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Extracellular adenosine deprivation induces epithelial differentiation of HT29 cells: evidence for a concomitant adenosine A_1/A_2 receptor balance regulation

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Abstract

HT29 cells display an undifferentiated phenotype in culture. However, numerous treatments are able to induce both epithelial differentiation and cell growth inhibition. We have previously demonstrated that adenosine and its analogues act through specific adenosine receptors to modulate cell proliferation in HT29 and other human colon adenocarcinoma cell lines. Among the treatments tested, the most potent inhibition of HT29 cell growth was induced by deprivation of extracellular adenosine using adenosine deaminase. Here, we investigated the capacity of adenosine deaminase to initiate epithelial differentiation. After 1 month of daily addition of 10 U/ml adenosine deaminase to the culture medium, HT29 cells were cloned by limited dilution. Among the clones obtained, we focused our attention on clone 13. Microscopic visualization and proliferation studies indicated that cells from this clone grew very slowly and in a pseudo-monolayer, in marked contrast with the situation observed in the mother HT29 cell line. In addition, clone 13 cells displayed epithelial features that mimic the enterocytic differentiation of Caco-2 cells. These modifications were accompanied by dramatic changes in the activity of adenosine receptors, as demonstrated by pharmacological studies. In contrast to the original HT29 cells, clone 13 as well as Caco-2 cells displayed (i) a very low number of adenosine A_1 receptors, and (ii) increases in intracellular cAMP levels when challenged with adenosine analogues. It is hypothesized that a loss of adenosine A_1 receptors, with no change or a concomitant increase in adenosine A_2 receptors, results in the emergence of adenosine A_2 receptor-mediated differentiation and inhibition of proliferation, through a cAMP-dependent pathway.

Keywords: Adenosine receptor; Colon adenocarcinoma; HT29 cell; Adenosine deaminase; Epithelial differentiation

1. Introduction

Four different adenosine receptors have been characterized, namely A_1, A_{2A}, A_{2B} and A_3, in terms of gene and protein sequences, of specificity for adenosine analogues, of intracellular signaling pathways and tissue localization (Londos et al., 1980; Williams, 1991; Linden, 1994; Olah and Stiles, 1995). Through its interaction with these specific receptors, adenosine triggers a variety of changes within the cells, including regulation of ion channel activity, membrane potential, neurotransmitter and hormone release (Stone et al., 1995). Beside these actions, a few reports describe adenosine as a novel modulator of cell proliferation and differentiation.

Adenosine has been shown to regulate the proliferation of tumor cell lines from astrocytoma (Rathbone et al., 1992), epidermoid carcinoma (Tey et al., 1992), leukemia and breast tumors (Colquhoun and Newsholme, 1997), and colon adenocarcinomas (Lelièvre et al., 1998a,b). Moreover, the inhibition or stimulation of proliferation observed was correlated to the adenosine receptor subtype involved. This point has been particularly highlighted in the human

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epidermoid carcinoma A431 cell line, where both adenosine A_1 and A_2 receptor subtypes are expressed and where extracellular adenosine triggers cell growth inhibition through A_1 receptors and stimulation through its interaction with adenosine A_2 receptors (Tey et al., 1992).

Adenosine is also involved in cell differentiation. In pheochromocytoma and neuroblastoma, adenosine stimulates neuronal differentiation through the adenosine A_2 receptor subtype (Abbracchio et al., 1989; Roskoski and Roskoski, 1989). Conversely, in kidney epithelial cells, over-expression of adenosine A_1 receptors results in a dramatic loss of differentiation features (Saunders et al., 1996).

Using human adenocarcinoma cell lines, we have previously demonstrated that adenosine analogues and extracellular adenosine deprivation regulate cell proliferation (Lelièvre et al., 1998a,b). We also observed that endogenous adenosine interacted with both adenosine A_1 and A_2 receptors. It was suggested that the relative expression of adenosine receptor subtypes might be associated with the state of epithelial differentiation. We demonstrate here that long-term treatment with adenosine deaminase, to remove adenosine from the culture medium, induced epithelial differentiation of HT29 cells and a dramatic reduction of cell growth. Data from binding studies and cAMP measurements indicated that such a treatment also resulted in a large reduction of adenosine A_1 receptor expression and activity. Finally, we observed that HT29 cells challenged for 1 month with adenosine deaminase appeared, in many respects, more closely related to Caco-2 cells (used as a model of enterocytic differentiation (Zweibaum et al., 1991) than to the mother cell line.

2. Materials and methods

2.1. Chemicals and materials

Cyclopentyl-1,3-dipropyl[2,3-^{3}H]xanthine: [^{3}H]DPCPX (100Ci/mmol), 5′-*N*-[adenine-2,8-^{3}H]-ethylcarboxamidoadenosine: [^{3}H]NECA (25.7 Ci/mmol) and the [^{125}I]cAMP radioimmunoassay kit were from NEN Dupont Research Products (Les Ulis, France). Bacitracin, fetal calf serum, glutamax I Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and trypsine-EDTA came from Gibco (Life Technologies, Eragny, France). Optiphase II scintillation cocktail was purchased from EG&G Division Instruments (Evry, France). Polycarbonate culture inserts (0.2 μm porosity: Millicell-PCF) were obtained from PolyLabo (Strasbourg, France). Adenosine deaminase type VI from calf intestinal mucosa: EC 3.5.4.4, bovine serum albumin, N6-cyclopentyladenosine (CPA), cyclopentyl-1,3-dipropylxanthine (DPCPX), dimethylsulfoxide, 5′-(*N*-ethyl)-carboxamidoadenosine (NECA), forskolin, HEPES, phenylmethyl-sulfonyl-fluoride and Triton X-100 were from Sigma Chimie, (Saint Quentin, France). Trichloracetic acid was purchased from Merck-Clévenot (Nogent-sur-Marne, France) and xanthine amine congener was from RBI (Bioblock Scientific, Illkirch, France).

