Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 2317–2326
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Induction of alkaline phosphatase in the inflamed intestine: a novel pharmacological target for inflammatory bowel disease

Fermín Sánchez de Medina[a,1,*], Olga Martínez-Augustin[b,1], Raquel González[a], Isabel Ballester[a], Ana Nieto[c], Julio Gálvez[a], Antonio Zarzuelo[a]

[a]*Department of Pharmacology, School of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain*
[b]*Department of Biochemistry, School of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain*
[c]*Department of Animal Medicine and Surgery, School of Veterinary, Universidad Complutense, Ciudad Universitaria, 28040 Madrid, Spain*

Received 2 March 2004; accepted 7 July 2004

Abstract

This study demonstrates the upregulation of alkaline phosphatase and the mechanisms involved in experimental colitis. All models of ileal and colonic inflammation examined, which were characterized by significant oxidative stress and neutrophil infiltration, resulted in an increase in alkaline phosphatase activity which was attributable to both epithelial cells and cells of the lamina propria, mainly leukocytes. The increase in alkaline phosphatase sensitivity to the inhibitors levamisole and homoarginine, together with changes in the apparent molecular size and in the sialization of the enzyme, indicated a change in the isoform expressed. An increase in tissue non-specific alkaline phosphatase expression was observed by Western blotting. Treatment with the bone/kidney alkaline phosphatase inhibitor levamisole or a monoclonal antibody resulted in significant protection from colonic inflammation. Taken together, these results indicate that the kidney isoform is a marker of intestinal inflammation and that it might even constitute a target for pharmacological intervention.

Keywords: Alkaline phosphatase; Oxidative stress; Inflammatory bowel disease; Levamisole; Enterocyte; Neutrophil

1. Introduction

Mammalian alkaline phosphatases (ortophosphoric monoester phosphohydrolase-alkaline optimum, EC 3.1.3.1) are a family of enzymes that cleave phosphate moieties at alkaline pH. At least four genes loci have been described in humans: the tissue nonspecific or bone/liver/kidney, plus the intestinal, the placental, and the germ cell or placental-like isoforms, which are expressed predominantly in these tissues and thus are 'tissue specific'. All isoforms are glycoproteins that differ in their glucidic residues as well as in their linkage to the protein [1]. The tissue nonspecific gene gives rise to the bone, kidney and liver isoforms, which differ at the messenger RNA (mRNA) level, so that the first exon (exon 1A) of the bone isoform is different from that of the other two isoforms (exon 1B) [2]. However, the three isoforms are identical at the aminoacid level because the first exon is untranslated. In addition, there are differences in terms of sensitivity to chemical inhibitors and heat, which are ascribed to differences in the glycosylation pattern [3]. Thus, the liver enzyme is more resistant to levamisole or homoarginine inhibition than either the kidney or bone isoforms [3]. The placental and intestinal enzymes have an 87–90% similarity at the genomic level [4] and are generally similar in their sensitivity to chemical inhibitors, displaying only minor differences [5,6]. It should be noted however that the rat equivalents of the placental and germ cell isoforms have not been detected to date.

AP distribution is ubiquitous among cell types and tissues, but its physiological function is largely unknown. In the bone AP is thought to mediate phosphate assimilation,

Abbreviations: AP, alkaline phosphatase; TNBS, trinitrobenzenesulfonic acid; MPO, myeloperoxidase; LAP, aminopeptidase; PBS, phosphate buffered saline

* Corresponding author. Tel.: +34 958 243888; fax: +34 958 248964.
E-mail address: fsanchez@ugr.es (F. Sánchez de Medina).
[1] Both authors contributed equally to this work.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.07.045

since patients lacking this enzyme as well as knockout mice display important skeletal abnormalities [7,8]. In the intestine AP is localized to the brush border membrane and has been classically assumed to participate in nutrient absorption. Thus it has been proposed to participate in the intestinal transport of lipids [9] and nucleotides. Similarly, AP may play a role in the renal transport of phosphate [10]. A possible role in phosphate binding has also been proposed [11]. On the other hand, AP may play a role in inactivation or 'rundown' of the cystic fibrosis transmembrane conductance regulator [12]. Interestingly, lipopolysaccharide has been shown to be a substrate for AP although the specific isoenzyme was not identified [13]. Furthermore, a protective role was suggested by the deleterious effect of the AP inhibitor levamisole in rats infected intraperitoneally with *Escherichia coli*. Intestinal AP activity is not exclusive of enterocytes but is also present in intestinal cells located in the lamina propria, including neutrophils and other leukocytes [14], although its possible function here is unclear.

The small intestine of the adult mouse is thought to express exclusively intestinal alkaline phosphatase isoenzyme, although the tissue nonspecific type is transiently expressed in the fetal stage [15]. On the other hand, the large bowel does express tissue nonspecific alkaline phosphatase, which is secreted by the colonocytes in surfactant-like particles [16]. This isoform seems to be important in the maintenance and/or function of some intestinal structures [17], but its specific physiological role is unknown. Of note, treatment with specific antibodies directed against the surfactant-like particles seems to be beneficial in experimental colitis [18].

We have previously reported that colonic AP activity was invariably augmented in two models of induced inflammation [19,20]. The significance of these findings is uknown. Therefore, the present study was undertaken to characterize the nature of this event and the mechanisms involved. Furthermore, the possible pathophysiological significance of AP changes was explored using an enzyme inhibitor as well as a specific antibody.

2. Materials and methods

2.1. Animals

Female Wistar rats (200–230 g) and Swiss-Ofi female mice (20–24 g) provided by the Laboratory Animal Service of the University of Granada were used for all experiments, except for the HLA-B27/β_2 transgenic rats and age matched Fischer control rats, which were provided by Taconic. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was approved by the Animal Welfare Committee of the University of Granada.

2.2. Materials and reagents

Except where indicated, all reagents were obtained from Sigma. The polyclonal antibody against murine intestinal AP was the generous gift of Dr. Tsukamoto of the Aichi Cancer Center Research Institute. The B4-78 antibody against human tissue nonspecific AP developed by Dr. Katzmann [21] was obtained from the Development Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. All other antibodies were purchased from Santa Cruz Biotechnology and Sigma.

