Adenosine Down-Regulates the Surface Expression of Dipeptidyl Peptidase IV on HT-29 Human Colorectal Carcinoma Cells

Implications for Cancer Cell Behavior

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Dipeptidyl peptidase IV (DPPIV) is a multifunctional cell-surface protein that, as well as having dipeptidase activity, is the major binding protein for adenosine deaminase (ADA) and also binds extracellular matrix proteins such as fibronectin and collagen. It typically reduces the activity of chemokines and other peptide mediators as a result of its enzymatic activity. DPPIV is aberrantly expressed in many cancers, and decreased expression has been linked to increases in invasion and metastasis. We asked whether adenosine, a purine nucleoside that is present at increased levels in the hypoxic tumor microenvironment, might affect the expression of DPPIV at the cell surface. Treatment with a single dose of adenosine produced an initial transient (1 to 4 hours) modest (~10%) increase in DPPIV, followed by a more profound (~40%) depression of DPPIV protein expression at the surface of HT-29 human colon carcinoma cells, with a maximal decline being reached after 48 hours, and persisting for at least a week with daily exposure to adenosine. This down-regulation of DPPIV occurred at adenosine concentrations comparable to those present within the extracellular fluid of colorectal tumors growing *in vivo*, and was not elicited by inosine or guanosine. Neither cellular uptake of adenosine nor its phosphorylation was necessary for the down-regulation of DPPIV. The decrease in DPPIV protein at the cell surface was paralleled by decreases in DPPIV enzyme activity, binding of ADA, and the ability of the cells to bind to and migrate on cellular fibronectin. Adenosine, at concentrations that exist within solid tumors, therefore acts at the surface of colorectal carcinoma cells to decrease levels and activities of DPPIV. This down-regulation of DPPIV may increase the sensitivity of cancer cells to the tumor-promoting effects of adenosine and their response to chemokines and the extracellular matrix,

facilitating their expansion and metastasis. (*Am J Pathol* 2004, 165:319–330)

Dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) is a 110-kD cell-surface peptidase that is normally expressed on epithelial cells of the intestine, kidney, liver, lung and prostate; fibroblasts; melanocytes; and certain leukocyte subsets.^{1–3} The enzyme cleaves NH₂-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position, including glucagon-like peptides and certain chemokines and neuropeptides.^{4,5} DPPIV usually acts on such molecules to reduce their biological activities, but sometimes may enhance their action.⁶ DPPIV also interacts with extracellular matrix molecules such as collagen^{7,8} and fibronectin (FN),^{9,10} in which context it plays a role in cell spreading⁸ and migration.¹¹ It is also the major binding protein for adenosine deaminase (ADA), and is identical to the ADA-complexing protein or ADA binding protein described in previous reports.^{12,13} DPPIV is also identical to the lymphocyte activation marker CD26, which has been shown to participate in the signal transduction process of T-cell activation.^{3,14}

DPPIV is frequently expressed aberrantly in solid cancers, and may influence both the genesis of the primary tumor and its subsequent capacity to spread from the initial site. In many cases, tumorigenesis is accompanied by a reduction in the expression of DPPIV, as for example in melanoma^{1,15} and prostate cancer.^{16,17} This reduced DPPIV expression is directly associated with carcinogenesis, because inducible gene transduction of DPPIV into melanoma cells dramatically reverses the malignant phe-

Supported by grants to J.B. from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Cancer Care Nova Scotia (CCNS). E.Y.T. was the recipient of studentship awards from NSERC and the Killam Foundation; and M.M. was supported by studentship awards from CaRE/CCNS and the Nova Scotia Health Research Foundation.

Accepted for publication March 30, 2004.

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notype.¹⁸ Decreased levels of DPPIV have also been linked to increased invasion and metastasis.^{16,19,20}

In hepatocellular and colorectal carcinomas there is highly variable expression of DPPIV,^{21–23} suggesting that local influences may regulate the expression of DPPIV and therefore its biological effects. However, despite the numerous reports of variable expression of DPPIV *in vitro* and *in vivo*, very little is known about the endogenous factors that might regulate the surface expression of DPPIV on tumor cells.

The unique metabolic environment existing within solid tumors might influence the surface expression of DPPIV on cancer cells *in vivo*. Solid tumors frequently develop regions of ischemia and hypoxia, primarily as a consequence of a structurally and functionally disturbed microcirculation.²⁴ These conditions favor the breakdown of ATP via AMP through the 5'-nucleotidase pathway²⁵ and a decrease in formation of AMP through inhibition of adenosine kinase.²⁶ As a consequence, there is an increase in the levels of the purine nucleoside adenosine. We have previously demonstrated using microdialysis of mouse and human colorectal carcinomas that such tumor tissues have elevated concentrations (10^{-5} mol/L) of adenosine in the extracellular fluid.²⁷ Adenosine has been proposed to have multiple potential effects on the fate of the tumor, including suppression of the cell-mediated immune response,^{28–32} promotion of angiogenic activity,³³ stimulation of the motility of tumor cells,³⁴ and either stimulation^{35,36} or inhibition^{37,38} of tumor cell growth. In the present work, we have considered whether the high adenosine levels within tumors might impact on DPPIV expression on cancer cells. We report that chronic treatment with adenosine markedly down-regulates the surface expression of DPPIV protein on HT-29 human colorectal carcinoma cells.