2.2. Cell culture

Human colon adenocarcinoma HT29 and Caco-2 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 (100 U/ml and 0.1 mg/ml, respectively). Cultures were incubated at 37°C in a humidified atmosphere of air/CO_2 (95%/5%). Medium was changed every 3 days. For subcultures, cells were incubated in trypsin-EDTA solution (0.05%/0.53 mM, respectively) in Puck's buffer, for 10 min at 37°C, and then harvested.

One to three days before the day of the experiment, cells were seeded at the desired density (see below) in 24-well plates. The culture dishes were previously coated with HT29 extracellular matrix to optimize cell attachment and spreading, according to Bellot et al. (Bellot et al., 1985). In brief, HT29 cells (0.1×10^6) were seeded in 24-well dishes and cultured until confluence was reached. Cells were then removed using 0.3 ml of Triton X-100 (0.1% in phosphate-buffered saline, 0.05 M, pH 7.2). The wells coated with extracellular matrix were then washed three times with sterile phosphate-buffered saline. Precoated 24-well plates were kept at −18°C up to 3 months.

2.3. Adenosine deaminase treatment and cloning of HT29

HT29 cells cultured in 25-cm^2 flasks were treated daily with 10 U/ml adenosine deaminase. This dose was chosen because it caused maximal inhibition of HT29 cell proliferation in our previous experiments (Lelièvre et al., 1998a,b). Cells were diluted (1:5 dilution) every 10 days. After 1 month of such a treatment, cells were harvested, and a cell suspension (2 cells/ml) was seeded into 96-well plates (250 μl/well). Cloning medium contained 50% fresh medium and 50% filtered adenosine deaminase-treated conditioned medium of HT29 cells. Cultures were maintained until cell clones emerged. After two passages, these clones (cultured in the presence of adenosine deaminase) were frozen. Among the 31 clones obtained, clone 13 was further characterized after a second round of cloning.

2.4. Analysis of cell morphology

Fifty thousand cells (HT29, Caco-2, and clone 13) were seeded in 1-ml culture medium, in 24-well dishes on 0.4 μm microporous polycarbonate membranes for light or electron microscopy studies. At confluence, cells on the inserts were fixed for 1 h at room temperature with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4). After three washes in phosphate-buffered saline (0.05 M, pH 7.4), they were post-fixed in osmium tetroxide (1%

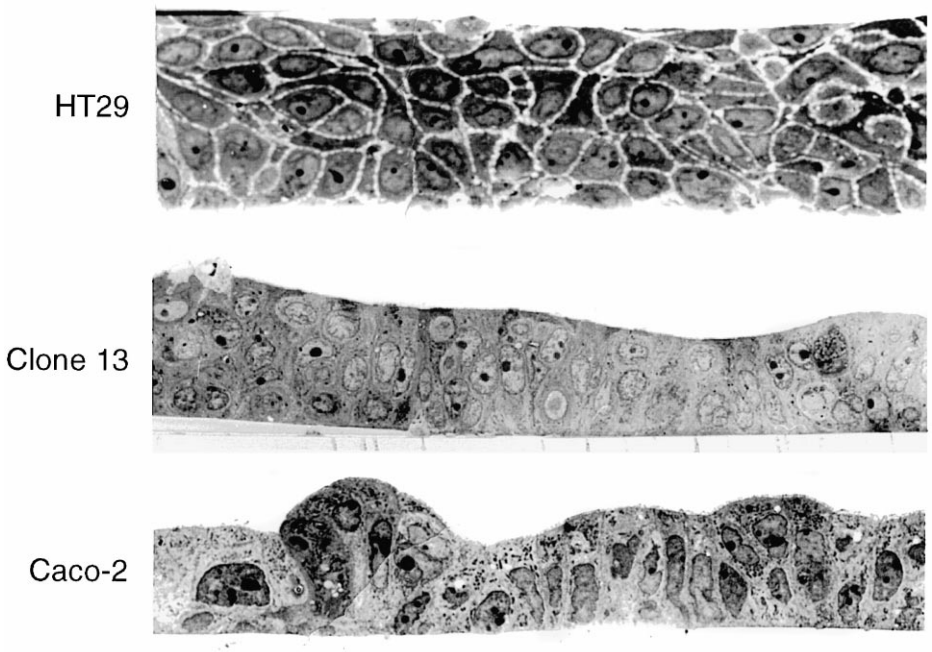

Fig. 1. Transverse section through cultured cells after glutaraldehyde fixation and araldite inclusion. Semi-thin slices of HT29, clone 13, and Caco-2 cells were stained with toluidine blue, ×750.

in sodium cacodylate buffer) and processed either for light microscopy or scanning electron microscopy. For light microscopy, cells were dehydrated in ascending acetone series and embedded in araldite. Semi-thin sections were stained with toluidine blue. Observation and photographs were carried out with a Zeiss Axioplan microscope with a ×100 immersion lens. For scanning electron microscopy, dehydrated cells were washed with acetone, desiccated under vacuum (in a Critical Point Dryer CPD 020, Balzers), coated with gold (metallization device, Balzers), and then observed under a JEOL JSM 35-CF scanning electron microscope.