2.3. Intestinal inflammation

Intestinal inflammation was induced in rats (and also in mice with TNBS) by several different methods:

(a) TNBS colitis: rats were fasted overnight and anaesthetized with halothane, and 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol were delivered intrarectally by means of a Teflon cannula inserted 8 cm through the anus [22]. Controls received phosphate buffered saline (PBS). TNBS colitis was similarly induced in mice with a 6 mg dose. Rat ileitis was also induced by TNBS injection in the ileal lumen, approximately 3 cm proximal to the cecum, in halothane-anaesthetized animals after laparotomy. Sham operated rats received an equal volume of phosphate buffered saline. Animals were sacrificed after 1 week.
(b) Acetic acid colitis: halothane anaesthetized rats received 2 ml of 4% (v/v) acetic acid intrarectally followed by 20 ml of phosphate buffered saline [23]. Controls received PBS. Animals were sacrificed after 4 days.
(c) Dextran sulfate colitis: colitis was induced by replacing drinking water with distilled water containing 5% (w/v) dextran sulfate sodium (M_r = 36–50 kDa, ICN Biochemicals) prepared daily [24]. Control rats received distilled water. Animals were sacrificed after 1 week.
(d) Iodoacetamide colitis: halothane anesthetized rats received 0.25 ml of iodoacetamide intrarectally (6 mg) [25]. Controls received PBS. Animals were sacrificed after 1 week.

In addition, the HLA-B27/β_2 rat model of spontaneous colitis was used [26]. Animals were sacrificed at 22 weeks of age.

2.4. Assessment of intestinal damage

Animals were killed by cervical dislocation. The ileum (10 cm proximal to the cecum) or colon was removed and

placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). A colonic damage score was assigned to each animal upon examination on a 0–25 scale according to the following criteria: adhesions (0–3), obstruction (0–2), thickening (0–2), hyperemia (0–3), fibrosis (0–3), necrosis (0–5), scarring and deformation (0–7). The intestinal segments were subsequently divided longitudinally in 2–3 pieces for biochemical determinations as described [27]. One sample was immediately weighed and immersed in 5% trichloroacetic acid for total glutathione determination [28]. Myeloperoxidase (MPO) activity, a marker of neutrophils, was measured spectrophotometrically [29]. The results are expressed as myeloperoxidase units (μmol/min) per gram of wet tissue.

2.5. Enzymatic determinations

AP activity was measured spectrophotometrically, using disodium nitrophenylphosphate (5.5 mM) as substrate in 50 mM glycine buffer with 0.5 mM $MgCl_2$ (pH 10.5) [30]. In some cases the sensitivity to AP inhibitors (levamisole, homoarginine) or heat (56 °C, 30 min) was tested. Leucine aminopeptidase (LAP) activity was measured spectrophotometrically [31,32]. Enzymatic activities are expressed as mU/mg protein [33].

2.6. Western blot

The samples were homogenized in ice cold buffer consisting of 0.1% sodium dodecylsulfate, 0.1% sodium deoxycholate, 1% Triton X-100 in phosphate buffered saline with freshly added protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, 1,10-phenanthroline). The protein content was measured by the bicinchoninic acid assay [34], using bovine serum albumin as standard. Samples were boiled for 4 min in Laemmli buffer, separated by 10% SDS–PAGE, electroblotted onto nitrocelulose membranes and blocked with 5% (w/v) nonfat dry milk in Tris buffered saline. After incubation with the proper antibodies the bands were detected by enhanced chemiluminescence (NEN).

2.7. In situ activity and sensitivity to neuraminidase

Samples were homogenized as above and then heated for 3 min at 37 °C in Laemmli buffer without β-mercaptoethanol nor SDS, separated by 7% SDS–PAGE and electroblotted onto nitrocelulose paper (Hybond, Amersham Pharmacia Biotech). AP activity was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium in a 100 mM Tris–HCl buffer with 100 mM NaCl and 5 mM $MgCl_2$ (pH 9.5). In some cases AP activity was detected by chemoluminiscence (Applied Biosystems). In the neuraminidase sensitivity analysis, the samples were separated in two aliquots, one of which was digested with neuraminidase (750 U/ml) for 3 h at 37 °C while the other was kept on ice. Both aliquots were analyzed as above.

2.8. Isolation of colonocytes

Whole colonic segments from control or TNBS rats were obtained and the mucosal layer was dissected as previously described [27]. The submucosa was homogenized for AP determination. Colonocytes were isolated by hyaluronidase digestion [35] and processed for AP determination. Purity was at least 90% in all samples. Cell viability was 85–90% (Trypan blue).

2.9. Immunohistochemistry

Immunolabelling was performed on formalin-fixed paraffin wax-embedded samples using the streptavidin–biotin peroxidase complex method and a high temperature pretreatment as antigen unmasking protocols. After the pretreatment, sections were incubated 15 min with 1% hydrogen peroxidase to block endogenous peroxidase and with protein blocking serum for 30 min to reduce nonspecific reactions. The primary antibody B4-78 was used (dilution of 1:10, incubated overnight at 4 °C). The developing system was Dako ChemMate™ Universal Kits (LSBA), following manufacturer's instructions. Immunoreactivity was visualized with 3-3′ diaminobenzidine tetrachloride (Sigma) and hydrogen peroxide (0.01%) and slides were counterstained 2 min with Gill's haematoxylin. Dilutions and washing were made in Tris buffered saline. Negative control slides were made by substituting the primary antibody with normal swine serum.

2.10. RNA isolation and northern blot analysis

Total RNA was isolated from rat tissues or cells as described elsewhere [36]. Equal amounts (30 μg) of total RNA were resolved using 1% agarose/formaldehyde gels, transferred to a nylon membrane (Hybond-XL™, Amersham Pharmacia Biotech) and crosslinked using a UV-Stratalinker (Stratagene). Prehybridization and hybridization with ^{32}P-labelled probes were carried out in ULTRAhyb solution (Ambion) at 42 °C. The intestinal AP probe was obtained by reverse transcription of mRNA isolated from rat colon using the First-strand cDNA synthesis kit (Amersham Pharmacia Biotech) followed by polymerase chain reaction (PCR) amplification of a 1057 bp fragment of intestinal alkaline phosphatase using the following primers: 5′ CTCGG ATGCA GACAT GCCT T C 3′ (sense oligo) and 5′ TCAGG ACACC ACCAA GGCTG 3′ (antisense oligo). The PCR reaction was performed at 94, 45 and 72 °C for 1 min for 45 cycles. The amplicon was then electroeluted and cloned in the TA vector pGEMT (Promega), sequenced and used to prepare the probe by digesting the construct with ApaI and NotI and purifying it by

electroelution. [α^{32}P]-deoxyadenosine triphosphate radiolabelled probes were obtained by the random priming labeling method. The probe recognizes both isoenzymes of the rat intestinal alkaline phosphatase [37].