Materials and Methods

Materials

HT-29 human colorectal carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Media and inosine were from ICN Biomedicals, Irvine, CA. Culture vessels (Nunc) and sera were purchased from Invitrogen Canada (Burlington, Ontario, Canada). Costar Transwell polycarbonate culture inserts (8- μ m pore size) were from Corning Inc. (Corning, NY). Adenosine, guanosine, 5'-iodotubercidin, dilazep, dipyridamole, S(4-nitrobenzyl)-6-thioinosine (NBTI), Gly-Pro-*p*-nitroaniline *p*-toluenesulfonate salt (Gly-Pro-*p*NA), human cellular fibronectin (cFN) and calf spleen ADA were from Sigma Chemical Co. (St. Louis, MO). Coformycin was from Calbiochem (San Diego, CA). Mouse anti-human monoclonal antibody (mAb) against DPPIV/CD26 (clone M-A261) and mouse IgG₁ (clone W3/25) isotype control were from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Rabbit anti-bovine ADA antibody was from Alpha Diagnostic International (San Antonio, TX). ¹²⁵I-labeled sheep anti-mouse IgG, F(ab')₂ fragment and ¹²⁵I-labeled goat anti-rabbit IgG, F(ab')₂ fragment were

obtained from Perkin-Elmer Life Sciences (NEN, Boston, MA), and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG was from PharMingen (San Diego, CA). High specific activity [³H]-labeled amino acid mixture (90 to 157 Ci/mmol) was from Amersham Biosciences Inc. (Baie d'Urfé, Quebec, Canada).

Cell Culture and Drug Treatments

HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, without antibiotics) supplemented with 10% v/v heat-inactivated newborn calf serum and maintained as stocks in 80-cm² flasks at 37°C in a humidified atmosphere of 90% air/10% CO₂. Cells for use in binding assays or for measurements of DPPIV enzyme activity were seeded into 48-well plates at 50,000 cells/well and allowed to adapt to culture for 48 hours. Cultures were then changed to medium containing 1% newborn calf serum for another 48 hours, and then treated with drugs or control vehicle for evaluation of changes in DPPIV protein expression. The common solvent dimethyl sulfoxide produced marked down-regulation of DPPIV at concentrations greater than 0.5% (v/v), and led to significant changes even at lower concentrations (10 to 12% at a final concentration of 0.25% (v/v) dimethyl sulfoxide). For this reason, the final dimethyl sulfoxide concentration was always kept less than 0.1% (v/v), or drugs were dissolved in ethanol, which did not affect DPPIV levels even when added to cultures at a final concentration of 5% (v/v).

Radioantibody Binding Assay for DPPIV

The culture plates were placed on ice, and all subsequent washes and incubations were performed at 4°C. Cells were washed with 250 μ l of phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and then incubated with 125 μ l of PBS containing 1% BSA and primary antibody or isotype control. After incubation for 60 minutes, the wells were washed twice as before and further incubated with 125 μ l of PBS containing 1% BSA and 1.2 μ Ci/ml ¹²⁵I-labeled sheep anti-mouse IgG, F(ab')₂ fragment, for 60 minutes. The monolayers were then washed three times and solubilized in 400 μ l of 0.5 mol/L NaOH for 60 minutes at room temperature before the counting of radioactivity. Figure 1 shows the binding of anti-DPPIV mAb to HT-29 cells with increasing antibody concentration. The specific binding of anti-DPPIV was better than 94% at saturating antibody concentrations. Maximal specific antibody binding was reached at 2 μ g/ml, which was the concentration used in all subsequent assays.

To exclude the possibility of interference from molecules such as ADA that might be bound to DPPIV, we used acid stripping to remove ADA and other bound ligands from the cell surface.^{39,40} Use of acetate buffer was avoided because this causes the release of intracellular ADA.⁴¹ Briefly, cell monolayers that had been treated with adenosine were washed and then incubated for 5 minutes at 4°C with 200 μ l of medium that had been adjusted to the required pH. After washing twice with PBS

Figure 1. Measurement of cell-surface DPPIV on HT-29 cells by indirect radioantibody binding assay. Confluent monolayer cultures of HT-29 cells were incubated with either anti-DPPIV mAb (total binding, ●) or isotype control (nonspecific binding, ○), followed by ¹²⁵I-labeled anti-mouse Ig, F(ab')₂ fragment as described in Materials and Methods. Points are mean values, *n* = 4. Standard errors fall within the symbols. The third curve (■) shows the specific binding (total binding – nonspecific binding).

containing 0.2% BSA, the assay was performed as usual. To assess the effect of previous ADA loading on DPPIV immunoreactivity, cell monolayers were incubated for 60 minutes at 4°C with 10 μg/ml of ADA before the DPPIV binding assay.

Assay for Cellular Binding of Exogenous ADA

The method used to measure the cellular capacity for ecto-ADA binding was a modification of the DPPIV radioantibody binding assay, except that the cells were first loaded with saturating concentrations of bovine ADA and the bound, exogenous ADA was then detected with an antibody selective for the bovine enzyme. Monolayer cultures of HT-29 cells in 48-well plates were first treated with 10 μg/ml of calf spleen ADA in medium for 60 minutes at 37°C. (Preliminary experiments had shown this concentration of calf spleen ADA to saturate the unfilled binding capacity of HT-29 cells.) The plates were then washed and assayed for bound ADA using a rabbit anti-bovine ADA antibody and a ¹²⁵I-labeled goat anti-rabbit secondary antibody (F(ab')₂ fragment) using the procedures described above. Data were corrected for binding in the absence of loading with exogenous ADA. The unloaded background binding was never more than twice the antibody isotype control value, and likely represented a low degree of DPPIV occupancy with ADA acquired from serum; or endogenous human ADA secreted from the cells, absorbed from the medium, and subsequently detected by the weakly cross-reacting anti-ADA antibody.