2.5. Proliferation assays

Cells were seeded at the initial density of 10,000 cells/ml into 24-well dishes. After 24 h, the specified drugs were added daily (10 μl from a 100-fold concentrated solution). Adenosine analogues were dissolved in dimethylsulfoxide (0.01% final concentration); an equiva-

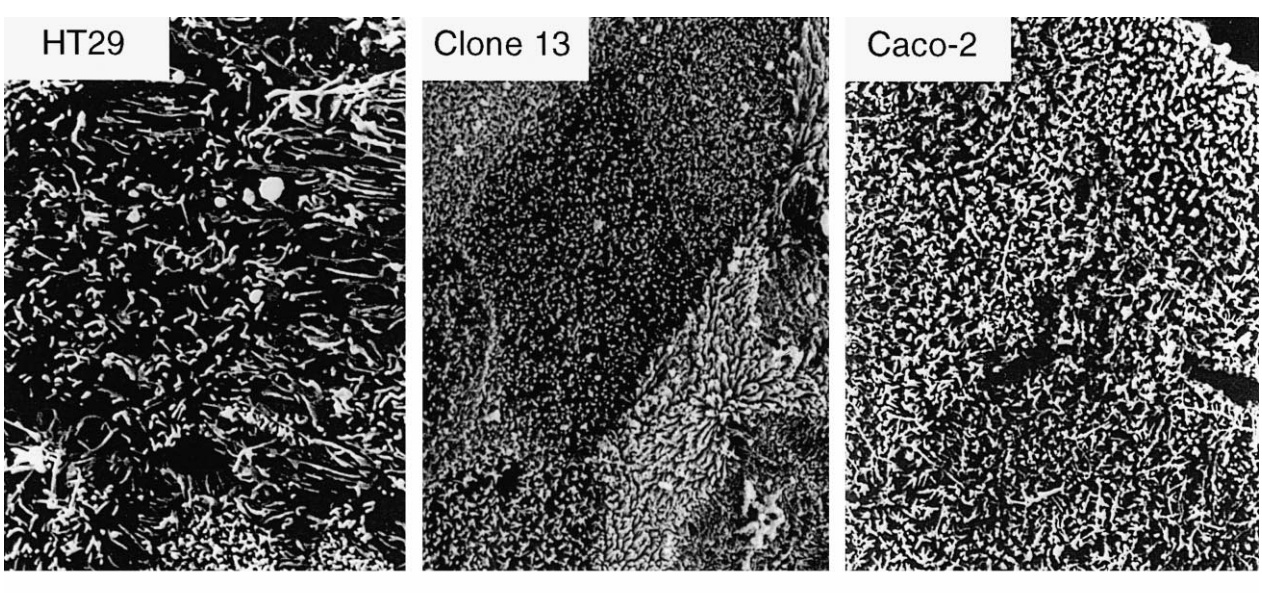

Fig. 2. Details of the cell surface of confluent HT29, clone 13, and Caco-2 cells in culture observed by scanning electron microscopy. Bar: 5 μm.

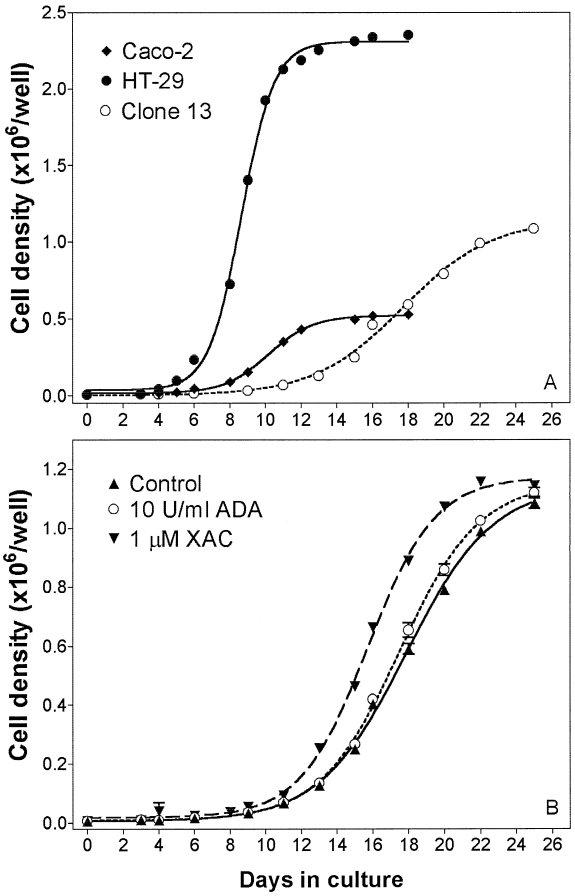

Fig. 3. (A) Proliferation curve for the three cell lines cultured in the absence of adenosine deaminase (ADA). As indicated in the Materials and Methods Section, cells were seeded in precoated 24-well plates at the initial density of 10,000 cell/well. Cell densities were assayed at the indicated times, by direct counting with a hematocytometer, after trypsinization. Data were means (±S.E.M.) of two independent experiments each performed in triplicate ($n = 6$). (B) Effects of 10 U/ml adenosine deaminase (ADA) and 1 μM xanthine amine congener (XAC) treatments on clone 13 cell growth. Drugs were added as indicated in Materials and Methods. Data are presented as means ± S.E.M. of two independent experiments each performed in triplicate ($n = 6$).

lent amount of carrier was added to control wells. Culture medium was replaced every 3 days. At the end of the treatments, cells were harvested and cell density was calculated by direct counting performed in duplicate. Cell viability was assessed by means of Trypan blue exclusion. The number of dead cells never exceeded 1%. Systematic observation of the supernatant indicated that detachment did not occur.

2.6. Measurement of intracellular cAMP levels in colon carcinoma cells

Cells were seeded at a density of 2×10^6 (HT29) or 3.5×10^6 (Caco-2 and clone 13) cells/well. On the third day of the culture, medium was replaced by fresh medium containing either vehicle, forskolin (5 μM) alone, or forskolin and CPA (50 nM). After 20 min, different concentrations of the adenosine agonist NECA were added to the wells. Fifteen minutes later, the plates were placed on ice, and the medium was replaced with 300 μl of trichloracetic acid (6% aqueous solution). Cells were lysed by direct sonication in the wells and the extracts were evaporated. The resulting pellets were re-suspended in sodium acetate buffer (0.05 M, pH = 5.9). After a short centrifugation, the cAMP in the supernatant was quantified by means of a commercially available cAMP radioimmunoassay kit, using [^{125}I]cAMP as a tracer and a γ-counter (Wallac; EG&G).

