ELSEVIER

European Journal of Pharmacology 341 (1998) 289–297

ejp

Adenosine modulates cell proliferation in human colonic adenocarcinoma. I. Possible involvement of adenosine A_1 receptor subtypes in HT29 cells

Vincent Lelièvre [a,b], Jean-Marc Muller [a], Jack Falcón [b,*]

[a] *Laboratoire de Biologie des Interactions Cellulaires, CNRS UMR 6558, Université de Poitiers, 40 avenue du Recteur Pineau, 86022 Poitiers Cédex, France*

[b] *Laboratoire de Neurobiologie Cellulaire, CNRS UMR 6558, Université de Poitiers, 40 avenue du Recteur Pineau, 86022 Poitiers Cédex, France*

Received 24 April 1997; revised 21 October 1997; accepted 24 October 1997

Abstract

Several lines of evidence suggest that extracellular adenosine interacting with specific cell surface receptors may influence cell growth and differentiation of cancer cells in culture. The data presented here demonstrate that various treatments of human colonic adenocarcinoma HT29 cells in the presence of exogenously added adenosine deaminase, which converts extracellular adenosine into inosine, resulted in a significant decrease of the proliferation. Cell growth inhibition was also observed in the presence of adenosine A_1 receptor antagonists. These various treatments also induced a significant elevation of basal intracellular cAMP levels. This strongly indicated that extracellular adenosine was maintaining low intracellular cAMP levels in HT29 cells. A partial pharmacological characterization of the binding of the adenosine A_1 receptor agonist [^{3}H]CCPA (2-chloro-*N*^{6}-cyclopentyl[2,3,4,5-^{3}H]adenosine), and the adenosine A_1 receptor antagonist [^{3}H]DPCPX (cyclopentyl-1,3-dipropyl[2,3-^{3}H]xanthine), to HT29 cells is also provided. Together the data support the idea that A_1-adenosine receptors are expressed in HT29 cells and might mediate part of the above described effects of adenosine on cell proliferation. © 1998 Elsevier Science B.V.

Keywords: Adenosine; Adenosine deaminase; HT29 cell; Cell growth; Adenosine A_1 receptor

1. Introduction

Colorectal cancer is a very common and lethal malignancy in western countries (Greenberg and Baron, 1993). Colonic tumorigenesis is now recognized as a multistep process associated with profound but quite reproducible genetic alterations (specific gene mutations, deletions of chromosomal regions and loss of heterozigosity) (Fearon and Vogelstein, 1990). Concurrently, metabolic disorders leading to modifications in the function and expression of various cellular components, such as proto-oncogenes involved in intracellular signalling, transcription factors or cell to cell recognition molecules, take place during this process (Rodrigues et al., 1990; Nagai et al., 1992).

In this context, the study of in vitro behavior of human colonic adenocarcinoma cell lines, like HT29, appears particularly relevant to the investigation of the molecular mechanisms of colonic tumorigenesis. In spite of their undifferentiated aspect in glucose-containing medium, HT29 cells, first isolated by Fogh and Trempe (1975), display some interesting properties in culture: secretion of extracellular matrix, rapid growth (with a generation time of about 24 h), expression of cell surface receptors for a variety of extracellular messengers and ability to set up adaptative processes in response to their environment (Zweibaum et al., 1991). The last property, which allows the cells to develop phenotypic features of enterocytes, is usually correlated to a reduced growth rate. This phenomenon has been widely explored by several research groups, leading to the observation of enterocyte-like differentiation of HT29 cells in glucose-free or sodium butyrate medium and to the isolation of HT29 subclones displaying enterocytic phenotypes of mucus-secreting or absorptive cells (Huet et al., 1987; Phillips et al., 1988). To date, most of the drugs tested for their ability to trigger differentiation

* Corresponding author. Tel.: +33-549453977; fax: +33-549454051; e-mail: falcon@cri.univ-poitiers.fr.

and to modulate growth of HT29 cells were components which interfered with cell metabolism and energy (Rousset et al., 1979; Weinhouse, 1982). These drugs include chemotherapeutic substances like methotrexate (Lesuffleur et al., 1990). We were here interested in investigating whether HT29 cells in culture may be influenced by extracellular adenosine.

Adenosine is an essential and ubiquitous nucleoside, taking part in the metabolism of nucleic acids, second messengers, cofactors of methyltransferases and ATP regeneration (Meghji, 1991). Furthermore, adenosine is also a well-known extracellular messenger. This auto/paracrine factor binds cell-surface receptors coupled with adenylate cyclase: The A_1 and A_3 receptor subtypes mediate inhibition, while the A_2 receptor subtype mediates activation of the cyclase. They all belong to the so called 'R7G' (seven transmembrane spanning domains, G protein coupled) receptor family (Olah and Stiles, 1995).

There are indications that adenosine may modulate cell proliferation and differentiation in human endothelial, astrocytoma and epidermoid carcinoma cells in culture (Rathbone et al., 1992; Tey et al., 1992; Ethier et al., 1993). In addition, A_1 and A_2 adenosine receptors have been characterized in the digestive tract (Reymann and Gniess, 1988; Barrett et al., 1989; Strohmeier et al., 1995). These observations prompted the present investigation with human HT29 cells. We analyzed the effects of extracellular adenosine depletion and of adenosine A_1 receptor antagonists on HT29 cAMP levels and proliferation. We also report that an adenosine A_1-selective receptor agonist and an adenosine A_1-selective receptor antagonist bind specifically to these cells.