Flow Cytofluorimetric Detection of DPPIV

HT-29 cells were washed and resuspended (10⁶ cells) in filter-clarified PBS with 2.5% BSA and 0.2% sodium azide containing anti-DPPIV mAb (1 μg/10⁶ cells) for 45 minutes at 4°C. The cells were washed twice with the same buffer and then incubated with fluorescein isothiocyanate secondary mAb conjugate (1 μg/10⁶ cells) for 40 minutes

at 4°C. After three further washes the cells were fixed in 1% paraformaldehyde and stored in the dark at 4°C until analyzed. Flow cytofluorimetric analysis was performed with a FACScan (BD Immunocytometry Systems, Mountain View, CA) flow cytometer equipped with a 15-mW argon laser operating at a wavelength of 488 nm and detection at 680 nm. Data were analyzed using Lysis II software (BD Biosciences, San Jose, CA).

Assay of Cellular DPPIV Enzymatic Activity

DPPIV enzyme activity was measured spectrophotometrically using Gly-Pro-pNA as a DPPIV substrate. The culture plates were placed on ice and the wells were washed twice with 500 μl of ice-cold PBS; then given 500 μl of 2 mmol/L Gly-Pro-pNA in 100 mmol/L HEPES buffer (pH 7.6) containing 0.12 mol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO₄, 8 mmol/L glucose, and 10 mg/ml BSA and incubated for 60 minutes at 37°C. After incubation, 100 μl of the supernatant was transferred to a 96-well flat-bottomed microtiter plate and the absorbance (resulting from *p*-nitroaniline release) was measured at 405 nm. The enzyme activity was calculated after subtraction of the absorbance values for cell-free controls.

Assay of Cell Adhesion to cFN

Cells for use in adhesion assays were seeded into 80-cm² flasks at 10⁶ cells/flask and cultured and treated with adenosine as for other approaches. Before adhesion assays, HT-29 cells were first labeled for 3 hours with 1 μCi/ml ³H-labeled amino acids in amino acid-free medium³⁰ containing 1% fetal calf serum. Four-well plates were coated overnight at 4°C with cFN at a concentration of 5 μg/ml (~1 μg/cm²) in PBS. Before their use in assays, plates were treated with 2% BSA in serum-free DMEM to reduce nonspecific binding. Radiolabeled HT-29 cells (50,000) in serum-free DMEM were added to wells and incubated for 2 hours at 37°C. At the end of the incubation period, plates were gently washed twice by inverting into PBS (containing Ca²⁺ and Mg²⁺) for 10 minutes at 4°C to remove nonadherent cells. The bound cells were dissolved in 500 μl of 0.1 mol/L NaOH containing 1% sodium dodecyl sulfate and the measured radioactivity (after correction for the specific activity of labeling of the cells) was used as a measure of cell adhesion.

Assay of Cellular Motility on cFN

Cells were cultured in 80-cm² flasks and treated with adenosine as before. Transwell culture inserts were coated overnight at 37°C with 5 μg/ml of cFN and washed three times with serum-free DMEM before use. Motility assays were performed with the coated inserts in 24-well plates using DMEM medium supplemented with 0.5% fetal calf serum. Control and adenosine-treated cells were released from monolayer culture by trypsinization, added to the upper chamber (250,000 cells/insert), and incubated for 18 hours at 37°C. At the end of that

time, spontaneously migrating cells that had reached the lower surface of the membrane were fixed in ethanol and stained with Mayer's hematoxylin. Nonmigrated cells were scraped from the upper surface of the membrane chamber with a cotton-tipped applicator, and the filter was washed and mounted for counting of the migrated cells. Counts were performed with the observer blinded.

Results

Time-Dependent Modulation of Cell-Surface DPPIV Protein by Adenosine

We initially examined the effect of adenosine on the surface expression of DPPIV protein by HT-29 colorectal carcinoma cells after treating with a single high dose (300 $\mu\text{mol/L}$) of adenosine, in this way allowing for the rapid metabolism of adenosine in culture ($t_{1/2} \sim 120$ minutes for HT-29 cells⁴²) while avoiding the use of inhibitors of adenosine metabolism. Adenosine at this concentration did not affect cellular viability (E. Garcia del Busto, M. Mujoomdar, and J. Blay, unpublished data), as determined by either the MTT assay⁴³ or DNA fragmentation assay.⁴⁴ Adenosine (300 $\mu\text{mol/L}$) produced an early transient increase in DPPIV that was evident at 1 hour and persisted until the 4-hour time point, after which time no difference was evident (Figure 2a). This short-term, significant (although modest, $\sim 10\%$) increase in DPPIV was seen at both confluent and subconfluent cell densities. Given the transient nature of this initial increase in DPPIV expression we focused instead on the effect of adenosine on DPPIV expression on HT-29 cells throughout a more prolonged time course. With a single dose of adenosine, the amount of cell-surface DPPIV protein was significantly decreased after 12 hours and maximally suppressed by 48 hours, at which time DPPIV expression was reduced by 32% (Figure 2b). The baseline expression of DPPIV increased substantially in some studies (up to 127% after 48 hours of culture; data not shown). This was not simply because of increasing cell number. Under the incubation conditions used, the mean increase in cell number after 48 hours was only $8.0 \pm 0.36\%$ (mean $\pm$ SE, $n = 3$). The down-regulation of DPPIV by adenosine at 48 hours was also evident using flow cytometry of trypsinized cells in suspension (Figure 2c). The anti-DPPIV-stained cells showed an approximate threefold leftward shift in the mean cellular fluorescence after adenosine treatment, with no change in the fluorescence of the antibody isotype control. This reduced antibody binding to HT-29 cells in suspension shows that DPPIV is not masked within the cell monolayer after adenosine treatment and confirms that there is a decline in total cell-surface DPPIV protein.