2.11. Effect of alkaline phosphatase inhibition in vivo

The TNBS model of rat colitis was selected to test the effect of in vivo inhibition of AP activity. Rats were administered levamisole (25 mg/kg day i.p.) or B4-78 antibody (2.5 μg/kg day i.p.) starting 1 day before colitis induction. The TNBS and the control (noncolitic) group received sterile saline solution. Colitis was induced as described above.

2.12. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Differences among means were tested for statistical significance by one-way analysis of variance and a posteriori least significance tests on preselected pairs. Pair comparisons were performed with Student's two-tailed *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. AP activity is increased in several models of intestinal inflammation

Because we had previously reported the increase in AP activity in two models of colitis, our first aim was to check whether this phenomenon occurred in other models of intestinal inflammation. Thus colitis was induced in rats by four different methods, namely dextran sulfate sodium, iodoacetamide, TNBS and acetic acid, and also in mice by TNBS. The HLA-B27/β_2 transgenic rat model was also examined. The results, shown in Table 1, confirm that all models examined were characterized by a marked increase in AP activity.

The increase in AP activity was not restricted to the large bowel, since it was also detected in a model of ileitis induced with TNBS (Table 2). In this experiment the activity of other brush border enzyme, namely leucine aminopeptidase, was not increased (Table 2). These data establish that the increase in AP activity is a common consequence of intestinal inflammation, at least in the two species used. On the other hand, AP was shown to be increased in both inflamed and noninflamed tissue in TNBS colitic rats (data not shown).

In order to ascertain whether the increase in AP was attributable to leukocytes or epithelial cells, enterocytes were isolated from the colonic segments and the AP activity was measured. AP activity was higher in the samples obtained from inflamed tissue (35.7 ± 9.8 mU/mg protein versus 7.0 ± 0.6 mU/mg protein, $p < 0.05$, $n = 7$). Furthermore, the sensitivity to levamisole and homoarginine was also increased (Fig. 1A). In comparison, the AP activity of the submucosal layer was much more sensitive to the chemical inhibitors in basal conditions and experimented a lower increase as a result of inflammation (Fig. 1B). This is consistent with the presence of resident leukocytes in the colonic wall, particularly in the submucosa [38]. Immunohistochemical analysis using monoclonal antibodies against the tissue nonspecific isoform of AP detected a significant expression of this protein in enterocytes and secondarily also in lamina propria leukocytes (specially macrophages) (Fig. 2).

3.2. The tissue nonspecific isoform is responsible for the increase in AP activity in the inflamed intestine

The AP isoform expressed by the rat colon has been reported to be the tissue nonspecific type [16]. We used an array of molecular and biochemical techniques to identify the AP isoform expressed by the normal and inflamed

Table 1
AP activity in different models of colonic inflammation

	AP (mU/mg protein)		Myeloperoxidase (U/g)		Weight:length ratio (mg/cm)		Glutathione (nmol/g)	
	Control	Inflamed	Control	Inflamed	Control	Inflamed	Control	Inflamed
Rat colitis								
TNBS	5.6 ± 1.1	21.5 ± 3.6*	4.2 ± 2.0	56.1 ± 5.4*	74.0 ± 2.7	144.2 ± 14.9*	2142.3 ± 206.4	1597.7 ± 156.2*
Dextran sulfate	6.7 ± 0.5	15.1 ± 2.0*	5.6 ± 3.2	122.5 ± 21.3*	72.4 ± 2.1	111.9 ± 16.7*	1568.0 ± 149.1	1044.1 ± 141.6*
Acetic acid	6.3 ± 0.5	21.0 ± 2.1*	12.2 ± 3.0	35.4 ± 8.9*	75.6 ± 4.7	128.4 ± 7.8*	1736.2 ± 116.3	1006.8 ± 172.0*
Iodoacetamide	6.4 ± 2.1	28.8 ± 5.4*	2.0 ± .7.0	33.0 ± 3.8*	70.9 ± 5.1	178.0 ± 15.2*	1648.3 ± 27.1	1232.6 ± 76.1*
HLA-B27	9.2 ± 1.6	44.8 ± 6.1*	5.8 ± 2.4	19.1 ± 12.3*	69.1 ± 2.0	127.2 ± 9.8*	–	–
Mouse colitis								
TNBS	57.3 ± 6.2	120.8 ± 9.7*	2.9 ± 0.8	12.0 ± 6.9*	31.5 ± 2.1	43.6 ± 2.8*	1713.1 ± 69.9	1310.1 ± 127.3*

Experimental colitis was induced in rats with TNBS, acetic acid or iodoacetamide enemas or with dextran sulfate administered in drinking water. Colitis was also induced in mice with TNBS. Control animals received PBS enemas except those of dextran sulfate colitis, which received distilled water to drink. Spontaneous (HLA-B27/β_2) rat colitis was also studied (control: age matched Fischer rats). Myeloperoxidase was used as a marker of neutrophil infiltration. The weight:length ratio is the wet weight divided by the length of the colonic segment under a load of 2 g. Glutathione is used as an index of oxidative stress. Glutathione was not measured in HLA-B27/β_2 rats. Data are mean ± S.E.M. ($n \geq 6$).

* $p < 0.05$ vs. control.

Table 2
TNBS rat ileitis

	AP (mU/mg protein)	LAP (mU/mg protein)	Weight (mg/cm)
Control	72.5 ± 5.4	76.4 ± 8.1	49.1 ± 5.5
Sham operated	89.9 ± 9.3	81.5 ± 12.5	39.5 ± 3.8
TNBS	141.6 ± 16.6*	64.5 ± 10.0	148.1 ± 34.0*

Enzymatic activities, LAP: leucine aminopeptidase. Values are expressed as mean ± S.E.M. (n = 5–10).
* $p < 0.05$ vs. control.

tissue. Expression of the intestinal AP isoform must be discarded, as it seems to be restricted to the small intestine by both Western and Northern analysis and is not induced in the inflamed intestine (Fig. 3). The B4-78 antibody, which reacts with tissue nonspecific AP molecules [21], detects a corresponding 57 kDa band in the normal and inflamed rat colon, with a higher expression level in the latter (Fig. 3D). However, this antibody cannot differentiate between the three tissue nonspecific isoforms, i.e. bone, liver and kidney.