2. Materials and methods

2.1. Chemicals and materials

2-Chloro-N^6-cyclopentyl[2,3,4,5-^{3}H]-adenosine: [^{3}H]CCPA, was obtained from Amersham (Life Science S.A, Les Ulis), [^{125}I]cAMP radio-immunoassay kit and cyclopentyl-1,3-dipropyl[2,3-^{3}H]xanthine: [^{3}H]DPCPX, were from NEN Dupont Research Products (Les Ulis). Bacitracin, fetal calf serum, Glutamax I Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, trypsin-EDTA were obtained from GibcoBRL (Life technologies, Eragny). Optiphase II scintillant was from EG and G Division Instruments (Evry). Adenosine deaminase type VI from calf intestinal mucosa: EC 3.5.4.4, bovine serum albumin, 2-chloroadenosine (CLAD), N^6-cyclohexyladenosine (CHA), N^6-cyclopentyladenosine (CPA), cyclopentyl-1,3-dipropylxanthine (DPCPX), dimethylsulfoxide, HEPES, 5′-(*N*-ethyl)-carboxamidoadenosine (NECA), *S*-(*p*-nitrobenzyl)-6-thioinosine, N^6-phenylisopropyladenosine (R-PIA), phenylmethyl–sulfonyl–fluoride, 8-phenyltheophylline (8-PT), Triton X100, were purchased from Sigma (Saint Quentin). Trichloracetic acid was from Merck-Clévenot S.A. (Nogent-sur-Marne). Xanthine amine congener (XAC) was from RBI (Bioblock Scientific, Illkirch).

2.2. Cell culture

HT29 cells were routinely cultured in 75 cm^2 culture flasks in Glutamax I DMEM (25 mM glucose, without sodium pyruvate) supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin (respectively, 100 U/ml and 0.1 mg/ml), in a humidified atmosphere of air/CO_2 (95%/5%), at 37°C. Culture medium was removed and replaced by fresh medium every 3 days. For subcultures, cells were harvested in trypsin-EDTA solution (respectively, 0.05%/0.53 mM) in Puck's buffer, for 10 min at 37°C. One to 3 days before the day of the experiment, cells were seeded at the desired density (see below) on 24-well plates. The culture dishes used were previously coated with HT29 extracellular matrix to optimize cell attachment and spreading, according to Bellot et al. (1985). In brief, HT29 cells (0.1×10^6) were seeded in 24-well dishes and cultured to reach confluency. Cells were then removed by solubilization in 0.3 ml of Triton X100 (0.1%) in phosphate buffered saline (PBS). The wells coated with extracellular matrix were then washed 3 times with sterile PBS. Precoated 24-well plates were kept at −18°C for several months.

2.3. Proliferation assays

Dishes were seeded with 10000 cells/well in 1 ml of culture medium. After 24 h, a daily addition (10 μl from a 100-fold concentrated solution) of specified drugs, or of vehicle, was initiated. The vehicle was glycerol (0.3% final concentration) in experiments using adenosine deaminase, and dimethylsulfoxide (0.01% final concentration) in experiments using adenosine analogs. The culture medium was replaced every 3 days. At the end of the treatments, the cells were harvested in 500 μl trypsin/EDTA. Protease activity was then stopped by the addition of 500 μl of culture medium and the cells were counted by double direct counting. Each treatment was run in triplicate. Cell viability was evaluated with the trypan blue extrusion test. Mortality never exceeded 1%. Cell counting in the supernatant indicated that detachment had not occurred.

2.4. Binding of adenosine analogs in HT29 cells

Dishes were seeded at the density of 2×10^5 cells per well. After three days, medium was removed and the cells were incubated with fresh culture medium supplemented with adenosine deaminase 2 U/ml in order to convert endogenous extracellular adenosine into the pharmacologically inactive inosine. One hour later, the medium was removed and the cells were incubated in the binding

Table 1
Growth parameters (latency and doubling time) of HT29 cells under control conditions or in the presence of either adenosine deaminase or xanthine amine congener or 8-phenyltheophylline

	Latency (h)	Doubling time (h)
Control	26	25
ADA 2 U/ml	36[b]	26[c]
ADA 5 U/ml	36[b,c]	35[b,c]
ADA 10 U/ml	52[b]	60[b]
XAC 1 nM	25[c]	39[b,c]
XAC 1 μM	39[b]	52[b]
ADA 5 U/ml + XAC 1 nM	42[b,c]	46[b,c]
ADA 10 U/ml + XAC 1 nM	50[b]	62[b]
8-PT 1 nM	32[b,c]	32[a,c]
8-PT 1 μM	40[b]	46[b]

Drugs were added daily in the culture medium. Calculations were made from data presented in Fig. 1. ADA: adenosine; XAC: xanthine amine congener, 8-PT: 8-phenyltheophylline.
Values were compared to the control ([a]$P < 0.002$, [b]$P < 0.00005$) or between them within a group of treatments ([c]$P < 0.00005$). The values for the [ADA 5 U/ml + XAC 1 nM] group were significantly different from those for the ADA 5 U/ml[a], and XAC 1 nM[b] groups.

buffer, consisting of 300 μl of DMEM containing 15 mM phenylmethyl–sulfonyl–fluoride, 150 μM HEPES, 2 U/ml adenosine deaminase, 0.1% bacitracin, 1% bovine serum albumine, and [^{3}H]CCPA (39 Ci/mmol) or [^{3}H]DPCPX (90 Ci/mmol) at the desired concentration. Typically, assays were performed in triplicate at 4°C for 150 min. In the kinetics and displacement experiments the radioligand concentration was 10 nM ([^{3}H]CCPA), or 5 nM ([^{3}H]DPCPX). Non-specific binding was determined by the addition of 50 μM of unlabelled CPA or DPCPX to the incubation medium. Other conditions are given in Section 3 and in the legend of the figures. At the end of the incubation, the cells were quickly washed 3 times with cold PBS (pH = 7.2). They were then lysed in 300 μl NaOH (0.5 M) for 30 min. Radioactivity in the extracts was measured with a β scintillation counter (Beckman LS6500), using the Optiphase II scintillation cocktail.

2.5. Analysis of intracellular cAMP levels in HT29 cells

Cells were seeded at the density of 1.5×10^6 cells/well. After 3 days, the culture medium was replaced by 270 μl of fresh medium supplemented or not with the drugs to be tested. At the end of the incubation period, the plates were placed on ice, and the medium was replaced with 300 μl of a 6% trichloroacetic acid solution. The cells were lysed by direct sonication in the wells and the extracts were evaporated. The resulting pellets were dissolved in sodium acetate buffer (0.05 M, pH = 5.9). After a short centrifugation, cAMP in the supernatants was quantitated using a commercially available cAMP radioimmunoassay, using [^{125}I]cAMP as a tracer. Counting was done in a γ-counter (Wallac; EG and G Instruments, Evry).