The Measured Decrease in DPPIV Is Not Because of Masking by DPPIV Binding Proteins

The 48-hour time point was used in further studies of DPPIV protein down-regulation. We considered whether

the decline in immunoreactivity in our radioantibody binding assay or flow cytometry reflected masking of the antibody epitope because of binding of ADA or another protein to DPPIV. We used two approaches to exclude this possibility. First, we examined whether the adenosine effect would be reduced by further ADA loading of DPPIV. To do this, we exposed HT-29 cell monolayers to 10 $\mu\text{g/ml}$ of ADA for 60 minutes at 4°C , conditions that saturate the ADA binding capacity of DPPIV on HT-29 cells (E.Y. Tan and J. Blay, unpublished data). ADA loading significantly interfered with the measurement of DPPIV ($47.6 \pm 2.7\%$ reduction in immunoreactivity, three independent experiments). Because ADA loading was performed at 4°C to exclude internalization, this decrease is likely because of partial interference of anti-DPPIV antibody binding to its target, rather than down-regulation of DPPIV. Nevertheless, ADA did not alter the depression of DPPIV observed in response to adenosine treatment (Figure 3). It was also possible that a protein other than ADA might be interfering with antibody access. In a second approach, we subjected the cells to acid stripping, a technique that is widely used to dissociate various ligands, in addition to ADA, from their cell-surface binding proteins.^{39–41,45,46} Acid stripping at pH values down to 2.6 reduced the recoverable DPPIV immunoreactivity somewhat (by 4.6%, 15.6%, and 18.3% at pH 4.2, 3.1, and 2.6, respectively), but had absolutely no effect on the measured decrease caused by adenosine (Table 1). These data argue strongly that the down-regulation of DPPIV protein observed using either the binding assay or flow cytometry represents an authentic decrease in cell-surface DPPIV protein and not blocking by DPPIV binding protein(s).

Adenosine Down-Regulates DPPIV at Concentrations Found within the Tumor Microenvironment

The dose-response relationship for the down-regulation of DPPIV using a single dose of adenosine at the beginning of the 48-hour period is shown in Figure 4a. The maximal extent of down-regulation of DPPIV observed was $\sim 40\%$. The greatest effect in this approach was typically seen at 300 $\mu\text{mol/L}$ of adenosine, although in some experiments the maximal effect was reached at 100 $\mu\text{mol/L}$. The EC_{50} for down-regulation (single dose) was $43.3 \pm 12.1 \mu\text{mol/L}$ (mean $\pm$ SE, five separate experiments). Such concentrations (up to 100 $\mu\text{mol/L}$) of adenosine are likely reached *in vivo* as a result of significant cell death within the tissue, a scenario that might occur during treatment with cytotoxic chemotherapeutic agents or anti-angiogenic strategies. However, the single dosing approach contrasts with the situation that would be more usual *in vivo*, in which adenosine production may be less dramatically raised throughout a period of some days (eg, sites of inflammation) or even longer (eg, hypoxic solid tumors). In these situations we would expect the adenosine signal to be more persistent *in vivo* than represented by a single dose *in vitro*, but not to reach such a high concentration. Figure 4b shows the result of extend-

Figure 2. Adenosine modulation of DPPIV on HT-29 colorectal carcinoma cells. **a** and **b**: DPPIV protein on cell monolayers. The surface expression of DPPIV on HT-29 cells was measured after incubation in the absence or presence of adenosine (300 $\mu\text{mol/L}$) for the times indicated. The data are mean values $\pm$ SE, $n = 3$. *, Significant change because of adenosine, $P < 0.05$. **, $P < 0.01$. **c**: DPPIV protein on isolated cells. HT-29 cells were incubated for 48 hours in the absence (i) or presence (ii) of 300 $\mu\text{mol/L}$ of adenosine and released by trypsinization. Cyt-ofluorimetric profiles for HT-29 cells stained with isotype control mAb (open peaks) and with DPPIV-specific mAb (shaded peaks) are shown.

Figure 3. Failure of ADA loading to eliminate the decline in DPPIV because of adenosine. HT-29 cells were incubated for 48 hours in the absence (open bars) or presence (hatched bars) of adenosine (300 $\mu\text{mol/L}$), washed, and further incubated for 60 minutes at 4°C with 10 $\mu\text{g/ml}$ of ADA before the binding assay for DPPIV. The data are mean values $\pm$ SE ($n = 4$). **, Significant reduction by adenosine, $P < 0.01$.

ing the additions of adenosine throughout the 48-hour treatment period to provide more consistent levels without the high initial concentration. Exposure to adenosine at 12.5 $\mu\text{mol/L}$ (12 doses during 48 hours) produced a down-regulation indistinguishable from the single 300- $\mu\text{mol/L}$ dose. This is consistent with our experience that a high concentration of adenosine is necessary when given as a single dose to provide sufficient levels of adenosine throughout an extended time period in the face of rapid metabolism. Our results imply that the reduction in DPPIV requires more than transient exposure of the cells to adenosine. At these dosage intervals the rapid metabolism of adenosine would exclude any net accumulation.⁴² This shows that the observed down-regulation of DPPIV can be achieved with concentrations of adenosine that are present within the tumor environment.²⁷

Down-Regulation of DPPIV Is Because of Adenosine and Not Its Deamination Product, Inosine

It was possible that the effect of adenosine in down-regulating DPPIV was mediated by its deamination product inosine, which itself has been reported to regulate a number of cellular responses.^{47,48} However, neither inosine nor the alternative purine nucleoside guanosine caused down-regulation of DPPIV at concentrations comparable to that for adenosine (Figure 5). Furthermore, blocking inosine production using 2.5 $\mu\text{mol/L}$ of coformycin (an inhibitor of ADA) did not abrogate the down-regulation of DPPIV. Indeed, the presence of coformycin shifted the apparent EC_{50} for adenosine (single dose) approximately fourfold from 43 ± 12.1 $\mu\text{mol/L}$ to 10.7 ± 4.0 $\mu\text{mol/L}$ (mean $\pm$ SE, three separate experiments; $P < 0.5$), likely because of increased adenosine availability.