2.7. Binding of adenosine analogues in colon carcinoma cells

Cells were seeded at a density of 2×10^6 cells (HT29) or 3.5×10^6 (Caco-2 and clone 13) cells/well in 24-well plates. After 3 days, they were incubated for 150 min at 4°C, in 300 μl of binding medium consisting of culture medium containing 15 mM phenyl-methyl-sulfoxyl-fluoride, 150 μM HEPES, 2 U/ml adenosine deaminase, 0.1% bacitracin, 1% bovine serum albumin and [^{3}H]DPCPX (5 nM) or [^{3}H]NECA (10 nM). Conditions were dictated by previous results (Lelièvre et al., 1998a,b). Non-specific binding was determined in the presence of an excess (5 μM) of the appropriate unlabelled ligand. NECA binds to both adenosine A_1 and A_2 receptor subtypes. Thus, binding of [^{3}H]NECA was also performed in the presence or in the absence of 50 nM of CPA in order to prevent the binding to the adenosine A_1 receptor subtypes, as previously described (Blazynski and Mc Intoch, 1993; Lelièvre et al., 1998a,b). Specificity was determined by increasing the concentration of adenosine analogues (in the range of 10 pM to 1 μM) in the reaction mixture to competitively displace [^{3}H]DPCPX and [^{3}H]NECA binding.

At the end of the incubations, the wells were quickly washed once with 1 ml cold phosphate-buffered saline

Table 1
Growth parameters of clone 13, HT29, and Caco-2 cells. Cells were cultured in the absence (control cultures) or in the presence of either adenosine deaminase (ADA, 10 U/ml) or xanthine amine congener (XAC, 1 μM). Latency and doubling time (DT) were derived from curves for the proliferation kinetics. Values were compared to their respective controls (ANOVA)

Cells	Clone 13		HT29		Caco-2	
	Latency (h)	DT (h)	Latency (h)	DT (h)	Latency (h)	DT (h)
Control	120	82	26	25	96	44
ADA 10 U/ml	112[a]	81	52[b]	60[b]	134[b]	66[b]
XAC 1 μM	108[c]	76[a]	39[b]	52[b]	82[b]	44

[a] $P < 0.05$.
[b] $P < 0.0005$.
[c] $P < 0.002$.

(0.05 M, pH = 7.2), and then lysed with 400 μl NaOH (0.5 M) for 10 min. The extracts were incubated overnight in 5 ml of Optiphase II scintillation fluid in counting vials. Radioactivity was measured in a β-scintillation counter (Beckman Instruments, Gagny, France).

2.8. Statistical analysis and curve plots

All the data shown represent the means of two or three independent experiments. Each experiment used triplicate determinations. Each determination used double counting in the proliferation experiments. All data were computed using the GRAPHPAD/PRISM™ iterative curve-fitting program, (ISI software). Data from the proliferation and displacement experiments were fitted to a Hill equation. For fitting more complex competitive inhibition as observed for [^{3}H]DPCPX or [^{3}H]NECA displacement, the two-site competitive binding curve equation was used. Data are described as a better fit by one model of ligand binding than another when a partial F-test comparing the two models indicated significant improvement in the residual sum of square as described previously (Wells, 1992). The F-value was calculated as detailed elsewhere (Lelièvre et al., 1998a,b).

In the proliferation experiments, latency was defined as the time necessary to reach the first doubling of the population. Values within this range were compared using an analysis of variance (ANOVA). To compare the doubling times, proliferation curves were linearized (Log[cell number] = $f(t)$) before running ANOVA.

3. Results

3.1. Morphology of clone 13 cells

Thirty-one clones were obtained after treatment of HT29 cells with adenosine deaminase. Our efforts focused on