2.6. Statistical analysis and curve plots

All data were analyzed using the iterative curve-fitting program GRAPHPAD™ (ISI software). Data from the proliferation and displacement experiments were fitted to a Hill equation. Those from the kinetics and saturation experiments were fitted to the equation of the rectangular hyperbola. In the proliferation experiments, latency was defined as the time necessary to reach the first doubling of the population, and the doubling time was determined after linearization (log[cell number] = $F(t)$). The data were compared using an analysis of variance (Anova).

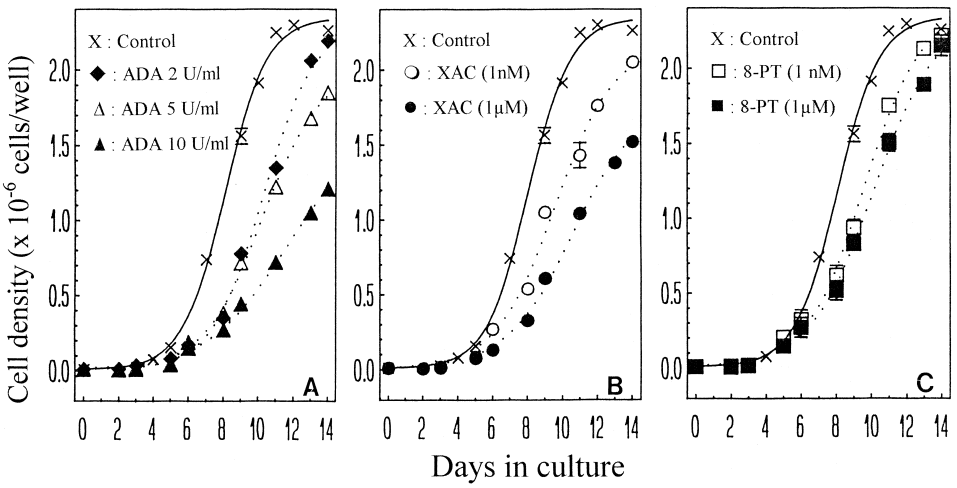

Fig. 1. Action of adenosine deaminase and of adenosine A_1 receptor antagonists on the proliferation of HT29 cells in culture. Cells (10,000/well) were seeded on extracellular matrix coated dishes and drugs were added daily as indicated in Section 2: adenosine deaminase (A), XAC (B, xanthine amine congener), 8-PT (C, 8-phenyltheophylline). Data are the means ± S.E.M. of 3 independent experiments, each performed in triplicate, and curve-fitting was computer-assisted according to a Hill plot equation (GRAPHPAD™).

3. Results

3.1. Effects of adenosine deaminase and of adenosine A_1 receptor antagonists on HT29 cell proliferation

In order to facilitate the reading of this section, the values of the SNEDECOR F and corresponding levels of significance have not been included in the text. However, the reader should keep in mind that all significant differences had $P < 0.05$, $P < 0.002$ or $P < 0.00005$ (see Table 1).

The number of cells in the control dishes, low during the first 3 days, increased rapidly from day 6 to reach confluency after day 11 (Fig. 1A). Adenosine deaminase induced a dose-dependent decrease in cell proliferation, which was apparent from day 5 (Fig. 1A). Thus, at day 11 of culture, the number of cells in the treated groups was 75% (adenosine deaminase 2 U/ml), 71% (adenosine deaminase 5 U/ml) and 42% (adenosine deaminase 10 U/ml) of the number of cells in the controls. These effects were also evident when one considered the doubling time and the latency (Table 1). For example, at the higher concentration of adenosine deaminase, these parameters were twice as high as in the controls (Table 1). The presence of the antagonists xanthine amine congener ($A_1 \gg A_2$) or 8-phenyltheophylline ($A_1 = A_2$), also resulted in a significant reduction of cell proliferation, at the concentration of 1 nM or 1 μM (Fig. 1B and C), accompanied by an increase in doubling time and latency (Table 1). Xanthine amine congener was always more potent than 8-phenyltheophylline: at the concentration of 1 μM, xanthine amine congener induced a 41% reduction in the number of cells counted at day 11 of the culture, whereas the presence of 8-phenyltheophylline induced a 19% reduction (Fig. 1B and C). The effects of xanthine amine congener (1 nM) and adenosine deaminase were additive

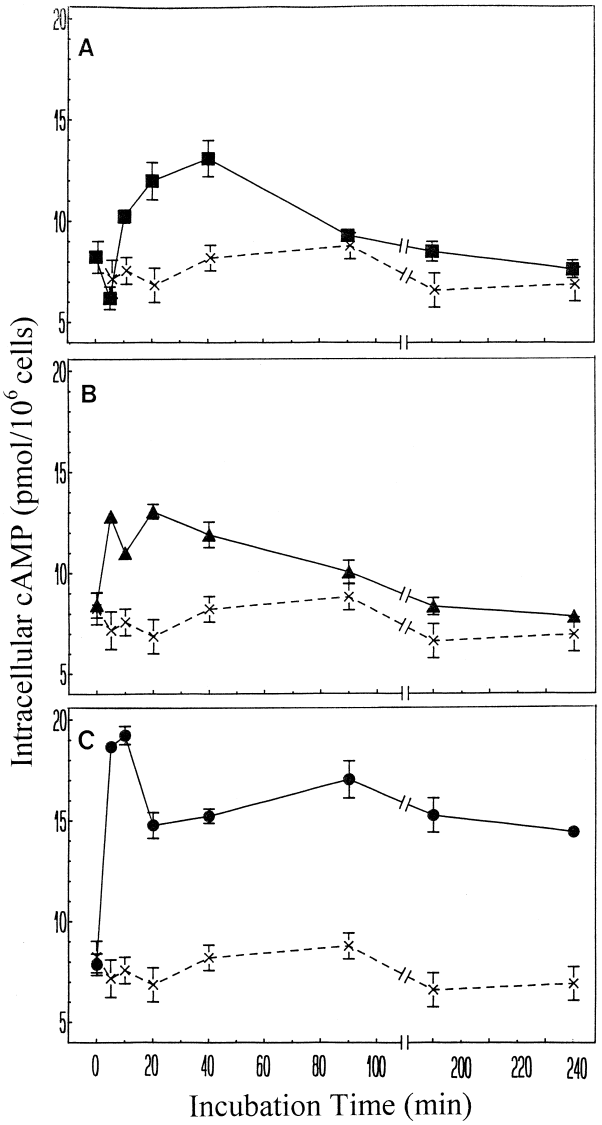

Fig. 2. Effects of adenosine deaminase on intracellular cAMP production in HT29 cells. Cells (150,000/well) were seeded in 24-well dishes and cultured for 3 days, then adenosine deaminase was added in the culture medium at the concentrations of 2 (■; A), 5 (▲; B) or 10 (●; C) U/ml. Basal cAMP levels were determined in each experiment (×). Data are the means ± S.E.M. of 2 independent experiments, each performed in triplicate.