Table 1. Effect of Acid Stripping on the Detection of DPPIV after Adenosine Treatment

	Conditions	Treatment*		% Decrease
		Control	Adenosine	
I.	Control [†]	5,282 ± 91 [‡]	4,105 ± 29 [§]	22
	pH 4.2	5,041 ± 160	3,891 ± 73 [§]	23
II.	Control	4,531 ± 92	4,132 ± 107 [¶]	9
	pH 3.1	3,825 ± 125	3,393 ± 69 [¶]	11
III.	Control	4,034 ± 38	3,441 ± 55 [§]	15
	pH 2.6	3,295 ± 79	2,742 ± 23 [§]	17

* HT-29 cells were treated for 48 hours with adenosine (300 μ mol/L) or vehicle control.

[†] Prior to the binding assay, cells were incubated for 5 minutes at 4°C either at pH 7.4 (control), or in buffer at the pH indicated.

[‡] Data are mean values $\pm$ SE for three to four replicate determinations.

[¶] Significant reduction by adenosine relative to control treatment, $P < 0.05$.

[§] $P < 0.01$.

Taken together, these data indicate that metabolism of adenosine to inosine is not required for DPPIV down-regulation to occur, and that the response is not generalized to other nucleosides.

The Down-Regulation of DPPIV Does Not Require Cellular Uptake of Adenosine

To determine the cellular location at which adenosine acts to modulate DPPIV, we used the lowest maximally effective single dose of adenosine (30 μ mol/L) in the presence of 2.5 μ mol/L of coformycin to reduce adenosine metabolism through ADA. This allowed assessment of the effects of pharmacological agents on the adenosine response. We used adenosine uptake inhibitors to assess the possibility that adenosine might be mediating down-regulation of DPPIV by intracellular effects on nucleoside metabolism, or by action on the P-site of intracellular adenylyl cyclase (IC_{50} , ~ 80 μ mol/L⁴⁹), or by interfering with the transport of other nucleosides into the cell.⁵⁰ Neither dilazep (10 μ mol/L; Figure 6a) nor NBTI or dipyridamole (each at 1 μ mol/L; Figure 6b) were able to block the down-regulation caused by adenosine. These concentrations are in excess of the IC_{50} values for nucleoside transport inhibition.^{51–53} Indeed, the combination of NBTI with dipyridamole, which concurrently inhibits both NBTI-sensitive and NBTI-insensitive transport⁵⁴ did not prevent the down-regulation of DPPIV caused by aden-

Figure 4. Dose dependence of adenosine down-regulation of DPPIV expression on HT-29 cells. **a:** Single dosing. HT-29 cells were incubated at 37°C with adenosine at the indicated concentrations for 48 hours and surface expression of DPPIV was then measured. **b:** Multiple dosing. HT-29 cells were incubated at 37°C with the indicated concentrations of adenosine (μ mol/L) and number of doses (in parentheses) throughout the 48-hour treatment period. The data are mean values $\pm$ SE ($n = 3$). *, Significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

Figure 5. Lack of effect of inosine and guanosine on the surface expression of DPPIV. HT-29 cells were incubated at 37°C with medium alone or in the presence of 100 μ mol/L of adenosine, inosine, or guanosine. Forty-eight hours later cell-surface DPPIV was measured. The data are mean values $\pm$ SE ($n = 4$). **, Significant reduction by adenosine, $P < 0.01$.

Figure 6. Lack of requirement for adenosine uptake or adenosine phosphorylation for down-regulation of DPPIV. HT-29 cells were pretreated for 30 minutes with control vehicle or 10 μ mol/L of dilazep (**a**); 1 μ mol/L NBTI, 1 μ mol/L dipyrindamole (DP), or 1 μ mol/L NBTI and DP (**b**); or 1 μ mol/L iodotubercidin (IodoT) (**c**). The cells were then incubated with medium alone (open bars) or adenosine at 10 μ mol/L (hatched bars) or 30 μ mol/L (shaded bars), plus 2.5 μ mol/L coformycin. Forty-eight hours later surface expression of DPPIV was measured. The data are mean values $\pm$ SE ($n = 3$). *, Significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

osine. Similarly, 5'-iodotubercidin (1 μ mol/L), which inhibits intracellular phosphorylation of adenosine through adenosine kinase, failed to alter the adenosine down-regulation of DPPIV (Figure 6c). Thus, neither the uptake

Figure 7. Persistent depression of DPPIV protein and dipeptidase activity with long-term exposure to adenosine. HT-29 cultures were incubated in control media (open bars) or with adenosine (100 μ mol/L) added daily (hatched bars). The cultures were then assayed for the presence of cell-surface DPPIV protein (**a**) or its associated dipeptidase activity (**b**). The data are mean values $\pm$ SE ($n = 4$). *, Significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

nor the intracellular phosphorylation of adenosine is necessary for this effect, and indicates that adenosine acts at a cell-surface site to down-regulate DPPIV on HT-29 cells.