The AP of the inflamed rat colon had an apparent molecular weight slightly higher than that of the control tissue (detected as the dimer in nondenaturing conditions, Fig. 4) and, more importantly, gave rise to two bands upon neuraminidase digestion, with an apparent increase in molecular size. Of note, the AP of kidney homogenates was of identical size on the blots and showed a similar behaviour when digested with neuraminidase, whereas the enzyme present in the bone and liver had different molecular weights (Fig. 4). Neuraminidase digestion of normal colonic samples had little if any effect on the electrophoretic mobility (Fig. 4). These results indicate that there are differences in the glycosylation pattern of the AP isoforms expressed by the normal and inflamed rat colonic segments, and that the latter is similar, if not identical, to kidney AP. The three tissue nonspecific AP protein products are considered to be identical at the aminoacid level and to differ extensively in the glycosidic fraction. This in turn gives rise to marked differences in their response to chemical inhibitors. Therefore we examined the sensitivity of tissue homogenates to levamisole and homoarginine.

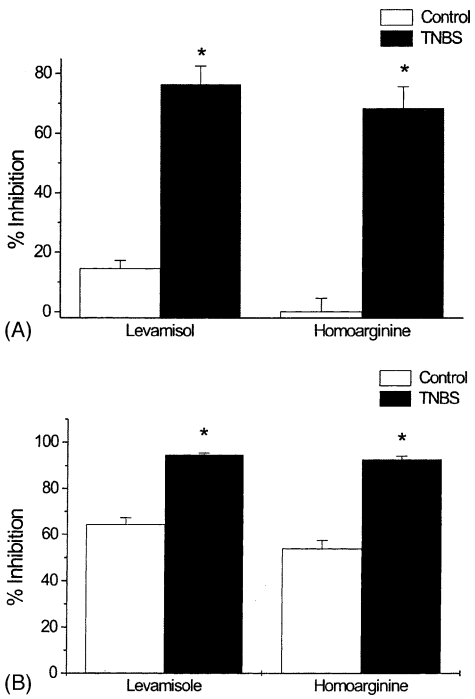

Fig. 1. Sensitivity of alkaline phosphatase in the normal and inflamed rat colon. Colitis was induced with TNBS. (A) Sensitivity of AP activity in isolated colonocytes. (B) Sensitivity of the submucosal layer. The chemical inhibitors were used at 1 mM (levamisole) and 0.1 M (homoarginine). Data are mean ± S.E.M. (n = 7). *$p < 0.05$ vs. control.

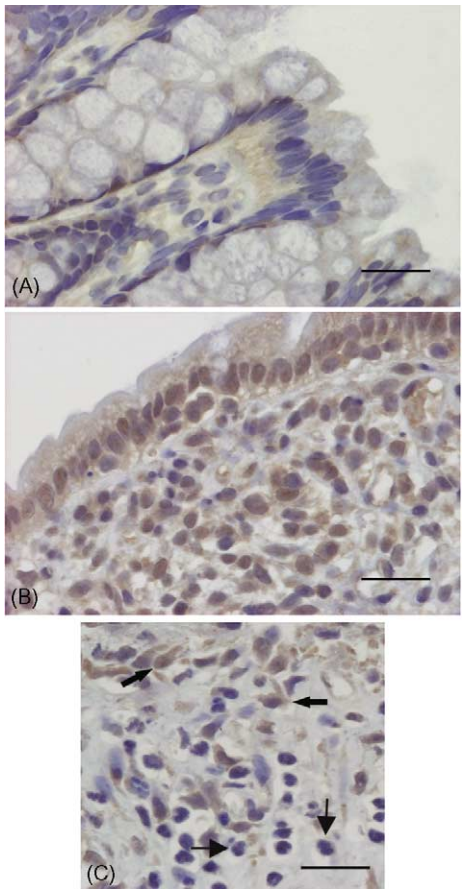

Fig. 2. Immunohistochemistry of tissue nonspecific AP in TNBS rat colitis. Immunoreactivity to the B4-78 antibody against tissue nonspecific AP is shown: (A) normal colon; (B) inflamed colon; (C) detail of inflamed colon. Colonocytes appear uniformly stained in the inflamed tissue (brown), while a significant number of lamina propria cells, notably macrophages (thick arrows) also show significant expression. Neutrophils display little staining (fine arrows). In contrast, the normal mucosa shows a much lower level of expression, particularly in the epithelium. Bar: 40 μM.