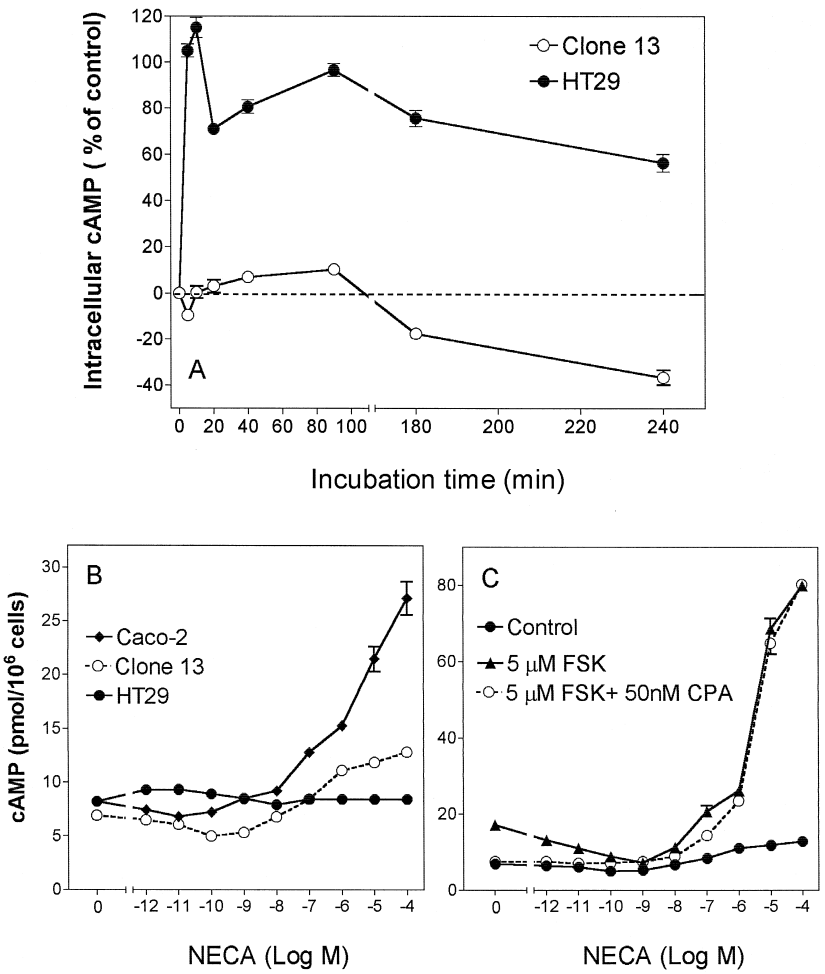

Fig. 4. (A) Time-course variations in intracellular cAMP content recorded in HT29 and clone 13 cells in response to 10 U/ml of adenosine deaminase (ADA). Treatments were done at 37°C. Data are presented as means ± S.E.M. of two independent experiments each performed in triplicate (n = 6). (B) Variations in intracellular cAMP content measured in HT29, clone 13 and Caco-2 cells in response to increasing concentrations of NECA (from 0.1 pM to 100 μM). Cells were treated for 15 min at 37°C. Data are the means ± S.E.M. of two independent experiments each performed in triplicate (n = 6). (C) Variations in intracellular cAMP content measured in clone 13 cells in response to increasing concentrations of NECA (from 0.1 pM to 100 μM) in the presence of 5 μM FSK either alone or with 50 nM CPA. Cells were treated as described in (B). Data are the means ± S.E.M. of two independent experiments each performed in triplicate (n = 6).

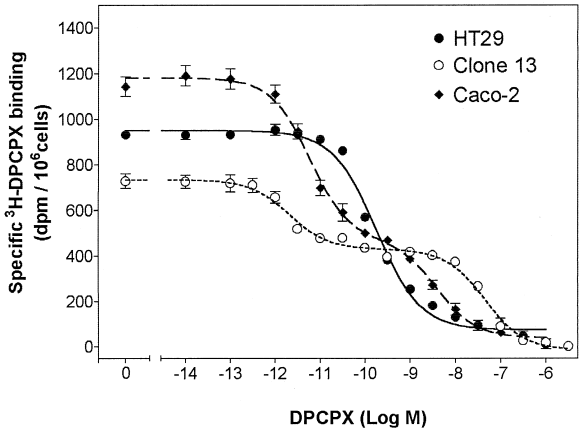

Fig. 5. Competitive displacement of [^{3}H]DPCPX (5 nM/100 Ci/mmol) from intact HT29, clone 13, and Caco-2 cells by increasing concentrations of DPCPX. Cells were incubated for 150 min at 4°C with the radiotracer and the specified concentrations of the unlabelled analogue. Data are the means ± S.E.M. of three independent experiments each performed in triplicate ($n = 3$). A partial *F*-test indicated that the Hill equation was the best fit for HT29 cells, whereas for clone 13 and Caco-2, a two-site competition model was more appropriate to describe the interaction between ligands and binding sites.

clone 13. Cells from clone 13 appeared homogeneously small and poorly refringent under the phase contrast light microscope (data not shown). Transverse sections of fixed clone 13 cells revealed they grew in a pseudo-monolayer (Fig. 1). The cells displayed an apical pole covered with microvilli, as seen under scanning electron microscopy (Fig. 2). The morphological features of the undifferentiated mother HT29 cells and those of the more differentiated Caco-2 cells, cultured under similar conditions, are shown for comparison (Figs. 1 and 2). The organization and general appearance of clone 13 cells resembled those of Caco-2 cells more than those of the mother HT29 cells.

3.2. Proliferative behavior of clone 13 cells

The growth of clone 13, HT29, and Caco-2 cells was assessed in parallel (Fig. 3A), and data were computerized to calculated growth parameters (Table 1). Latency was longer (5-fold), and doubling time was higher (3-fold), in clone 13 cells than in HT29 cells. Clone 13 cells reached confluence with half as many cells as with HT29 cells. These data made clone 13 cells appear more like Caco-2 cells than HT29 cells, except with regard to confluence.

We also investigated the effects of adenosine deaminase and the adenosine receptor antagonist xanthine amine congener on cell proliferation. While these compounds appeared to be potent inhibitors of proliferation in HT29 and Caco-2 cells (see also Lelièvre et al., 1998a,b), in clone 13 cells adenosine deaminase was almost ineffective in reducing cell growth and xanthine amine congener slightly stimulated proliferation (Fig. 3B, Table 1).

3.3. Effects of adenosine deaminase and adenosine receptor agonist on cAMP production

Adenosine deaminase-induced extracellular adenosine deprivation resulted in a dramatic and long lasting elevation of intracellular cAMP content in HT29 cells. In contrast, no elevation was observed in clone 13 cells; rather, cAMP levels started to decrease after 90-min incubation, and reached 30% of the initial concentration after 240 min (Fig. 4A).

Cyclic AMP levels of clone 13 cells increased significantly in response to increasing concentrations of NECA (a non-selective adenosine receptor agonist) ranging from 10^{-9} to 10^{-4} M (Fig. 4B). An increase was also observed in Caco-2 cells but not in HT29 cells. Moreover, in the presence of forskolin (5 μM), the NECA-induced modulation of intracellular cAMP content appeared bimodal (Fig. 4C): inhibition was observed with concentrations ranging from 10^{-12} to 10^{-9} M, and a dramatic increase was seen with concentrations over 10^{-9} M. Interestingly, the inhibitory part of the dose–response curve was no longer observed in the presence of 50 nM CPA (an A_1 preferring agonist), as a result of an overall decrease in basal cAMP levels (Fig. 4C).