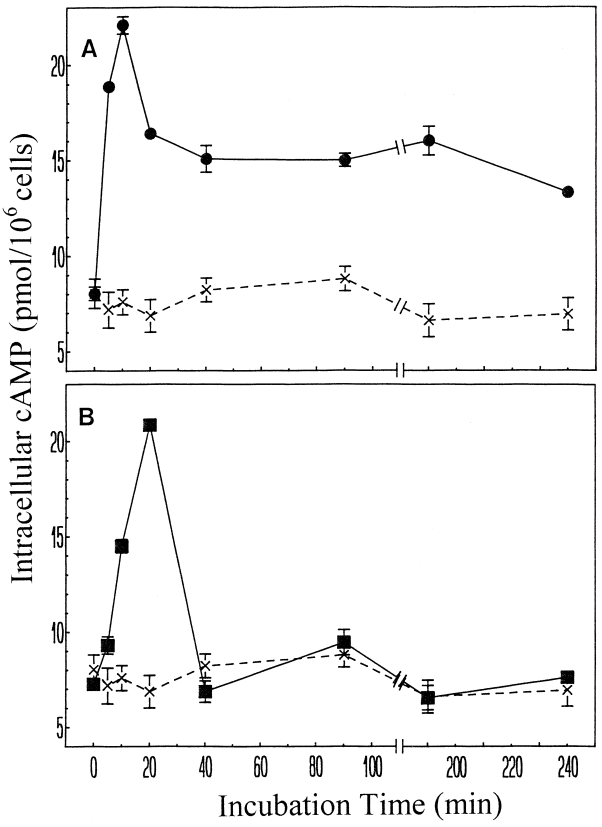

Fig. 3. Effects of adenosine A_1 receptor antagonists on intracellular cAMP production in HT29 cells. Same conditions as in Fig. 2. xanthine amine congener (●; A) or 8-phenyltheophylline (■; B) was used at the concentration of 1 μM. Basal cAMP levels were determined in each experiment (×). Data are the means ± S.E.M. of 2 independent experiments, each performed in triplicate.

at an adenosine deaminase concentration of 5 U/ml but not 10 U/ml (Table 1). Also, at the concentration of 10 nM, both CPA and NECA were able to counteract, albeit partially, the effects of adenosine deaminase (5 U/ml) (data not shown). Under similar conditions, the adenosine transmembrane carrier inhibitor (nitrobenzyl-6-thioinosine, 1 μM) had no significant effect on cell proliferation (data not shown).

3.2. Effects of adenosine deaminase and adenosine A_1 receptor antagonists on intracellular cAMP levels

The effect of adenosine deaminase (Fig. 2) or xanthine amine congener and 8-phenyltheophylline (Fig. 3) on intracellular cAMP accumulation was assayed with incubation times ranging from 0 to 240 min, at 37°C. Cyclic AMP levels in control cells remained rather stable during the whole incubation time, with mean basal levels of about 8 pmol/10^6 cells. In the presence of adenosine deaminase 2 U/ml, cAMP levels increased significantly after a lag time of 10 min, to reach a maximal level after 40 min of incubation. Thereafter the cAMP content decreased progressively, returning to its basal values after 90 min of incubation. Rather similar results were obtained in the presence of adenosine deaminase 5 U/ml. The major difference concerned the absence of latency (compare Fig. 2A and B) and the amplitude of the response that was slightly increased. Adenosine deaminase 10 U/ml induced the strongest response in terms of amplitude, with almost no detectable latency (Fig. 2C). Furthermore, under these conditions the cAMP levels remained high, and never returned to their basal levels during the entire duration of the experiment.

The adenosine receptor antagonist, xanthine amine congener (1 μM), induced an immediate and strong increase in cAMP content, reaching maximal levels at 10 min,

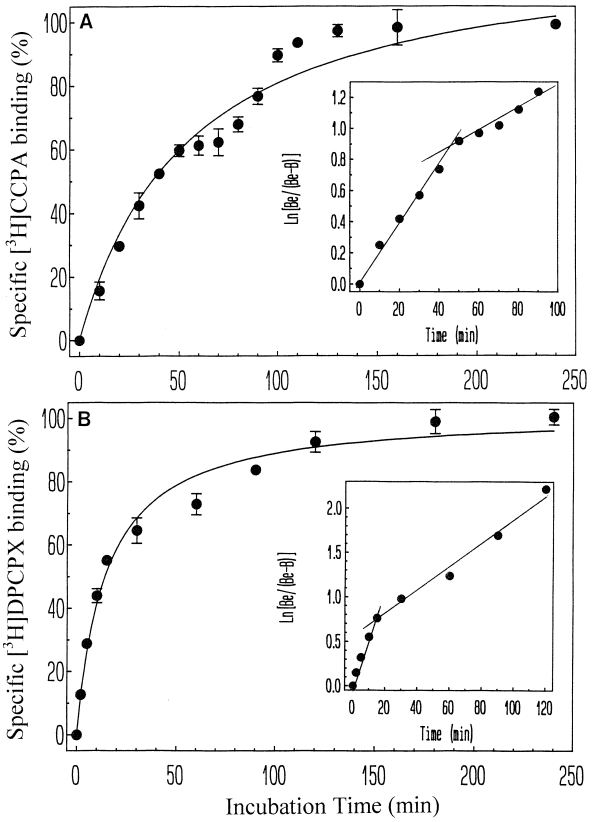

Fig. 4. Time course of [^{3}H]CCPA and of [^{3}H]DPCPX binding to HT29 cells. Cells (200,000/well) were seeded in 24-well dishes and cultured for 3 days. Then, they were incubated in presence of 10 nM [^{3}H]CCPA (A) or 5 nM [^{3}H]DPCPX (B) for the indicated times, at +4°C. Data are in percent of the maximal specific binding and represent the means ± S.E.M. of 3 independent experiments, each performed in triplicate. The non-specific interaction was measured in the presence of 50 μM of unlabelled cyclopentyl-adenosine (A) or cyclopentyl-1,3-dipropylxanthine (B) (not shown; it did not exceed 10% of the specific binding). Data were fitted to the rectangular hyperbola equation (Graphpad™) or linearized (insets) as indicated in Section 2.