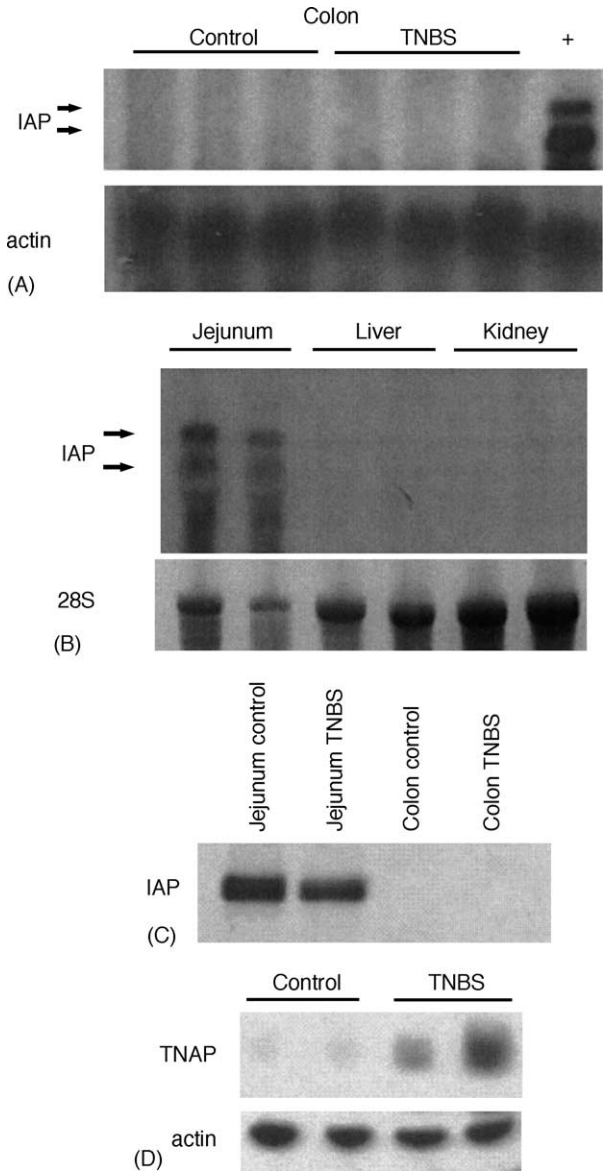

Fig. 3. Expression of intestinal AP. (A) Northern blot of samples from control or TNBS colitic rats. (B) Northern blot of samples from several rat tissues showing expression restricted to the small intestine. (C) Western blot of rat tissues confirming expression restricted to the small intestine. The blots are representative of at least two separate experiments ($n \geq 4$). 28S ribosomal RNA and β-actin are included as loading controls. The arrows indicate the two different intestinal AP isoforms expressed by the rat small intestine. IAP: intestinal alkaline phosphatase; (+): positive control (rat small intestine). (D) Expression of tissue nonspecific AP. A Western blot showing increased expression of this isoform, detected as a 57 kDa band in the colon of TNBS-treated rats compared with the controls. The blot is representative of a total of five experiments (n = 12–14). Actin is shown as loading control.

The AP of the inflamed colon was clearly more sensitive to these chemical inhibitors (Fig. 5), as well as to heat (not shown). Of note, the AP of ileitis samples was also more sensitive to levamisole (not shown). These results are consistent with an induction of the kidney isozyme (see Fig. 6 for the sensitivity profiles of the AP from different tissues).

3.3. Effect of tissue nonspecific AP inhibition on TNBS colitis

In order to verify whether the induced AP in the inflamed intestine plays a role in the inflammatory response, rats were pretreated with the inhibitor levamisole or the B4-78 antibody and 1 day later colitis was induced with TNBS. The treatments were maintained until the animals were sacrificed. The results obtained are summarized in Table 3. Both levamisole and the B4-78 antibody markedly reduced colonic myeloperoxidase activity when compared with the TNBS group. This was accompanied by some evidence of macroscopic benefit, which did not reach statistical significance. This was associated with a reduced AP colonic activity in the levamisole group, while the effect did not reach statistical significance in the rats treated with the antibody.

4. Discussion

The present study was designed to explore the nature of the AP increase observed in the inflamed intestine and its possible pathophysiological significance. We have extended our previous observations to demonstrate that inflammation of the intestine is consistently associated with a significant increase of AP activity, independently of the intestinal segment, experimental model and rodent species. Therefore, this phenomenon is characteristic of intestinal inflammation rather than specific of a given experimental model. We selected the TNBS model of rat colitis to identify the mechanism of this effect.

Our data clearly indicate that the increase in AP activity is associated with a change of isoform, evidenced by the shift in molecular weight, sensitivity to chemical inhibitors and heat, and differential response to neuraminidase digestion. The constitutive AP isoform expressed by the rat colon has been previously identified as tissue nonspecific [16]. Our data are in agreement with this hypothesis. Thus the AP of the normal colon was recognized by the tissue nonspecific AP antibody B4-78 and showed relatively low sensitivity to chemical inhibitors as well as heat, and had an apparent molecular size of ∼160 kDa (for the dimer). The sensitivity profile is consistent with the liver isoform but not with the kidney or bone enzymes (Fig. 6). In contrast, the inflamed colon had increased levels of tissue nonspecific AP as assessed by Western blot but it was very sensitive to chemical inhibitors and heat. This indicates augmented expression of either the bone or kidney isoforms, both of which share the same sensitivity profile (Fig. 6) [39,40]. Because the bone, liver and kidney isoforms have the same amino acid sequence, this effect implies an increase in expression as well as a change in the glycosylation pattern. The latter was evidenced by the change in electrophoretic mobility under nondenaturing conditions as well as by the shift observed after neuraminidase