Chronic Exposure of Cells to Adenosine Produces a Sustained Decrease in Both DPPIV Protein and DPPIV Enzyme Activity

Having established that adenosine acts directly at the cell surface to cause a net down-regulation of DPPIV protein, we examined the consequences of this phenomenon for regulation of its enzyme activity. First, we determined whether longer-term exposure to adenosine was able to produce a sustained depression of cell-surface DPPIV. In the absence of any addition, DPPIV protein increased with time in culture as before, even after correction for changes in cell number (Figure 7a). However, daily supplementation of media with 100 μ mol/L of adenosine produced a sustained depression of the amount of DPPIV protein per cell. The decline in DPPIV continued to day 3 with repetitive dosing, and this depression was maintained throughout the culture period (Figure 7a). The

maximum effect on DPPIV was a ~40% decrease from control. We next measured the enzyme activity of DPPIV (Figure 7b). The dipeptidase activity of DPPIV increased substantially through the 6-day culture period, the activity per cell nearly doubling at the end of this time. However, in the presence of daily 100- μ mol/L treatments with adenosine, there was a relative decline compared with control that closely paralleled the time course of change in immunoreactive protein (Figure 7b). Therefore, the enzyme activity of DPPIV is persistently decreased by adenosine treatment.

The Adenosine Down-Regulation of DPPIV Leads to a Reduction in Ecto-ADA Binding Capacity

DPPIV has been shown to bind ADA.^{12,13} We therefore quantified the ecto-ADA binding in cells that had been treated (48 hours) with adenosine to down-regulate DPPIV. We measured the ADA binding capacity of the cells by first loading with bovine ADA, and then using an indirect radioantibody binding assay incorporating a rabbit antibody against the bovine enzyme. Although we did not first strip the cells free of bound endogenous ADA, two sets of observations suggest that the baseline occupancy of DPPIV with endogenous human ADA on HT-29 cells is low when the cells are maintained in low-serum conditions. Firstly, we have found only moderate levels of endogenous ADA when using this assay approach with anti-ADA antibodies of varying species cross-reactivities (E.Y. Tan and J. Blay, unpublished data). Secondly, exogenous bovine ADA causes a significant shift in DPPIV immunoreactivity in both binding assays (Figure 3) and fluorescence-activated cell sorting methods (E.Y. Tan and J. Blay, unpublished data), indicating that there is a substantial loading of the DPPIV with ADA. We are therefore confident that our method is a valid measure of the cellular capacity for binding of ADA. Using this assay, we found that adenosine-pretreated cells show a reduced capacity for binding of ADA (Figure 8b) and that the reduced ADA binding capacity is quantitatively comparable to the measured reduction in DPPIV protein in paired cell cultures (Figure 8a). This confirms our expectation that the adenosine down-regulation of DPPIV, which is identical to ADA complexing protein¹³ or ADA binding protein,¹² will reduce the amount of cellular ecto-ADA.

The Adenosine Down-Regulation of DPPIV Compromises the Ability of the Cells to Interact with cFN

DPPIV associates with the extracellular matrix proteins collagen and FN.⁷⁻¹⁰ In particular, it binds preferentially to cell-surface FN even in the presence of the soluble plasma form of this protein.¹⁰ The linkage through DPPIV to cFN on adjacent tumor cells might be one way in which tumor cells are contained within the tumor cell population and restrained from leaving the primary site to move to a different location. We therefore investigated the capacity

Figure 8. The adenosine-induced down-regulation of DPPIV is accompanied by a decline in the ability of the cells to bind exogenous ADA. HT-29 cells were exposed for 48 hours to control conditions or with a single addition of 300 μ mol/L of adenosine. Parallel cultures were then assayed for the presence of cell-surface DPPIV (**a**), or for their ability to bind exogenous ADA (**b**). The data are mean values $\pm$ SE ($n = 3$). **, Significant reduction by adenosine, $P < 0.01$.

of HT-29 cells to interact with cFN, both in terms of their initial binding to cFN and their behavior on that extracellular matrix protein (measured by an assay of motility on a cFN substratum). Measurement of HT-29 cell adhesion to cFN-coated surfaces showed that adenosine-pretreated cells had a reduced capacity to bind cFN (Figure 9a). In addition, adenosine-pretreated cells seeded onto one side of a porous filter that had been coated with cFN showed a reduced capacity to move from that location to the other side of the filter (Figure 9b).

Although cells may bind cFN through integrins as well as DPPIV, it has been shown that the integrin binding of HT-29 cells to FN occurs through α V β 6 (rather than the more usual β 1 integrins).⁵⁵ An assay of α V subunit on HT-29 cells using the anti- α V monoclonal antibody AMF7 revealed that the amount of cell-surface α V is not altered by adenosine pretreatment of the HT-29 cells (data not shown). This provides a strong argument that the effects of adenosine on FN binding occur independently of α V β 6, and is consistent with our view that the down-regulation of DPPIV leads to reductions in cFN binding and cellular responsiveness on cFN. These data, together with our observations of reduced ADA binding and dipeptidase activity, show clearly that the decline in DPPIV protein leads to changes in cellular functions that will have a significant impact on the behavior of the carcinoma cell population.