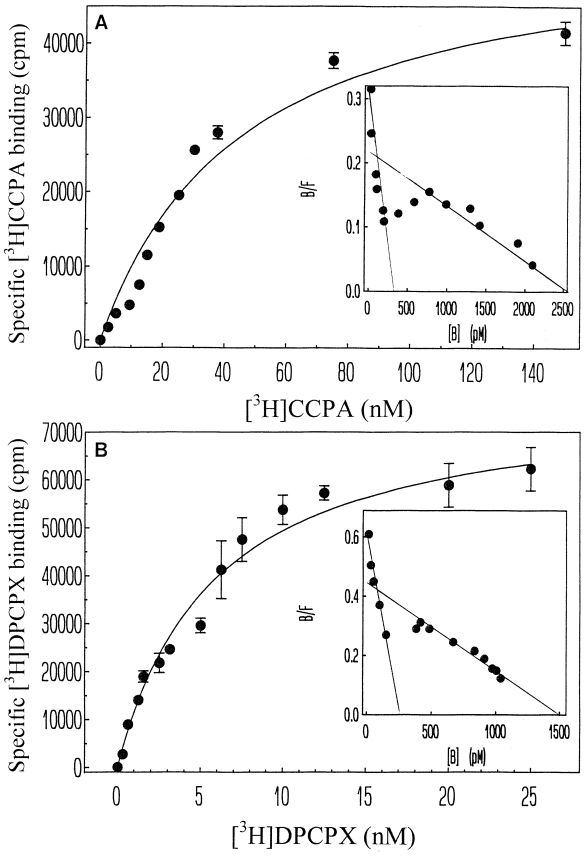

Fig. 5. Saturation isotherm and Scatchard transformation of the binding of [^{3}H]CCPA and [^{3}H]DPCPX to HT29 cells. Cells (200,000/well) were seeded in 24-well dishes and cultured for 3 days. Then, they were incubated in presence of increasing concentrations of [^{3}H]CCPA (A) or [^{3}H]DPCPX (B) for 150 min, at 4°C. Plots correspond to the specific binding (total minus non-specific). The non-specific interaction was measured in the presence of 50 μM of unlabelled cyclopentyl-adenosine (A) or cyclopentyl-1,3-dipropylxanthine (B) (not shown; it did not exceed 10% of the specific binding). Mean ± S.E.M. of 2 independent experiments, each performed in triplicate. Data were fitted to the rectangular hyperbola equation (Graphpad™), or linearized according to the Scatchard transformation (insets).

followed by a slow decay. Then the effect of xanthine amine congener persisted for the whole duration of the experiment (Fig. 3A). Addition of the antagonist, 8-phenyltheophylline (1 μM), induced a transient increase in cAMP content, starting at 5 min and ending at 40 min of incubation, with a peak value at 20 min (Fig. 3B).

Cell treatment in the presence of the transmembrane adenosine transport inhibitor nitrobenzyl-6-thioinosine (1 μM) never modified the basal intracellular cAMP levels under our experimental conditions (data not shown).

3.3. Binding of the A_1-receptor specific radioligands [^{3}H]CCPA and [^{3}H]DPCPX to HT29 cells

Both [^{3}H]CCPA (Fig. 4A) or [^{3}H]DPCPX (Fig. 4B) bound specifically to HT29 cells, in a time-dependent and saturable fashion. The non-specific binding never exceeded 10% of the specific binding. Under the experimental conditions chosen, equilibrium was reached after 150 min at 4°C. The kinetics of the association revealed two components which were clearly evidenced after linearization of the data (insets in Fig. 4). The apparent association constants (Kobs) obtained after computer assisted analysis of the data were 0.018 ± 0.0009 min^{-1} and 0.008 ± 0.001 min^{-1} for [^{3}H]CCPA, and 0.05 ± 0.0036 min^{-1} and 0.0015 ± 0.0019 min^{-1} for [^{3}H]DPCPX.