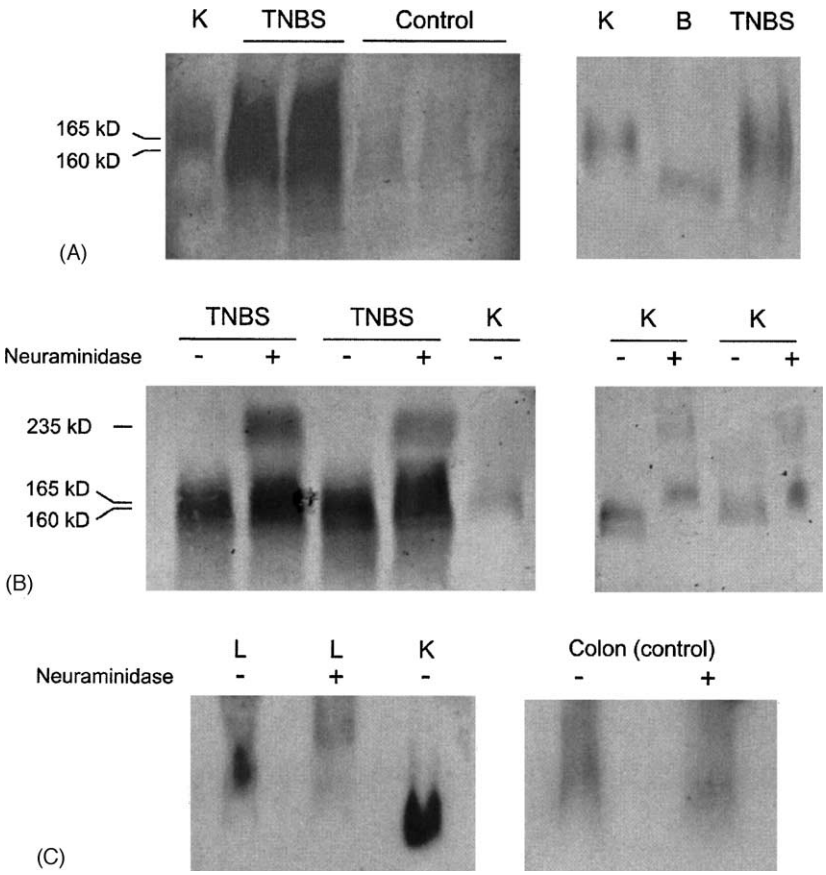

Fig. 4. In situ AP activity. (A) AP activity was detected in blots containing normal and TNBS rat colonic samples (left). AP in the TNBS samples was markedly higher and had a slight increase in molecular size than that of the controls (representative of three blots, $n = 6$–8). Bone AP (right) had a lower molecular weight than either the kidney or the inflamed colon ($n = 3$). (B) Response of the AP of TNBS colitic rats to neuraminidase. The original band is divided in two bands of lower electrophoretic mobility which are comparable to those of kidney homogenates ($n = 4$). The arrows correspond to the same molecular weights in the right and left panels. (C) In situ activity of liver (left) and uninflamed rat colon AP (right) and response to neuraminidase digestion. Liver AP has a lower electrophoretic mobility than the kidney isoform which is shifted by neuraminidase, while that of the rat colon is apparently unaffected. L, K, B: liver, kidney or bone homogenates.

digestion, which catalyzes the hydrolysis of terminal sialyl linkages from oligosaccharides. These characteristics were similar to those of the AP of kidney but not bone (or liver) homogenates. It should be noted that this shift in electrophoretic mobility does not reflect a change in molecular weight, but a result of the removal of sialic acid, with the resultant lower anionic charge [41]. Considered together, our results indicate that the rat colon expresses the liver AP isoform in the basal state and that the kidney isoform is induced in inflammatory conditions. It is also possible that the latter is expressed at low levels in the uninflamed colon, since its sensitivity to levamisole and homoarginine are intermediate between that of the liver and the kidney homogenates (compare Figs. 5 and 6).

Once the AP isoform induced in the inflamed intestine has been identified, the next unresolved question is the cell source of the enzyme. Biochemical and immunohistochemical analyses indicate that both colonocytes and infiltrating leukocytes account for the increase in AP in the inflamed colon. However, while neutrophils, which are actively recruited to the inflammatory site in TNBS colitis and inflammatory bowel disease (IBD) [42], express tissue nonspecific AP of the levamisole/homoarginine sensitive type [14] constitutively, colonocytes undergo a change of

Table 3
Effect of inhibition of tissue nonspecific AP on TNBS colitis

	MPO (U/g)	Score	AP (mU/mg protein)	Sensitivity to 1 mM levamisole (%)
Control	3.8 ± 1.9	0 (0)	34.8 ± 0.7	28.2 ± 4.1
TNBS	60.1 ± 8.5	6 (4.8–6)	78.5 ± 0.8	71.3 ± 3.9
Levamisole	30.7 ± 6.6*	5 (4–5.8)	55.7 ± 7.2*	66.9 ± 2.9
B4-78	31.7 ± 10.4*	5 (2–5)	61.7 ± 8.4	65.4 ± 5.5

Values are expressed as mean ± S.E.M. ($n = 7$). All means in the TNBS and levamisole groups are significantly different from those of the control group.
* $p < 0.05$ vs. TNBS.

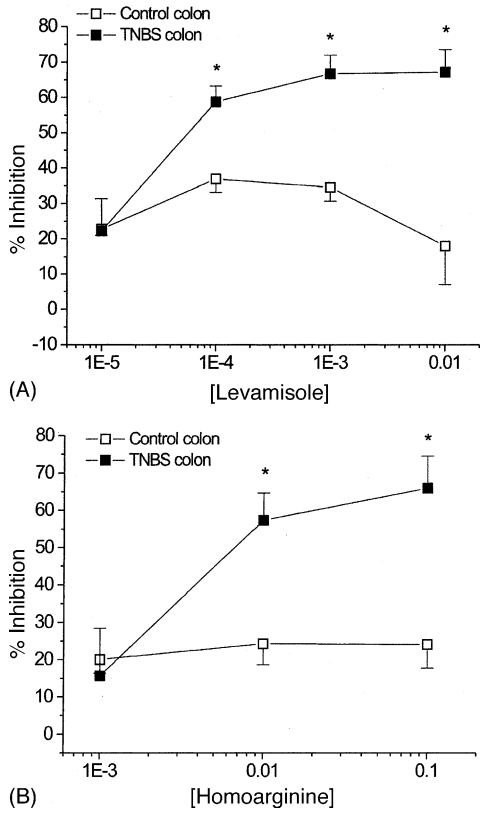

Fig. 5. Sensitivity of AP in rat colonic tissue to chemical inhibitors. The concentrations are expressed in mol/l. The enzymatic activity was (mU/mg protein): 34.8 ± 3.6 (control), 78.5 ± 11.0 (TNBS). There is a significant increase in the sensitivity to levamisole (A) and homoarginine (B) in the inflamed colon. Data are mean ± S.E.M. ($n \geq 10$). *$p < 0.05$.

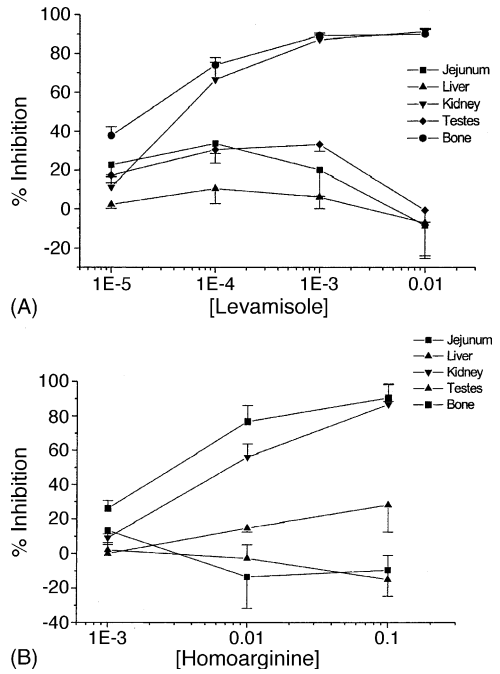

Fig. 6. Sensitivity of AP in rat tissue homogenates to chemical inhibitors. The concentrations are expressed in mol/l. The enzymatic activity was (mU/mg protein): 683.7 ± 23.1 (testes), 75.5 ± 6.8 (jejunum), 227.5 ± 9.4 (kidney), 9.4 ± 2.3 (liver), 682.4 ± 11.2 (bone). The AP of kidney and bone homogenates is clearly more sensitive to levamisole (A) and homoarginine (B) than other tissues. Data are mean ± S.E.M. ($n \geq 4$). Statistical significance has been omitted for clarity.

