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Effects of azathioprine and its metabolites on repair mechanisms of the intestinal epithelium in vitro

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Abstract

Erosions and ulcerations of the intestinal epithelium are hallmarks of inflammatory bowel diseases (IBD). Intestinal epithelial cell migration (restitution) and proliferation are pivotal mechanisms for healing of epithelial defects after mucosal injury. In addition, the rate of apoptosis of epithelial cells may modulate intestinal wound healing.

The purine antagonists azathioprine (AZA) and 6-mercaptopurine (6-MP) are widely used drugs in the treatment of IBD. In the present study, the hitherto unknown effects of AZA as well as its metabolites 6-MP and 6-thioguanine (6-TG) on repair mechanisms and apoptosis of intestinal epithelia were analysed.

Intestinal epithelial cell lines (human Caco-2, T-84 and HT-29 cells, rat IEC-6 cells) were incubated with AZA, 6-MP or 6-TG for 24 h (final concentrations 0.1 – 10 μM). Migration of Caco-2 and IEC-6 cells was analysed by in vitro restitution assays. Caco-2 and IEC-6 cell proliferation was evaluated by measurement of [^{3}H]thymidine incorporation into DNA. Apoptosis of Caco-2, T-84, HT-29 and IEC-6 cells was assessed by histone ELISA, 4′6′diamidino-2′phenylindole-dihydrochloride staining as well as flow cytometric analysis of Annexin V/propidium iodide (PI)-stained cells. Cell cycle progression was evaluated by PI staining and flow cytometry.

Epithelial restitution was not significantly affected by any of the substances tested. However, proliferation of intestinal epithelial cells was inhibited in a dose-dependent manner (maximal effect 92%) by AZA, 6-MP as well as 6-TG. In HT-29 cells, purine antagonist-effected inhibition of cell proliferation was explained by a cell cycle arrest in the G2 phase. In contrast, AZA, 6-MP and 6-TG induced no cell cycle arrest in Caco-2, T-84 and IEC-6 cells. AZA, 6-MP as well as 6-TG induced apoptosis in the non-transformed IEC-6 cell line but not in human Caco-2, T-84 and HT-29 cells.

In summary, AZA and its metabolites exert no significant effect on intestinal epithelial restitution. However, they profoundly inhibit intestinal epithelial cell growth via various mechanisms: they cause a G2 cell cycle arrest in HT-29 cells, induce apoptosis in IEC-6 cells and dose-dependently inhibit intestinal epithelial proliferation.

Keywords: Intestinal epithelium; Proliferation; Apoptosis; Restitution; Immunosuppression; Azathioprine; Inflammatory bowel diseases

1. Introduction

Azathioprine (AZA) and its first metabolite 6-mercaptopurine (6-MP) are important pharmacologic agents used for the therapy of a variety of diseases, including autoimmune hepatitis [1], rheumatoid arthritis [2,3], collagenoses [4,5], multiple sclerosis [6,7] and myasthenic syndromes [8] as well as for immunosuppression following organ transplantation [9]. 6-Thioguanine (6-TG), an important metabolite of AZA, is frequently used in the treatment of leukemia [10,11].

Abbreviations: AZA, azathioprine; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell scanner; FITC, fluorescein (isothiocyanate form); IBD, inflammatory bowel disease; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine.

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The purine analogues AZA and 6-MP are commonly used immunosuppressive drugs for therapy of patients with steroid-resistant or steroid-dependent courses of chronic active inflammatory bowel diseases (IBD), as well as for maintenance of induced remission [12–14]. 6-TG is currently discussed as a therapeutic alternative for AZA and 6-MP in the treatment of IBD since 6-TG has been shown to be effective in patients resistant or intolerant to therapy with AZA or 6-MP [15–19].

Erosions and ulcerations of the intestinal mucosa are typical defects in patients with IBD. Damage to the epithelium can be caused by numerous factors, including mechanical injury, irradiation, bacteria, viruses, toxins or drugs [20].

After injury of the intestinal mucosa, continuity of the intestinal barrier is reestablished in a well regulated healing process involving migration of epithelial cells from the wound margin into the denuded area, a process termed restitution [20–23] followed by cell proliferation [21,24]. In addition, apoptosis of intestinal epithelial cells may influence mucosal wound healing [20].

Since AZA and its metabolites are widely used therapeutics for IBD, it is of great interest to study their effects on wound healing mechanisms of intestinal epithelial cells. This appears to be clinically relevant because treatment with AZA has been shown to promote healing of intestinal lesions in IBD [12,25] and to decrease intestinal permeability in a small cohort of patients with Crohn's disease [26].