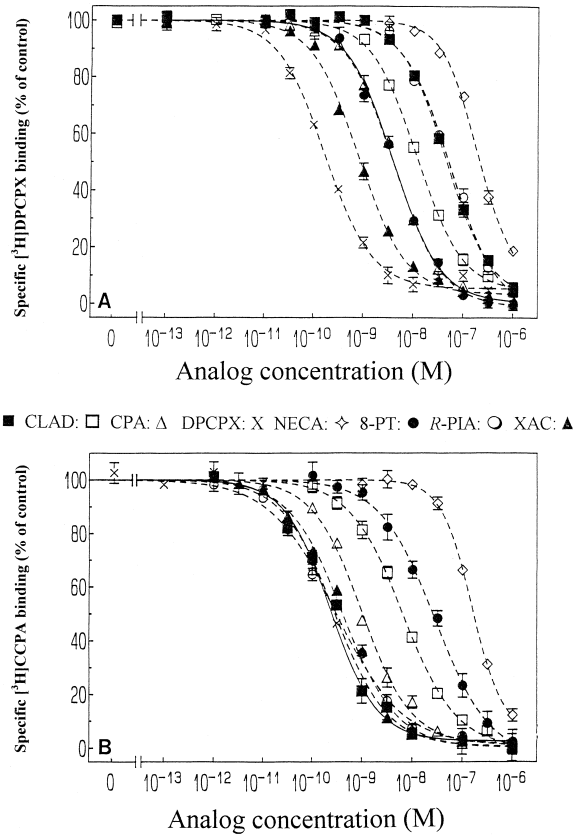

Fig. 6. Competitive inhibition of [^{3}H]CCPA or [^{3}H]DPCPX binding by adenosine analogs in HT29 cells. Cells (200,000/well) were seeded in 24-well dishes and cultured for 3 days. Then cells were incubated for 150 min at 4°C, in the presence of 10 nM of [^{3}H]CCPA (A) or 5 nM [^{3}H]DPCPX (B), and of increasing concentrations of either cyclohexyl–adenosine (CHA; ■), 2-chloro-adenosine (CLAD; □), cyclopentyl–adenosine (CPA; △), *N*-ethylcarboxamido–adenosine (NECA; ◇), *R*-phenylisopropyl–adenosine (R-PIA; ○), xanthine amine congener (XAC; ▲), cyclopentyl-1,3-dipropylxanthine (DPCPX; ×), or 8-phenyltheophylline (8-PT; ●). Results are expressed as the percentage of the maximal specific binding obtained in absence of unlabelled analogs. Non-specific binding was assessed in the presence of 50 μM unlabelled CPA (A) or DPCPX (B). Data are the means of four independent experiments, each performed in triplicate. Curve fitting was computed according to a Hill plot equation (GRAPHPAD™).

Table 2
Displacement of the [^{3}H]CCPA (10 nM) or [^{3}H]DPCPX (5 nM) binding by unlabelled adenosine analogs

Adenosine analogues	[^{3}H]CCPA		[^{3}H]DPCPX	
	IC_{50} (nM)	Hill number	IC_{50} (nM)	Hill number
CHA	58 ± 0.050	0.90	0.28 ± 0.056	0.81
CLAD	10.8 ± 0.043	0.87	7.4 ± 0.075	0.73
CPA	4.3 ± 0.061	0.91	0.95 ± 0.071	0.87
DPCPX	0.17 ± 0.085	0.92	0.2 ± 0.084	0.62
NECA	500 ± 0.061	0.85	152 ± 0.096	0.94
8-PT	4.5 ± 0.067	0.89	46 ± 0.077	0.85
R-PIA	62 ± 0.052	0.94	0.3 ± 0.082	0.71
XAC	0.5 ± 0.065	0.88	0.46 ± 0.056	0.81

IC_{50} ($\pm$S.E.M.) and Hill values were obtained from the curves presented in Fig. 6.
CCPA: 2-Chloro-N^6-cyclopentyl-adenosine, CLAD: 2-chloroadenosine, CHA: N^6-cyclohexyladenosine, CPA: N^6-cyclopentyladenosine, DPCPX: cyclopentyl-1,3-dipropylxanthine, NECA: 5′-(*N*-ethyl)-carboxamidoadenosine, R-PIA: N^6-phenylisopropyladenosine, 8-PT: 8-phenyltheophylline, XAC: Xanthine amine congener.

After 150 min of incubation at 4°C, the non-specific binding of [^{3}H]CCPA and of [^{3}H]DPCPX increased linearly with increasing concentrations of radioligand (not shown). Under these conditions, the specific binding was saturable (Fig. 5). Scatchard replotting of the data revealed two classes of binding sites. The affinity constants for [^{3}H]CCPA binding were 6.225 ± 0.009 and 77 ± 0.02 nM, corresponding, respectively, to 34,000 and 373,000 sites/cell. The affinity constants for [^{3}H]DPCPX binding were 0.52 ± 0.015 and 12.3 ± 0.056 nM, corresponding, respectively, to 18,200 and 270,000 sites/cell.

Competitive displacement experiments were run for 150 min at 4°C using 10 [^{3}H]CCPA (Fig. 6A) or 5 nM [^{3}H]DPCPX (Fig. 6B). Increasing concentrations of unlabelled analogues displaced [^{3}H]CCPA binding, with the following order of potency (Table 2):

DPCPX > XAC > CPA > CLAD > 8-PT > CHA $\approx$ *R*-PIA $\gg$ NECA.

As evidenced in Table 2, Hill coefficients were between 0.85 and 0.94 for all compounds tested. The same analogues displaced [^{3}H]DPCPX binding, with the following order of potency (Table 2):

DPCPX > CHA $\approx$ *R*-PIA > XAC > CPA $\gg$ CLAD $\gg$ 8-PT $\gg$ NECA

As evidenced in Table 2, Hill coefficients between 0.6 and 0.94 were obtained for all compounds tested.

4. Discussion

The aim of the present study was to investigate whether endogenous extracellular adenosine could modulate, in an autocrine/paracrine fashion, cell proliferation in the human colonic adenocarcinoma cell line HT29. This was studied using adenosine deaminase which converts adenosine into its catabolite, inosine. In these experimental series, the medium was changed only every three days, but adenosine deaminase was added daily to compensate for possible degradation of the enzyme. Assuming that adenosine deaminase was not inactivated at all during each daily addition, this treatment did not result in an exaggerated final concentration of the enzyme (at the most 3-fold the initial one). These experiments were run, concurrently, under the same conditions, in presence of adenosine A_1 receptor antagonists.

Adenosine deaminase reduced cell proliferation efficiently in a dose-dependent manner. This phenomenon appeared to result both from an increase in the lag time and, at least for the higher adenosine deaminase concentrations, from a decrease in growth rate. Neither enhanced cell detachment, as indicated by counting of the cells in the culture medium, nor an increased cell death, as evaluated by the trypan blue exclusion method, could account for these effects. Because extracellular adenosine acts through cell surface receptors, the effects of adenosine A_1 receptor antagonists were also investigated on HT29 cell proliferation. Both xanthine amine congener (highly selective for the adenosine A_1 receptor subtype), and 8-phenyltheophylline (non-selective for the adenosine A_1 and A_2 receptor subtypes) mimicked the action of adenosine deaminase by inducing a marked reduction of cell proliferation. Since neither compound is supposed to enter the cells, it is strongly suggested that the effects of xanthine amine congener and of 8-phenyltheophylline were due to their antagonistic interaction with cell surface adenosine receptors. In other words, it may be suggested that in HT29 cells, extracellular adenosine might modulate proliferation through its autocrine/paracrine action on specific cell surface receptors. In HT29 cells, the very strong cell growth inhibition observed in the presence of extracellular adenosine deaminase, together with the fact that low concentrations of antagonists were poorly efficient to reduce cell proliferation, might reflect an exacerbated production of adenosine by these cells. Moreover, the effects of xanthine amine congener and adenosine deaminase were additive except at the higher concentration of adenosine deaminase used. This further supports our above conclusions and suggests that only the higher concentration of adenosine deaminase was able to suppress extracellular adenosine totally. Our results agree with previous observations describing the modulation of astroglial cell proliferation by adenosine analogs (Christjanson et al., 1993; Abbracchio et al., 1994).