Table 2
Binding parameters (IC_{50}, representativity) of the [^{3}H]DPCPX (adenosine A_1-preferring receptor antagonist) and of the [^{3}H]NECA analogues (non-selective adenosine A_1/A_2 receptor agonist) in clone 13, HT29, and Caco-2 cells. The parameters were calculated from the displacement curves. NA (not available)

Cells	Clone 13			HT29			Caco-2		
Radioligand	DPCPX	NECA	NECA + 50 nM CPA	DPCPX	NECA	NECA + 50 nM CPA	DPCPX	NECA	NECA + 50 nM CPA
Site1: IC_{50} (pM)	2.1 ± 0	55 ± 0.85	NA	NA	NA	NA	5.5 ± 0.05	1.8 ± 0.09	NA
Site2: IC_{50} (nM)	4.1 ± 1.45	5 ± 0.45	0.95 ± 0.5	0.2 ± 0.08	25 ± 1.23	4 ± 1.14	3.3 ± 0.06	0.7 ± 0.06	0.9 ± 0.85
Hill number			0.91	0.65	0.85	0.92			0.76
Representativity	40/60	40/60	100	100	100	100	60/40	60/40	100
Site1/Site2 (%)			Site2	Site2	Site2	Site2			Site2

3.4. Binding of adenosine analogues

Displacement of [^{3}H]DPCPX binding by increasing concentrations of unlabelled DPCPX (Fig. 5) indicated the presence of at least two specific binding sites in clone 13 cells, namely high- (IC_{50} = 2.1 pM; 40%) and low- (41 nM; 60%) affinity sites (Table 2). These results are rather similar to those obtained with Caco-2 cells, but differ markedly from those obtained with HT29 cells, where only one component was observed (Fig. 5 and Table 2).

The displacement of [^{3}H]NECA binding to clone 13 cells by increasing concentrations of NECA also revealed two components (Fig. 6A): the IC_{50} was 5 pM for the high-affinity component, and 5 nM for the low-affinity component (Table 2). The high affinity sites were no longer detectable in the presence of 50 nM CPA (Fig. 6A). As for [^{3}H]DPCPX binding, the proportion of these high-affinity CPA-sensitive binding sites accounted for 40% of the total binding (Table 2). These results are more similar to those obtained with Caco-2 cells than to those obtained with HT29 cells (Fig. 6B).

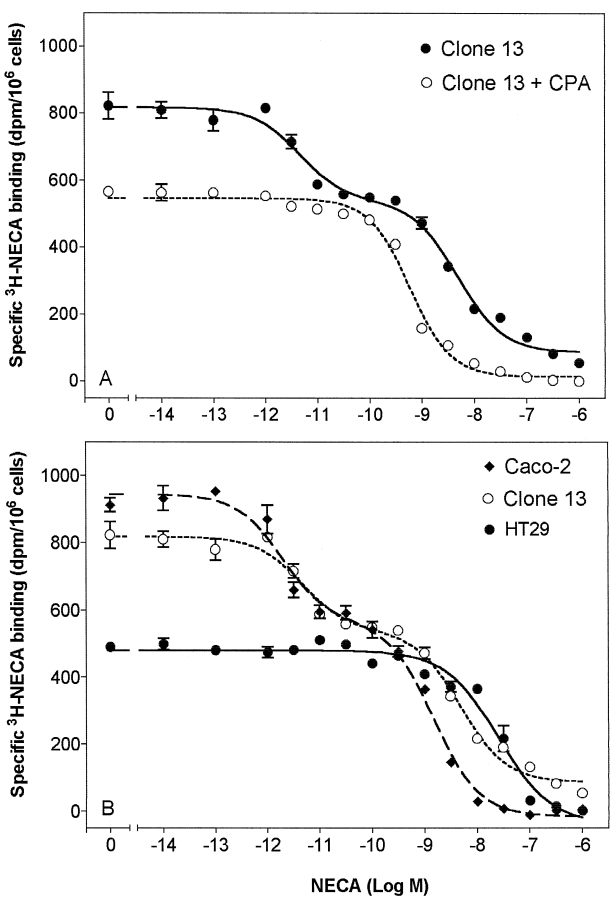

Fig. 6. (A) Competitive displacement of [^{3}H]NECA (10 nM/25.7 Ci/mmol) from intact clone 13 cells by increasing concentrations of NECA. Cells were incubated for 150 min at 4°C with the radiotracer and the specified concentrations of the unlabelled analogue, in the presence or absence of 50 nM CPA. Data are means ± S.E.M. of three independent experiments each performed in triplicate (n = 3). A partial F-test indicated that the Hill equation was the best fit for HT29 cells, whereas a two-site competition model was more appropriate in clone 13 and Caco-2. (B) Competitive displacement of [^{3}H]NECA (10 nM/25.7 Ci/mmol) from intact HT29, clone 13, and Caco-2 cells by increasing concentrations of NECA. Cells were incubated in the absence of CPA as described in (A). Data are the means ± S.E.M. of three independent experiments each performed in triplicate (n = 3). A partial F-test indicated that the Hill equation was the best fit for HT29 cells, whereas a two-site competition model was more appropriate for clone 13 and Caco-2.