The purine analogues AZA, 6-MP and 6-TG interfere with purine neosynthesis, second messengers, RNA and DNA synthesis [27–29] and $CD4^+$ T lymphocyte apoptosis [30]. However, the exact mode of action of AZA and its metabolites is not fully understood. Their effects on wound healing mechanisms of the intestinal epithelium is largely unknown.

In the present study, the effects of AZA and its metabolites 6-MP and 6-TG on migration, proliferation and apoptosis of intestinal epithelial cells were evaluated.

2. Materials and methods

2.1. Reagents used

AZA and 6-MP were dissolved in PBS containing 0.1 mM NH_4OH; 6-TG was dissolved in PBS containing 0.4 mM NH_4OH (all reagents from Sigma, Deisenhofen, Germany). Drugs were prepared at a stock concentration of 20 mM; all purine analogues were used at final concentrations of 0.1 μM–10 μM. NH_4OH was used as vehicle control at corresponding concentrations. Deoxycholic acid (DC), camptothecine and mitomycin C were purchased from Sigma. Recombinant human hepatocyte growth factor (HGF, from R&D Systems, Wiesbaden, Germany) was used as positive control for proliferation and restitution assays. DC was dissolved at a stock concentration of 20 mM in 0.05 M NaOH and used at a final concentration of 250 μM. Camptothecine was dissolved in 0.5 M NaOH at a concentration of 2.9 mM (1 mg ml^{-1}) and used at a final concentration of 11.5 μM (4 μg ml^{-1}). Mitomycin C was dissolved in DMSO at a concentration of 3.0 mM (1 mg ml^{-1}) and used at a final concentration of 6.0 μM (2 μg ml^{-1}).

2.2. Cell culture

The intestinal epithelial model cell lines used in this study (human Caco-2, T-84 and HT-29 cells, rat IEC-6 cells) are established cell lines for the study of intestinal wound healing mechanisms [31–34].

Caco-2 cells (HTB 37, American Type Culture Collection, ATCC) were cultured in Minimum Essential Medium (MEM) containing 4 mM L-glutamine, 100 IU ml^{-1} penicillin/100 μg ml^{-1} streptomycin and 20% (vol/vol) heat-inactivated fetal bovine serum (FBS) (all from Invitrogen/Life Technologies, Karlsruhe, Germany) and used at 20–26th passages. IEC-6 cells (CRL 1592, ATCC) derived from rat jejunum crypt epithelium were used at 20–25th passages and cultured in Dulbecco's modified Eagle medium (DMEM) containing 4 mM L-glutamine, 100 IU ml^{-1} penicillin/100 μg ml^{-1} streptomycin, 5% (vol/vol) heat-inactivated FBS and 5 μg ml^{-1} insulin (Sigma). T-84 cells (CCL 248, ATCC) were cultured in DMEM nut mix® containing 4 mM L-glutamine, 100 IU ml^{-1} penicillin/100 μg ml^{-1} streptomycin and 5% (vol/vol) heat-inactivated FBS and used at 77–85th passages. HT-29 cells (HTB 38, ATCC) were cultured in McCoy's medium containing 4 mM L-glutamine, 100 IU ml^{-1} penicillin/100 μg ml^{-1} streptomycin and 10% (vol/vol) heat-inactivated FBS and used at 134–140th passages. All cells were cultured at 37 °C and 5% CO_2 (vol/vol) (Inkubator HeraCell, Hermes, Germany).

2.3. Intestinal epithelial in vitro restitution assays

Restitution assays were performed by a modification of a previously described technique [31,34–36]. Before use, multiple parallel reference lines were stencilled across the outer bottom of 6-well plates (Greiner, Frickenhausen, Germany). Intestinal epithelial cells (Caco-2 and IEC-6, respectively) were cultured to confluency, washed and then cultured in serum-deprived (containing 0.1% FBS) medium for 15 h. Four parallel standardized wounds perpendicular to the positioning marks were generated using a sterile pipette tip (diameter 1.0 mm). Immediately after scraping the monolayer, supernatants were aspirated in order to remove cellular debris followed by addition of fresh serum-deprived medium containing AZA, 6-MP, 6-TG or control medium. Cells stimulated with 25 ng ml^{-1} HGF were used as positive controls. The distance of the wound margins was measured with a micrometer at predetermined locations (to avoid observer bias) immediately and 24 (Caco-2) or 18 (IEC-6) h,