Adenosine A_1 receptors are negatively coupled to adenylate cyclase, while A_2 receptors are positively coupled to it. Thus, removal of endogenous adenosine by adenosine deaminase should result in an increased intracellular cAMP production if the nucleoside was interacting strictly with adenosine A_1 receptors, while a decreased intracellular cAMP concentration should be expected if it was interacting with adenosine A_2 receptors. In our experiments, treatment of HT29 cells with adenosine deaminase resulted in a time- and dose-dependent increase in intracellular cAMP content. This suggested that the major impact of endogenous extracellular adenosine was to maintain low intracellular cAMP levels, possibly by interacting with adenosine A_1 receptors. Long lasting effects of adenosine deaminase on the stimulation of cAMP production were observed only at the maximal concentration used (10 U/ml). This indicates that adenosine removal may be transient with lower concentrations, i.e., 2 and 5 U/ml. Alternatively, it is possible that the enzyme added in the extracellular medium was susceptible to degradation, resulting in faster inactivation at low adenosine deaminase concentrations. The fact that xanthine amine congener and 8-phenyltheophylline also induced a time-dependent increase in cAMP content, further supports the idea that adenosine A_1 receptors were involved. As observed in proliferation experiments, xanthine amine congener was more potent than 8-phenyltheophylline. These discrepancies might have resulted from the differential potencies of the antagonists towards the adenosine receptors ($A_1 \gg A_2$ for xanthine amine congener and $A_1 \approx A_2$ for 8-phenyltheophylline) (Williams, 1991). Although, this argues in favor of the involvement of adenosine A_1 receptors, the presence of A_2 receptors cannot be excluded.

Because our data strongly suggested the involvement of adenosine A_1 receptors, we looked for the presence of adenosine binding sites in HT29 cells, using the agonist [^{3}H]CCPA and the antagonist [^{3}H]DPCPX, both highly specific for the adenosine A_1 receptor subtype (Bruns et al., 1987; Lohse et al., 1988). With both radioligands, the kinetics of the association appeared biphasic, displaying a fast and a slow component. Such a complex phenomenon has already been reported to occur for the binding of [^{3}H]DPCPX and [^{3}H]CHA on membrane preparations from CHO cells (Cohen et al., 1996). In these cells, kinetics of the association became monophasic in the presence of GTP; the authors suggested the presence of two sub-populations of adenosine A_1 receptors, differing by their coupling state to G proteins (Cohen et al., 1996). This could well be the case for intact HT29 cells. It is also possible that the two components reflected the presence of two distinct populations of binding sites. It has been demonstrated that A_1 and A_2 receptors are present in human normal and tumoral colonic epithelia (Khoo et al., 1996), and A_2 receptors are present in T84 human colonic adenocarcinoma cells (Strohmeier et al., 1995). Our results with HT29 cells also suggest that 2 populations of binding sites were labeled by [^{3}H]DPCPX and [^{3}H]CCPA. Concerning

the specificity of the tritiated analogs used, together with the rank order of potency of the analogs to displace the specific binding, it is highly probable that the high-affinity sites correspond to the A_1 subtype. The second population of binding sites might be related to A_2 or A_3 receptors. However, the binding to A_3 receptors is unlikely to occur under our present conditions. Indeed it has been shown that A_3 receptors display little affinity for xanthine antagonists such as DPCPX and xanthine amine congener (Zhou et al., 1992).

The present study provides various lines of evidence demonstrating the importance of extracellular local adenosine in the regulation of human colonic cancer cell proliferation in culture. Indeed, removal of extracellular adenosine or addition of adenosine A_1 receptor antagonists which do not enter the cells, resulted in the reduction of proliferation. Although no clear causal relationship could be established, it appeared that treatments which reduced proliferation also resulted in an increase in intracellular cAMP, suggesting the involvement of A_1 receptors. The partial characterization of the [^{3}H]DPCPX and [^{3}H]CCPA binding sites further supports this view. It remains to be established which level(s) of the adenosine metabolism is (are) affected in HT29 cells. Is there an over-production of extracellular adenosine, a deficient expression in adenosine deaminase, as evidenced in severe combined immunodeficiency disease (Blaese et al., 1993), or a detuned balance of the adenosine A_1/A_2 receptor influence? Looking at the complexity of the radioligand binding to HT29 cells, a complete characterization of the adenosine receptors expressed by these cells vs. normal colonic epithelial cells might help to determine whether one, or several, of these receptors could be involved in the proliferative process.

Acknowledgements

This work was supported by the University of Poitiers, the CNRS and the 'Fondation Langlois' (Rennes). We are very grateful to Professor Yves Cenatiempo (IBMIG, CNRS URA 1172, Université de Poitiers) who allowed us to use the facilities of his laboratory. We also wish to thank F. Chevalier for her very helpful and kind technical assistance.

References

Abbracchio, M.P., Saffrey, M.J., Höpker, V., Burnstock, G., 1994. Modulation of astroglial cell proliferation by analogues of adenosine and ATP in primary cultures of rat striatum. Neuroscience 59 (1), 67–76.

Barrett, K.E., Huott, P.A., Shah, S.S., Dharmsathaphorn, K., Wasserman, S.I., 1989. Differing effects of apical and basolateral adenosine on colonic epithelial cell line T84. Am. J. Physiol. 256, C197–C203.

Bellot, F., Pichon, J., Luis, J., El Battari, A., Secchi, J., Cau, P., Marvaldi, J., 1985. Extracellular material secreted by human colonic adenocarcinoma cell lines (HT29 and HRT18) promotes their own very rapid spreading in serum free medium and induces neurite outgrowth of PC 12 cells. Int. J. Cancer 36, 609–615.