4. Discussion

Long-term culture of HT29 cells in the presence of specific selective agents (galactose substituted for glucose), *N,N*,dimethylformamide, sodium butyrate, methotrexate, 5-fluorouracile), followed by cell cloning, triggers the emergence of differentiated cell types (Augeron and Laboisse, 1984; Louvard et al., 1984; Wice et al., 1985; Lesuffleur et al., 1990). The selected clones displayed either an enterocytic or a mucoid phenotype (Zweibaum et al., 1991). In the present study, we used adenosine deaminase as a selecting agent, to modulate extracellular levels of adenosine produced locally. This choice was dictated by previous observations indicating that adenosine deaminase can modulate cell growth in colon cancer cell lines (Lelièvre et al., 1998a,b), and that agents that trigger HT29 cell differentiation (*N,N*,dimethylformamide, sodium butyrate) also trigger modifications in the activity of enzymes associated with purine metabolism, including adenosine deaminase (Dexter et al., 1981). All the clones we obtained displayed a slow proliferation rate, and homogenous cell morphology in culture. As illustrated by clone 13, most of them were non-refringent cells, displaying an epithelial phenotype, including monolayer formation and the presence of apical microvilli. A few other clones were composed of small refringent cells showing an apical pole coated by a mucus-like substance (scanning electron microscopy, data not shown). The cytoplasm of these cells displayed an intense Periodic Acid Schiff (PAS) staining indicating the presence of mucus-secreting vesicles (data not shown). These results agree with previous reports indicating that either enterocytic or mucoid differentiation may occur after treatment of HT29 cells (Zweibaum et al., 1991).

In two previous reports (Lelièvre et al., 1998a,b), we have demonstrated that in colon adenocarcinoma cell lines, including HT29 and Caco-2, adenosine A_1 and A_2 receptors were co-expressed and participated in cell growth regulation. The activation of adenosine A_1 receptors was followed by an increase in proliferation, while adenosine A_2 receptors were involved in its inhibition. These two cell lines, known to display different states of differentiation, also showed different levels of expression and activity of adenosine receptors. Poorly differentiated HT29 cells

seemed to over-express adenosine A_1 receptors, while enterocytic Caco-2 cells functionally balanced the expression of these two receptors (Lelièvre et al., 1998b).

When compared to the growth of the original HT29 cells, the growth of clone 13 cells was characterized by a dramatic increase in latency and doubling time. Furthermore, the proliferation of clone 13 cells was unaffected by adenosine deaminase-induced adenosine depletion or by the non-permeant adenosine A_1 receptor antagonist xanthine amine congener. This contrasts with the situation observed in HT29 cells where both adenosine deaminase and xanthine amine congener treatments inhibited cell proliferation (Lelièvre et al., 1998a,b and the present study). Taken globally, these results may suggest that, in contrast to HT29 cells, clone 13 cells are characterized by a functional adjustment of adenosine receptor expression.

To test this hypothesis, we measured cAMP levels in response to either adenosine deaminase or NECA addition. Since adenosine A_1 receptors are usually negatively coupled to adenylate cyclase and A_2 receptors are positively coupled to adenylate cyclase, cAMP levels were measured to shed light on the relative activity of each receptor subtype. In the presence of forskolin, we observed a biphasic modulation of cAMP levels upon addition of increasing concentrations of NECA. This supports the idea that adenosine A_1 and A_2 receptors are co-expressed. While we observed a functional dominance of the adenosine A_1 receptors in the less differentiated phenotype (HT29), dramatic responses to adenosine A_2 receptor stimulation were recorded in the more differentiated phenotypes (Caco-2 and clone 13). This further supports the idea that adenosine receptors are involved in enterocytic differentiation.

Binding experiments conducted using either the adenosine A_1 receptor antagonist DPCPX, or the non-selective adenosine receptor agonist NECA as radiotracers, confirmed the presence of both adenosine A_1 and A_2 receptor subtypes in clone 13 cells. Data from the displacement experiments fitted a two-site competition equation, as was also observed for Caco-2 cells, but in marked difference with the data obtained from the original HT29 cells, where only one site was detected (Lelièvre et al., 1998b and present study). Moreover, the treatment with adenosine deaminase resulted in a 66% loss of the total amount of adenosine A_1 receptor binding (900 DPM/10^6cells bound in HT29 cells vs. 300 DPM/10^6cells in clone 13 cells), whereas binding at adenosine A_2 receptors was mostly unaffected (500 DPM/10^6 cells bound in HT29 cells vs. 550 DPM/10^6 cells in clone 13 cells). This suggests that long-term treatment with adenosine deaminase induced a dramatic reduction in the adenosine A_1 receptor number in clone 13 cells.

Taken together, our results suggest that a loss of A_1 adenosine receptors, with no change or a concomitant increase in A_2 adenosine receptors, results in the emergence of adenosine A_2 receptor-mediated inhibition of proliferation through a cAMP-dependent pathway. Caco-2 and SW403 cell lines that showed a balanced expression and function of adenosine receptor subtypes, also had slower growth rates than those observed in HT29 cells (Lelièvre et al., 1998b). It is noteworthy that adenosine A_2 receptors have been associated with functions of differentiated enterocytes (Barrett et al., 1989). It is relevant to know whether the A_2 receptors involved are of the A_{2A} and/or A_{2B} subtype, because NECA does not allow discrimination between these two subtypes. Previous studies had indicated that only A_1 and A_{2B} receptors, but not A_{2A} and A_3 receptors, are present in the intestine of mammals, including human (Barret et al., 1989; Strohmeier et al., 1995; Peachey et al., 1996; Prentice and Hourani, 1997), and that T84 human intestinal epithelial cells express exclusively the A_{2B} subtype in both their apical and basal part (Strohmeier et al., 1995). Our results are in keeping with these observations, and we suggest that adenosine receptors of the A_{2B} subtype are involved in colon adenocarcinoma. Indeed, in our hands, the A_{2A} selective agonist CGS21680 was unable to significantly displace the binding of [^{3}H]NECA, and [^{3}H]CGS21680 binding was less than 10% of the [^{3}H]NECA binding (data not shown).