isoform, as evidenced by the change of sensitivity to heat and chemical inhibitors. We have found that AP is not induced in rat neutrophils obtained from peripheral blood after 1 h exposure to lipopolysaccharide, a time frame sufficient to augment myeloperoxidase activity (data not shown). Furthermore, macrophages rather than neutrophils seem to be the main leukocyte expressing tissue nonspecific AP in the inflamed mucosa (Fig. 2C). The increase of AP activity in enterocytes has been recently confirmed microscopically in the dextran sulfate sodium model [43].

Finally, we have established the value of AP as a marker of intestinal inflammation, but the reason why it is upregulated in inflammatory conditions is unclear. We hypothetized that AP may be involved in the pathogenesis of intestinal inflammation and we attempted to modulate TNBS experimental colitis with a tissue nonpecific AP inhibitor (levamisole), as well as with a specific antibody (B4-78). The results show a striking reduction of colonic MPO activity, together with some macroscopic amelioration of the intestinal tissue (which did not reach statistical significance). This beneficial effect was associated with a significant decrease in intestinal AP activity in the case of the inhibitor, but not the antibody, a circumstance that may be related to the relatively high affinity of levamisole for the enzyme (data not shown). In fact, the relatively modest effects observed on AP may reflect bioavailability problems, suggesting that a greater antiinflammatory effect is possible. It is important to remember that levamisole has a number of other properties that may be involved in its antiinflammatory activity, such as potassium channel inhibition, activation of the cystic fibrosis transmembrane conductance regulator (CFTR), induction of apoptosis, inhibition of NF-κB, etc. [44–46]. Nevertheless, the fact that both levamisole and the B4-78 antibody had a positive effect on colonic inflammation certainly suggests that AP is involved in the response and that it might constitute a relevant target for drug therapy. It should be noted however that these results are largely preliminary and that new tests using more specific probes will be required. In addition, the cellular source of the enzyme plausibly targeted by the treatments as well as the mechanisms involved are unknown. Further clarification of the exact function of induced AP in intestinal inflammation is warranted, particularly considering that some investigators have demonstrated changes in AP expression in ulcerative colitis and Crohn's disease in humans [47].

Acknowledgements

The authors are grateful to Dr. Tsukamoto for the intestinal AP antibody and to Dr. González for her tech-

nical assistance. This work was supported by the Instituto de Investigación Carlos III (FIS 01/0936 and PI021732), the Ministerio de Ciencia y Tecnología (SAF2002-02592 and the Ramón y Cajal program), and the Fundación Ramón Areces. Part of these data were presented at the Meeting of the Spanish Society of Biochemistry and Molecular Biology (2000).