Blaese, R.M., Culver, K.W., Anderson, W.F., Nienhuis, A., Dunbar, C., Chang, L., Mullen, C., Carter, C., Leitman, S., 1993. Treatment of severe combined immunodeficiency disease (SCID) due to adenosine deaminase deficiency with CD34+ selected autologous peripheral blood cells transduced with a human ADA gene. Hum. Gene Ther. 4, 521–527.

Bruns, R.F., Fergus, J.H., Badber, E.W., Bristol, J.A., Santay, L.A., Hartman, J.D., Hats, S.J., Huang, C.C., 1987. Binding of the A_1-selective adenosine A_1 receptor antagonist [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. Naunyn-Schmiedeberg's Arch. Pharmacol. 335, 59–63.

Christjanson, L.J., Middlemiss, P.J., Rathbone, M.P., 1993. Stimulation of astrocytes proliferation by purine and pyrimidine nucleotides and nucleosides. Glia 7, 176–182.

Cohen, F.R., Lazareno, S., Birdsall, N.J.M., 1996. the effects of saponin on the binding and functional properties of the human adenosine A_1 receptor. Br. J. Pharmacol. 117, 1521–1529.

Ethier, M.F., Chander, V., Dobson, J.G. Jr., 1993. Adenosine stimulates proliferation of human endothelial cells in culture. Am. J. Physiol. 265, H131–138.

Fearon, E.R., Vogelstein, B., 1990. A genetic model for colorectal tumorigenesis. Cell 61, 759–767.

Fogh, J., Trempe, G., 1975. Human tumor cells in vitro. In: Fogh, J. (Ed.), Human Tumor Cells in Vitro. Plenum Publishing Corp. New York, pp. 115–159.

Greenberg, E.R., Baron, A., 1993. Prospects preventing colorectal cancer death. J. Natl. Cancer Inst. 85 (15), 1182–1184.

Huet, C., Sahuquillo-Merino, C., Coudrier, E., Louvard, D., 1987. Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. J. Cell. Biol. 105, 345–357.

Khoo, H.-E., Ho, C.-L., Chhatwal, V.J.S., Chan, S.T.F., Ngoi, S.-S., Moochhala, S.M., 1996. Differential expression of adenosine A_1 receptors in colorectal cancer and related mucosa. Cancer Lett. 106, 17–21.

Lesuffleur, T., Barbat, A., Dussaulx, E., Zweibaum, A., 1990. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. Cancer Res. 50, 6334–6339.

Lohse, M.J., Klotz, K.-N., Schwabe, U., Cristalli, G., Vittori, S., Grifantini, M., 1988. 2-chloro-N^6-cyclopentyladenosine: A high selective agonist at adenosine A_1 receptors. Arch. Pharmacol. 337, 687–689.

Meghji, P., 1991. Adenosine production and metabolism. In: Stone, T. (Ed.), Adenosine in the Nervous System. Academic Press., London, pp. 25–42.

Nagai, M.A., Habr-Gama, A., Oshima, C.T.F., Brentani, M.M., 1992. Association of genetic alterations of c-myc, c-fos, and c-Ha-ras proto-oncogenes in colorectal tumors. Dis. Colon Rectum 35, 444–451.

Olah, M.E., Stiles, G.L., 1995. Adenosine receptor subtypes: Characterization and therapeutic regulation. Annu. Rev. Pharmacol. Toxicol. 35, 581–606.

Phillips, T.E., Huet, C., Bilbo, P.R., Podolsky, D.K., Louvard, D., Neutra, M.R., 1988. Human intestinal goblet cells in monolayer culture: Characterization of a mucus-secreting subclone derived from the HT-29 colon adenocarcinoma cell line. Gastroenterology 94, 1390–1403.

Rathbone, M.P., Middlemiss, P.J., Kim, J.-K., Gysbers, J.W., DeForge, S.P., Smith, R.W., Hughes, D.W., 1992. Adenosine and its nucleotides stimulate proliferation of chick astrocytes and human astrocytoma cells. Neurosci. Res. 13, 1–17.

Reymann, A., Gniess, A., 1988. Evidence for adenosine A_1 receptor action in rat jejunal mucosa. Eur. J. Pharmacol. 149, 155–158.

Rodrigues, N.R., Rowan, A., Smith, M.E.F., Kerr, I.B., Bodmer, W.F., Gannon, J.V., Lane, D.P., 1990. p53 mutations in colorectal cancer. Proc. Natl. Sci. USA 87, 7555–7559.

Rousset, M., Chevalier, G., Rousset, J.P., Dussaulx, E., Zweibaum, A., 1979. Presence and cell-growth related variations of glycogen in human colorectal adenocarcinoma cell lines in culture. Cancer Res. 39, 531–534.

Strohmeier, G.R., Reppert, S.M., Lencer, W.I., Madara, J.L., 1995. The A_{2b} adenosine receptor mediates cAMP responses to adenosine receptors agonists in human intestinal epithelia. J. Biol. Chem. 270, 2387–2394.

Tey, H.B., Khoo, H.E., Tan, C.H., 1992. Adenosine modulates cell growth in human epidermoid carcinoma (A431) cells. Biochem. Biophys. Res. Commun. 187, 1486–1492.

Weinhouse, S., 1982. Changing perceptions of carbohydrate metabolism in tumors. In: Arnott, M.S., Van Eys, J., Wang, Y.M. (Eds.), Molecular Interrelations of Nutrition and Cancer. Raven Press., New York, pp. 167–177.

Williams, M., 1991. Adenosine receptor agonists and antagonists. In: Stone, T. (Ed.), Adenosine in the Nervous System. Academic Press, London, pp. 138–166.

Zhou, Q.-Y., Li, C., Olah, M.E., Johnson, R.A., Stiles, G.L., Civelli, O., 1992. Molecular cloning and characterization of an adenosine receptor: The A_3 adenosine receptor. Proc. Natl. Acad. Sci. USA 89, 7432–7436.

Zweibaum, A., Laburthe, M., Grasset, E., Louvard, D., 1991. Use of cultured cell lines in studies of intestinal cell differentiation and function. In: Field, M., Frizzel, R.A. (Eds.), Handbook of Physiology, The Gastrointestinal System, Intestinal Absorption and Secretion, Vol. 4. American Physiological Society, Washington DC, pp. 223–255.