present study, the marked increase in plasma GSH levels were noted in tumor-bearing mice as compared to the control group. GSH synthesis can be upregulated during oxidative stress and inflammation [22]. Oxidants cause short-term falls in intracellular GSH which associate with higher oxidized glutathione (GSSG) levels; this is followed by increases in GSH levels *in vivo* and also *in vitro* models [24,25,33]. Therefore, oxidants and oxidant-generating systems can upregulate GSH synthesis-linked genes, thus providing paradoxically a protective mechanism against oxidative stress [22]. Sometimes antioxidants such as GSH trigger protection against oxidants by directly scavenging these molecules or by regulating intracellular GSH levels through the induction of γ-glutamate-cysteine ligase [7]. In addition, we have observed that Y-27632 pretreatment preserved the plasma GSH levels, and increased tumor NO levels. NO has dual role in the cell. NO acts as stimulator of tumor growth and metastasis on the other hand inhibit neoplasia by its antioxidant action, inhibition of angiogenesis, and enhancement of vasodilatation, differentiation and apoptosis. In addition, these effects are attributed especially to the local NO concentration. NO transforms to peroxynitrite. Peroxynitrite can oxidize a many biomolecules such as carbohydrates, proteins and lipids. Peroxynitrite exhibits unique chemical reactivities such as protein nitration, DNA-strand breakage, and guanine nitration, which may then bring about not only cytotoxic effect but also mutagenesis [19]. We also observed that Y-27632 administration in post-carcinoma group increased lipid peroxidation leading to the rise of plasma MDA and the decrease of GSH level. GSH provides powerful antioxidant protection to body systems heavily exposed to reactive oxygen species. GSH deficiency causes oxidant damage and greater lipid peroxidation which in turn leading to cell damage [29]. It is known that inoculation of the tumor cells does not induce production of H_2O_2 by peritoneal cells [10]. In addition, Y-27632 has been shown to depress in remarkable elevation in serum levels of proinflammatory cytokines; interleukin-6, keratinocyte chemoattractant and granulocyte colony-stimulating factor [4]. Pro-inflammatory cytokines and other molecules might be involved in increasing antitumor effect of Y-27632. Y-27632 and derivatives of its action may also hinder adhesion, proliferation, migration of

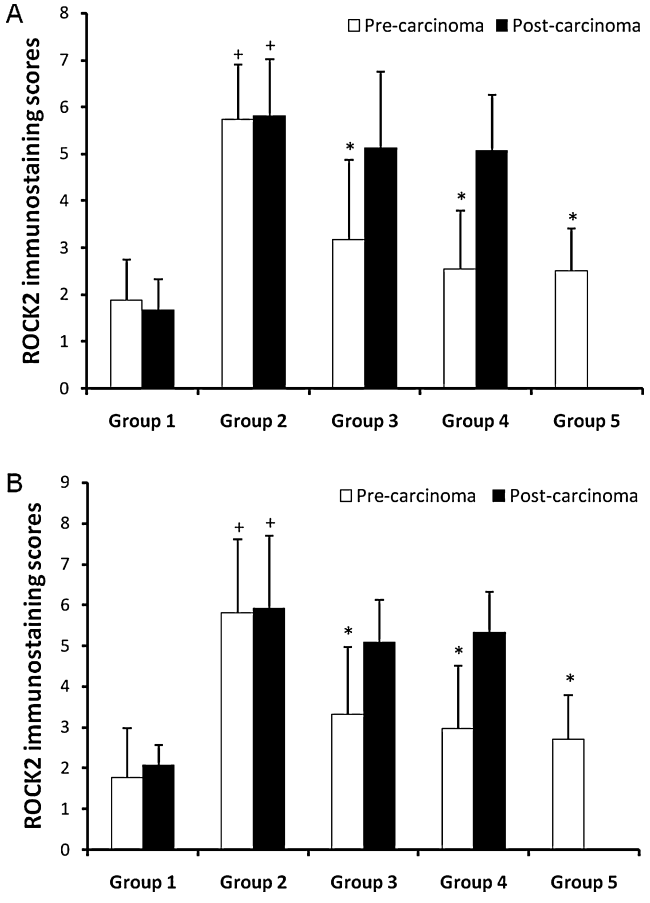

Fig. 8. Immunostaining scores for ROCK2 in small (A) and large (B) intestine. $^{+}p < 0.05$ when compared to Group 1, $^{*}p < 0.05$ when compared to Group 2.

cancer cells, deform tumor niche by *e.g.* changing surface molecules expression, and promote the apoptosis [31,36]. Collectively, these effects may induce antitumoral effect of Y-27632. Our findings may suggest that pretreatment, but not post-treatment, with a ROCK inhibitor is effective to observe antitumoral activity.

These data were supported by histopathological examination showed that Y-27632 administration to EAC-bearing mice diminished pathological structure, to 60–70% degree, toward to normal intact histological structure especially in pre-carcinoma inoculation regime. Respect to this, ROCK inhibition by Y-27632 decreased significantly tumor invasion and metastasis [20,32,37]. Our immunohistochemistry results showed that ROCK2 was mainly inhibited by Y-27632 in pre-carcinoma, but not in post-carcinoma, groups.

Our result was correlated with the other limited studies related with Y-27632 effectiveness *in vitro* and also *in vivo* [12,21,26]. Investigators reported that Y-27632 administration in tumor-bearing mice resulted in a reduction in melanoma tumor volume [26], attenuated the speed of tumor growth of the xenografts in nude mice [12] and suppressed tumor recurrence and prolonged survival time in a rat hepatocellular carcinoma model [21]. Moreover, it has been shown that ROCK inhibitor can reduce dissemination of cancer in the peritoneal cavity, reduce blood borne metastasis to the lung, and prevent early establishment of breast tumors in the mammary fat pad in animal models [37]. However, we found that Y-27632 had preventive effect but not suppressive effect compared to these articles. Although literature reports indicated suppressive efficacy of Y-27632 we did not find any effectiveness of Y-27632 after tumor inoculation. We thought that this might be related to the tumor characteristics. Because, EAC spreads in a short time (within 4–5 days) due to being a kind of more aggressive and metastatic experimental tumor.

In conclusion, this is the first study on the effectiveness of Y-27632 with experimental tumor modeling EAC providing direct evidence of decreased tumor formation with ROCK inhibition in an *in vivo* study. Our data suggest that Y-27632 might have preventive effect to the tumor formation and pretreatment with a ROCK inhibitor might be used to delay in tumor formation. Therefore, Rho-kinase inhibitors might be used in prophylactic agent for cancer treatment. ROCK inhibition might have preventive effect to the tumor formation and pretreatment with a ROCK inhibitor might be used to delay in tumor formation.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by a project (BAP TF.10.06) from the Scientific Research Project Council of the University of Gaziantep.

Acknowledgments

The authors thank to Lecturer Gregory Thomas Sullivan of the School of Foreign Languages, University of Ondokuz Mayis, Samsun, Turkey for his helpful comments and for editing the grammar and style of the manuscript.

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