In conclusion, adenosine deaminase-induced extracellular adenosine deprivation may result in the selection of differentiated cells. Two cell populations (enterocytic and mucoid) were identified. The enterocytic phenotype, represented by clone 13 cells, was associated with a strong reduction in proliferation. As a possible explanation for this phenomenon, we demonstrated that a concomitant down-regulation of adenosine A_1 receptors and up regulation of adenosine A_2 receptors occurred in cloned cells. Since it was recently reported that the adenosine A_1 receptor subtype is up-regulated in colon tumorigenesis (Khoo et al., 1996) and that adenosine deaminase induces a strong inhibition in many tumors (Colquhoun and Newsholme, 1997), the question of the role of adenosine metabolism in tumorigenesis appears especially important.

References

Abbracchio, M.P., Cattabeni, F., Clementi, F., Sher, E., 1989. Adenosine receptors linked to adenylate cyclase activity in human neuroblastoma cells: modulation during cell differentiation. Neuroscience 30, 819–825.

Augeron, C., Laboisse, C.L., 1984. Emergence of permanently differentiated cell clones in a human colon carcinoma cell line in culture after treatment with sodium butyrate. Cancer Res. 44, 3961–3969.

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.

Blazynski, C., Mc Intoch, H., 1993. Characterization of adenosine A_2 receptors in bovine retinal membranes. Exp. Eye Res. 56, 585–593.

Colquhoun, A., Newsholme, E.A., 1997. Inhibition of human tumor cell

proliferation by analogs of adenosine. Cell Biochem. Funct. 15, 135–139.

Dexter, D.L., Crabtree, G.W., Stoeckler, J.D., Savarese, T.M., Ghoda, L.Y., Rogler-Brown, T.L., Parks, R.E. Jr., Calabresi, P., 1981. *N*,*N*-dimethylformamide and sodium butyrate modulation of the activities of purine metabolizing enzymes in cultured human colon carcinoma cells. Cancer Res. 41, 808–812.

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.

Lelièvre, V., Muller, J.-M., Falcòn, J., 1998a. Adenosine modulates cell proliferation in human colonic carcinoma: I. Possible involvement of A_1 adenosine receptor subtypes in HT29 cells. Eur. J. Pharmacol. 341, 289–297.

Lelièvre, V., Muller, J.-M., Falcòn, J., 1998b. Adenosine modulates cell proliferation in human colonic carcinoma: II. Differential behavior of HT29, DLD-1, Caco-2 and SW403 cell lines. Eur. J. Pharmacol. 341, 299–308.

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 absortive and mucus-secreting cells. Cancer Res. 50, 6334–6339.

Linden, J., 1994. A_1 adenosine receptors. J. Biol. Chem. 269, 27900–27906.

Londos, C., Cooper, D.M., Wolff, J., 1980. Subclasses of extracellular adenosine receptors. Proc. Natl. Acad. Sci. U.S.A. 77, 2551–2554.

Louvard, D., Arpin, M., Coudrier, E., Huet, C., Prigault, E., Robine, S., Sahuquillo-Merino, C., 1984. Experimental manipulation of intestinal cell differentiation using a human adenocarcinoma cell line (HT29). J. Cell Biol. 99, 6a.

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

Peachey, J.A., Hourani, S.M., Kitchen, I., 1996. Differential development of adenosine A_1 and A_{2B} receptors in the rat duodenum. Br. J. Pharmacol. 119, 949–958.

Prentice, D.J., Hourani, S.M., 1997. Adenosine analogues relax guinea-pig taenia caeci via an adenosine A_{2B} receptor and a xanthine-resistant site. Eur. J. Pharmacol. 323, 103–106.

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

Roskoski, R., Roskoski, L.M., 1989. Adenosine receptor activation and the regulation of tyrosine hydroxylase activity in PC12 and PC18 cells. J. Neurochem. 53, 1934–1940.

Saunders, C., Keefer, J.R., Kennedy, A.P., Wells, J.N., Limbird, L.E., 1996. Receptors coupled to pertussis toxin-sensitive G-proteins traffic to opposite surfaces in Madin–Darby canine kidney cells. J. Biol. Chem. 271, 995–1002.

Stone, T.W., Collis, M.G., Williams, M., Miller, L.P., Karasawa, A., Hillaire-Buys, D., 1995. Adenosine: some therapeutic applications and prospects. In: Cuello, A.C., Collier, B. (Eds.), Pharmacological Sciences: Perspectives for Research and Therapy in the Late 1990s. Birkhäuser, Basel, pp. 304–309.

Strohmeier, G.R., Reppert, S.M., Lencer, W.I., Madara, J.L., 1995. The A_{2B} adenosine receptor mediates cAMP responses to adenosine receptor 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.

Wells, J.W., 1992. Analysis and interpretation of binding at equilibrium. In: Hulme, E.C. (Ed.), Receptor–ligand Interactions, a Practical Approach. IRL Press, Oxford, pp. 289–395.

Wice, B.M., Trugnan, M.G., Pinto, M., Rousset, M., Chevalier, G., Dussaulx, E., Lacroix, B., Zweibaum, A., 1985. The intracellular accumulation of UDP-*N*-acetylhexoamines is concomitant with the inability of human cancer cells to differentiate. J. Biol. Chem. 260, 139–146.

Williams, M., 1991. Adenosine receptor agonists and antagonists. In: Stone, T. (Ed.), Adenosine in the Nervous System. Acad. Press, London, pp. 137–171.

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., Frizzell, R.A. (Eds.), Handbook of Physiology, The Gastrointestinal System, Intestinal Absorption and Secretion. American Physiological Society, Washington, DC, pp. 223–255.