Discussion

We have identified a novel property of adenosine in tumor cell regulation and shown that it down-regulates the expression of the peptidase DPPIV on the surface membrane of HT-29 human colorectal carcinoma cells. The amount of DPPIV protein on the cell surface increased with time in culture, as did its enzymatic activity. This progressive increase in baseline DPPIV is consistent with the known ability of HT-29 cells to show increased expression and activity of DPPIV during acquisition of a

Figure 9. The adenosine-induced down-regulation of DPPIV is accompanied by a decline in the ability of the cells to bind to cFN (**a**), and exhibit normal motility on cFN (**b**). HT-29 cells were treated with adenosine (300 μ mol/L) or control vehicle as indicated, and tested for their ability to adhere to cFN (**a**) and to exhibit normal motility on a cFN substratum (**b**). Data are mean values $\pm$ SE with $n = 4$ (**a**) or $n = 5$ (**b**). **, Significant reduction by adenosine, $P < 0.01$.

more differentiated phenotype in response to time in culture, achievement of confluent density, and depletion of glucose.^{56–58} The DPPIV enzyme activity increased proportionally more with time relative to cell-surface protein. This suggests either that the specific activity of DPPIV is increasing with time in culture, or that the enzyme activity assay detects an increasing minor pool of DPPIV that is not recognized by our radioantibody detection procedure. This progressive change in the nature of the enzyme activity was seen for both untreated and adenosine-treated cells.

Relative to this background, we observed an initial, transient (1 to 4 hours) small (<10%) increase in DPPIV protein that was subsequently replaced by a prolonged (maximum reached at 48 hours) and more profound (typically ~40% under optimized conditions) decrease in cell-surface DPPIV expression. This decline in DPPIV protein was preceded by a decrease in the DPPIV mRNA signal measured by semiquantitative reverse transcriptase-polymerase chain reaction (data not shown), suggesting that the down-regulation occurs at least in part at the message level. The down-regulation of DPPIV was dose-dependent, with an EC_{50} for adenosine (single dose) of ~40 μ mol/L and a plateau at 100 to 300 μ mol/L. The down-regulation of immunoreactive DPPIV was not an artifact of epitope blocking because neither loading with the major DPPIV binding protein, ADA, nor acid stripping of surface ligands reduced the decrease caused by adenosine. The extent of DPPIV down-regulation produced by a single high dose of adenosine could also be matched by a dosing approach involving a more persistent exposure to adenosine at lower concentrations, comparable to those found within the extracellular fluid of solid tumor tissues.²⁷ Furthermore, daily exposure to moderate levels of adenosine produced a sustained decrease in both the amount of DPPIV protein at the cell surface and the measured dipeptidase activity. We therefore believe that the concentrations of adenosine that are present within the tumor have the capacity to down-regulate DPPIV levels and activity *in situ*.

We considered whether the effect of adenosine might be mediated by its metabolite inosine. The irreversible hydrolytic deamination of adenosine to inosine is catalyzed by ADA, and HT-29 cells express ecto-ADA activity at the cell surface (E.Y. Tan and J. Blay, unpublished data), which should lead to elevated levels of inosine. Inosine at high concentrations (~10 to 100 μ mol/L) has been shown to have cell regulatory roles, including inhibition of inflammatory cytokine production⁴⁸ and stimulation of mast cell degranulation.⁴⁷ These effects may in part be mediated by its binding to the A_3 adenosine receptor. However, inosine itself had no effect on DPPIV regulation. Furthermore, blocking inosine production with the ADA inhibitor coformycin did not abrogate the adenosine response, and indeed enhanced the potency of the response to adenosine. Guanosine was also without effect. Our data therefore point to a specific role for adenosine rather than a general effect of nucleosides.

We tested whether adenosine might be mediating its inhibitory effect on DPPIV expression intracellularly by interfering with signaling pathways⁴⁹ or by interfering with the uptake of essential nucleosides.⁵⁰ However, the nucleoside uptake inhibitors NBFI, dilazep, and dipyridamole each failed to reverse the effect of adenosine at concentrations sufficient to block uptake by both NBFI-sensitive (es) and NBFI-insensitive (ei) Na^+ -independent equilibrative transporters. The combination of NBFI and dipyridamole together, which rigorously prevents uptake by equilibrative transport,⁵⁴ was also without effect. Although Na^+ -dependent concentrative transporters are known to be expressed in certain cell types,⁵⁹ they are likely absent from human colorectal carcinoma cell lines.⁵⁴ The addition of 5'-iodotubercidin, which inhibits the phosphorylation of intracellular adenosine by adenosine kinase, also failed to prevent the adenosine effect. Thus, neither uptake nor intracellular phosphorylation of adenosine are required for adenosine to exert its effect on DPPIV. We conclude that adenosine acts at the cell surface to produce down-regulation of the DPPIV protein. Other work indicates that the effect of adenosine may be exerted through an unconventional receptor, or a co-operative action through more than one of the known adenosine receptor subtypes, and is signaled through kinase/phosphatase pathways (E.Y. Tan, D.W. Hoskin, H. Zhang, and J. Blay, manuscript in preparation).

We examined several aspects of the functional consequences of the down-regulation of DPPIV by adenosine. We would anticipate that the adenosine should have been completely degraded by the end of the 48-hour pretreatment period. As the half-time of decay of adenosine added to HT-29 cultures is ~120 minutes,⁴² even a large initial dose of 300 μ mol/L would be effectively cleared (<1 μ mol/L) in no more than ~17 hours. However, to completely exclude direct effects of adenosine, the adenosine-pretreated cells were washed thoroughly before the assays to remove any residual adenosine. The altered responses in functional assays were therefore the result of an intrinsic change in properties of the cells brought about by adenosine.