References

[1] Van Hoof VO, De Broe ME. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. Crit Rev Clin Lab Sci 1994;31:197–293.
[2] Matsuura S, Kishi F, Kajii T. Characterization of a 5′-flanking region of the human liver/bone/kidney alkaline phosphatase gene: two kinds of mRNA from a single gene. Biochem Biophys Res Commun 1990;168:993–1000.
[3] Martins MJ, Negrao MR, Hipólito-Reis C. Alkaline phosphatase from rat liver and kidney is differentially modulated. Clin Biochem 2001;34:463–8.
[4] Henthorn PS, Raducha M, Kadesch T, Weiss MJ, Harris H. Sequence and characterization of the human intestinal alkaline phosphatase gene. J Biol Chem 1988;263:12011–9.
[5] Mulivor RA, Hannig VL, Harris H. Developmental change in human intestinal alkaline phosphatase. Proc Natl Acad Sci USA 1978;75:3909–12.
[6] Ali NN, Rowe J, Teich NM. Constitutive expression of non-bone/liver/kidney alkaline phosphatase in human osteosarcoma cell lines. J Bone Miner Res 1996;11:512–20.
[7] Narisawa S, Frohlander N, Millan JL. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev Dyn 1997;208:432–46.
[8] Zurutuza L, Muller F, Gibrat JF, Taillander A, Simon-Buoy B, Serre JL, et al. Correlations of genotype and phenotype in hypophosphatasia. Hum Mol Genet 1999;8:1039–46.
[9] Young GP, Friedman S, Yedlin S, Alpers DH. Effect of fat feeding on intestinal alkaline phosphatase activity in tissue and serum. Am J Physiol 1981;241:461–8.
[10] Petitclerc C, Plante GE. Renal transport of phosphate: role of alkaline phosphatase. Can J Physiol Pharmacol 1981;59:311–23.
[11] Hirano K, Iiizumi Y, Mori Y, Toyoshi K, Sugiura M, Iino S. Role of alkaline phosphatase in phosphate uptake into brush border membrane vesicles from human intestinal mucosa. J Biochem Tokyo 1985;97:1461–6.
[12] Becq F, Jensen TJ, Chang XB, Savoia A, Rommens JM, Tsui LC, et al. Phosphatase inhibitors activate normal and defective CFTR chloride channels. Proc Natl Acad Sci USA 1994;91:9160–4.
[13] Poelstra K, Bakker WW, Klok PA, Kamps JAAM, Hardonk MJ, Meijer DKF. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997;151:1163–9.
[14] Smith GP, Harris H, Peters TJ. Studies of the biochemical and immunological properties of human neutrophil alkaline phosphatase with comparison to the established alkaline phosphatase isoenzymes. Clin Chim Acta 1984;142:221–30.
[15] Hoshi K, Amizuka N, Oda K, Ikehara Y, Ozawa H. Immunolocalization of tissue non-specific alkaline phosphatase in mice. Histochem Cell Biol 1997;107:183–91.
[16] Eliakim R, Goetz GS, Rubio S, Chailley-Heu B, Shao JS, Ducroc R, et al. Isolation and characterization of surfactant-like particles in rat and human colon. Am J Physiol 1997;272:G425–34.
[17] Shao JS, Engle M, Xie Q, Schmidt RE, Narisawa S, Millan JL, et al. Effect of tissue non-specific alkaline phosphatase in maintenance of structure of murine colon and stomach. Microsc Res Tech 2000;51:121–8.
[18] Eliakim R, Karmeli F, Cohen P, Rachmilewitz D, Shao JS, Alpers DH. Sulphydryl blocker-induced rat colitis is ameliorated by intravenous injection of antibody to colonic surfactant-like particle. Inflamm Bowel Dis 1999;5:16–23.
[19] Galvez J, Cruz T, Crespo E, Ocete MA, Lorente MD, Sanchez de Medina F. Rutoside as mucosal protective in acetic acid-induced rat colitis. Planta Med 1997;63:409–14.
[20] Sanchez de Medina F, Galvez J, Romero JA, Zarzuelo A. Effect of quercitrin on acute and chronic experimental colitis in the rat. J Pharmacol Exp Ther 1996;278:771–9.
[21] Lawson GM, Katzmann JA, Kimlinger TK, O'Brien JF. Isolation and preliminary characterization of a monoclonal antibody that interacts preferentially with the liver isoenzyme oh human alkaline phosphatase. Clin Chem 1985;31:381–5.
[22] Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 1989;96:795–803.
[23] MacPherson BR, Pfeiffer CJ. Experimental production of diffuse colitis in rats. Digestion 1978;17:135–50.
[24] Stucchi AF, Shofer S, Leeman S, Materne O, Beer E, McClung J, et al. NK-1 antagonist reduces colonic inflammation and oxidative stress in dextran sulfate-induced colitis in rats. Am J Physiol Gastrointest Liver Physiol 2000;279:1298–306.
[25] Rachmilewitz D, Karmeli F, Okon E. Sulfhydryl blocker-induced rat colonic inflammation is ameliorated by inhibition of nitric oxide synthase. Gastroenterology 1995;109:98–106.
[26] Hammer RE, Maika SD, Richardson JA, Tang JP, Taurog JD. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders. Cell 1990;63:1099–112.
[27] Sánchez de Medina F, Pérez R, Martínez-Augustin O, González R, Lorente MD, Gálvez J, et al. Disturbances of colonic ion secretion in inflammation: role of the enteric nervous system and cAMP. Pflügers Arch- Eur J Physiol 2002;444:378–88.
[28] Akerboom TP, Sies H. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol 1981;77:373–82.
[29] Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity: assessment of inflammation in rat and hamster models. Gastroenterology 1984;87:1344–50.
[30] Bessey OA, Lowry OH, Brook MJ. Rapid colorimetric method for the determination of alkaline phosphatase in five cubic mililiters of serum. J Biol Chem 1946;164:321–9.
[31] Maroux S, Louvard D, Baratti J. The aminopeptidase from hog intestinal brush-border. Biochim Biophys Acta 1977;321:282–95.
[32] Dahlqvist A. Assay of intestinal dissacharidases. Anal Biochem 1968;22:99–107.
[33] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding: measurement of protein using bicinchoninic acid. Anal Biochem 1976;72:248–54.
[34] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
[35] Roediger WE, Truelove SC. Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies. Gut 1979;20:484–8.
[36] Murnane MJ, Tam SW. Isolation and characterization of RNA from snap frozen tissues and cultured cells. J Nutr Biochem 1992;3:251–60.
[37] Yeh K, Yeh M, Holt PR, Alpers DH. Development and hormonal modulation of postnatal expression of the intestinal alkaline phosphatase mRNA species and their encoded isoenzymes. Biochem J 1994;301:893–9.

[38] James SP. Mucosal immune regulation. In: Targan SR, Shanahan F, editors. Inflammatory bowel disease: from bench to bedside. Baltimore: Williams and Wilkins; 1994. p. 65–77.
[39] Wada H, Niwa N, Hayakawa T, Tsuge H. Changes of serum alkaline phosphatase isoenzymes in fasted rats. J Nutr Sci Vitaminol Tokyo 1996;42:435–47.
[40] Cohen-Tanugi A, Bolle M, Boy-Lefevre ML, Anagnostou F, Forest N. Phenotypic characterization of the 3/A/1D-1M osteogenic cell line derived from in vivo transplantation of 3/A/1D-1 chondroprogenitor murine teratocarcinoma cells. Differentiation 1996;60:327–37.
[41] Komoda T, Koyama I, Nagata A, Sakagishi Y, DeSchryver-Kecskemeti K, Alpers DH. Ontogenic and phylogenic studies of intestinal, hepatic, and placental alkaline phosphatases. Evidence that intestinal alkaline phosphatase is a late evolutionary development. Gastroenterology 1986;91:277–86.
[42] Palmen MJ, Dieleman LA, van der Ende MB, Uyterlinde A, Pena AS, Meuwissen SG, et al. Non-lymphoid and lymphoid cells in acute, chronic and relapsing experimental colitis. Clin Exp Immunol 1995;99:226–32.
[43] Renes IB, Verburg M, van Nispen DJPM, Büller HA, Dekker J, Einerhand AW. Distinct epithelial responses in experimental colitis: implications for ion uptake and mucosal protection. Am J Physiol Gastrointest Liver Physiol 2002;283:G169–79.
[44] Kim JA, Kang YS, Lee SH, Lee EH, Yoo BH, Lee YS. Glibenclamide induces apoptosis through inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^{(-)}$ channels and intracellular $Ca^{(2+)}$ release in HepG2 human hepatoblastoma cells. Biochem Biophys Res Commun 1999;261:682–8.
[45] Artwohl M, Holzenbein T, Wagner L, Freudenthaler A, Waldhausl W, Baumgartner-Parzer SM. Levamisole induced apoptosis in cultured vascular endothelial cells. Br J Pharmacol 2000;131:1577–83.
[46] Liu CY, Lai YY, Lo CJ. Levamisole modulates prostaglandin E2 production and cyclooxygenase II gene expression in human colonic cancer cells. J Surg Res 2004;117:223–31.
[47] Gibson P, Rosella O, Nov R, Young G. Colonic epithelium is diffusely abnormal in ulcerative colitis and colorectal cancer. Gut 1995;36:857–63.