A major function of DPPIV is its binding of ADA, raising the possibility that a consequence of adenosine-induced

DPPIV down-regulation might (if it leads to a decline in ecto-ADA) be facilitation of the effects of adenosine itself. Indeed, we have confirmed here that adenosine-treated HT-29 cells have a reduced capacity for binding of ADA that is quantitatively comparable to the decrease in DPPIV. The resulting increased availability of adenosine through decreased ecto-ADA may facilitate the stimulation of cell growth that we have demonstrated in HT-29 and other colorectal carcinoma cell lines.⁴² The elevated concentrations of adenosine in the proximity of tumor cells would also impair the anti-tumor cell immune response. We and others have shown that adenosine can suppress the recognition/adhesion and effector phases of tumor cell destruction by cytotoxic lymphocytes.^{28–32} The action of adenosine that we describe here may therefore form the basis of a mechanism that amplifies the detrimental effects of adenosine in the context of tumorigenesis, providing a cascade of events that collectively

contribute a selective advantage to cells configured in a solid tumor.

Aside from autoregulation of adenosine levels, the reduction in DPPIV because of adenosine has the potential to have a number of effects that might facilitate invasion and metastasis. Firstly, the decline in net DPPIV enzyme activity will reduce the inactivation rate for certain chemokines. The chemokine that is most susceptible to DPPIV cleavage is the CXC chemokine stromal cell-derived factor-1- α (SDF-1 α , CXCL12).⁶⁰ SDF-1 α is rapidly ($t_{1/2}$, <1 minute) and effectively (K_m , 2 μ mol/L) truncated by DPPIV, causing loss of its chemotactic activity.^{61,62} Consequently, a reduction in DPPIV will facilitate the chemotactic activities of SDF-1 α and allow it to bind to its receptor CXCR4 at the cell surface.⁶⁰ CXCR4 is highly expressed on many cancer cells including colon carcinoma^{63,64} and has recently been identified as being associated with the metastatic process,⁶⁵ although in colorectal cancer it has been implicated in the outgrowth of established micrometastases, rather than in their initial movement to the metastatic site.⁶⁶ In either circumstance, increased adenosine concentrations in the primary tumor or metastatic tissue are predicted to down-regulate DPPIV and remove a constraint on SDF-1 α /CXCR4 action.

Altered DPPIV abundance at the carcinoma cell surface will also affect its interaction with the extracellular matrix.^{7–10} We chose to examine the interaction of HT-29 cells with cFN, because DPPIV binds preferentially to cell-surface FN even in the presence of the soluble form of this protein.¹⁰ Furthermore, the ability of HT-29 cells to interact with FN through alternative cell adhesion molecules is limited because integrin binding to FN occurs exclusively through $\alpha V\beta 6$ (rather than the more usual $\beta 1$ integrins).⁵⁵ Because we had shown that adenosine does not down-regulate αV immunoreactivity, we anticipated that any adenosine modulation of the interaction of HT-29 cells with cFN would occur through DPPIV. Using assays both of cell binding to adsorbed cFN and cell motility on a cFN-coated substratum, we were able to show that the adenosine down-regulation of DPPIV was accompanied by a reduction in cell:extracellular matrix interactions. Our data on the interaction of these cells with cFN suggest that the adenosine down-regulation of DPPIV may interfere with mechanisms that help to restrain cells at the initial tumor site. This may work in concert with the direct effect of adenosine on motility, because adenosine itself increases the migration of colorectal carcinoma cells (M. Mujoomdar and J. Blay, unpublished data). Together with our results that implicate reduced chemokine inactivation, these findings point to a possible facilitation of the process by which cells leave the primary site and are disseminated to other locations.

The ability of adenosine to modulate DPPIV levels in the way we have described may also explain variability in DPPIV expression in tumor tissues *in vivo*. Significant intratumoral variability in DPPIV immunoreactivity has been demonstrated in colorectal adenocarcinoma tissues, with areas of both high and low expression.^{21,22} These latter regions of reduced DPPIV expression in colorectal adenocarcinoma tumors might represent areas of DPPIV down-regulation because of increased

Figure 10. Possible scheme for the involvement of adenosine-mediated DPPIV down-regulation in altering cancer cell behavior. **a:** Enhancement of the tumor-promoting functions of adenosine through reduced local levels of bound ecto-ADA. Adenosine levels in hypoxic tumors are high.²⁷ Down-regulation of DPPIV leads to decreased ecto-ADA, adding to the rise in adenosine levels and therefore promoting events that facilitate tumor expansion.^{28–38} **b:** Implications of DPPIV down-regulation for other DPPIV functions. Down-regulation of DPPIV also leads to decreased dipeptidase activity and reduced binding to extracellular matrix (ECM) proteins. Increased chemokine activity and reduced ECM adherence, together with the effects of adenosine, may further act to enhance the spread of tumor cells.^{4,5,9,10,12,13}

adenosine production resulting from local hypoxia. In human colon adenocarcinoma xenografts, the overall fraction of hypoxic cells has been found to vary between 5% and 22%, and may exceed 80%.⁶⁷ These areas of tissue hypoxia should contain high levels of adenosine,²⁷ and on the basis of our results, would lead to a local reduction in cell-surface DPPIV expression resulting in localized changes in chemokine degradation and cell: extracellular matrix interactions.

In summary, our study shows that adenosine chronically down-regulates the surface expression of DPPIV on human colorectal carcinoma cells. This down-regulation is accompanied by reduced dipeptidyl peptidase enzyme activity, binding of ecto-ADA, and cellular interaction with the extracellular matrix. The potential impact of these alterations in cellular properties and functions are summarized in the schemes shown in Figure 10. A reduction in the level of ecto-ADA at the cell surface will facilitate cellular responses to adenosine that collectively promote tumor expansion (Figure 10a). Reductions in the other functions of DPPIV in chemokine processing and extracellular matrix association may further tend to promote processes that will lead to movement of cells from their primary location (Figure 10b). Further studies are required to determine the exact biological consequences of DPPIV down-regulation, particularly in terms of tumor growth and metastasis.

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