Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing ERK1/2 activity via a novel pathway

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Tan, Ernest Y., Cynthia L. Richard, Hong Zhang, David W. Hoskin, and Jonathan Blay. Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing ERK1/2 activity via a novel pathway. Am J Physiol Cell Physiol 291: C433–C444, 2006. First published April 12, 2006; doi:10.1152/ajpcell.00238.2005.—The multifunctional cell-surface protein dipeptidyl peptide IV (DPPIV/CD26) is aberrantly expressed in many cancers and plays a key role in tumorigenesis and metastasis. Its diverse cellular roles include modulation of chemokine activity by cleaving dipeptides from the chemokine NH2-terminus, perturbation of extracellular nucleoside metabolism by binding the ecto-enzyme adenosine deaminase, and interaction with the extracellular matrix by binding proteins such as collagen and fibronectin. We have recently shown that DPPIV can be downregulated from the cell surface of HT-29 colorectal carcinoma cells by adenosine, which is a metabolite that becomes concentrated in the extracellular fluid of solid tumors. Most of the known responses to adenosine are mediated through four different subtypes of G protein-coupled adenosine receptors: A1, A2A, A2B, and A3. We report here that adenosine downregulation of DPPIV from the surface of HT-29 cells occurs independently of these classic receptor subtypes, and is mediated by a novel cell-surface mechanism that induces an increase in protein tyrosine phosphatase activity. The increase in protein tyrosine phosphatase activity leads to a decrease in the tyrosine phosphorylation of ERK1/2 MAP kinase that in turn links to the decline in DPPIV mRNA and protein. The downregulation of DPPIV occurs independently of changes in the activities of protein kinases A or C, phosphatidylinositol 3-kinase, other serine/threonine phosphatases, or the p38 or JNK MAP kinases. This novel action of adenosine has implications for our ability to manipulate adenosine-dependent events within the solid tumor microenvironment.

CD26; deaminase binding protein; mitogen-activated protein kinases; nucleosides; tumor microenvironment

THE CELL-SURFACE PEPTIDASE, dipeptidyl peptide IV (DPPIV; EC3.4.14.5), is expressed at the surface of a variety of epithelial cells as well as on fibroblasts and certain leukocytes (19, 45). Its dipeptidase activity allows DPPIV to cleave 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 (14, 20). In addition to its own enzyme function, DPPIV is the major binding protein for the ecto-enzyme adenosine deaminase (ADA; 63), facilitating the scavenging of adenosine from the extracellular environment. DPPIV has also been found to directly interact with collagen and fibronectin (6, 11, 29, 57), playing a part in cellular interactions with the extracellular matrix. DPPIV is identical to the lymphocyte activation marker CD26, and participates in the process of T cell activation (45). This multifaceted protein therefore plays a major part in regulating the interaction of cells with both soluble and structural components of its immediate environment.

In cancer, tumorigenesis is often accompanied by a reduction in the expression of DPPIV (9, 46). This reduced DPPIV expression is directly associated with carcinogenesis because inducible gene transduction of DPPIV into melanoma cells has been shown to dramatically reverse the malignant phenotype (80). Decreased levels of DPPIV have also been linked to increased invasion and metastasis (18, 38, 55). In hepatocellular and colorectal carcinomas there is highly variable expression of DPPIV (70, 73, 74), suggesting that local influences may regulate the expression of DPPIV and therefore its participation in tumor progression.

We have recently identified a common tumor metabolite as a regulator of the cell surface expression of DPPIV protein (72). Adenosine, a purine nucleoside known best for its role in energy metabolism, is produced at increased levels in the extracellular fluid of solid tumors (8). This is a consequence of changes in the rates of production and removal of adenosine that are characteristic of hypoxic tissues like those in the poorly vascularized tumor (15, 31, 77). The adenosine that is produced is likely to have widespread actions within the tumor, including suppression of the cell-mediated immune response (33, 34, 40, 43, 44), promotion of angiogenic activity (4), stimulation of the motility of tumor cells (82), and stimulation of tumor cell growth (47, 48). We recently found that adenosine also downregulates the surface expression of DPPIV protein on HT-29 human colorectal carcinoma cells, and that this effect is accompanied by decreases in DPPIV dipeptidase activity, ADA binding, adhesion to fibronectin, and cellular motility (72). The downregulation of cell-surface DPPIV protein occurs at relatively high adenosine concentrations when a single dose of adenosine is used (EC50: 43.3 ± 12.1 μM) but repeated dosing to simulate steady-state levels takes the required dose down to the 10−5 M range (72), which approximates to the concentration present in the tumor extracellular fluid (8). The downregulation of DPPIV protein begins by 12 h following exposure to adenosine, reaches a maximum after 48 h, and is maintained if cultures are replenished with adenosine (72).

Address for reprint requests and other correspondence: J. Blay, Dept. of Pharmacology, Faculty of Medicine, Sir Charles Tupper Medical Bldg., Dalhousie Univ., 1459 Oxford St., Halifax, Nova Scotia, Canada B3H 1X5 (e-mail: jonathan.blay@dal.ca).

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Cellular responses to adenosine are usually effected through one or more of four different subtypes (A₁, A₂A, A₂B, and A₃) of G protein-coupled adenosine receptors (25), all of which are expressed to some extent on HT-29 cells (M. Mujoomdar and J. Blay, unpublished observations). We therefore anticipated that the downregulation of DPPIV would occur following adenosine occupation of such a receptor subtype(s). However, although we had shown that adenosine acts at the cell surface, extensive studies to implicate a classic adenosine receptor(s) were negative. In contrast, we show here that the adenosine downregulation of DPPIV on HT-29 cells is mediated by a novel mechanism that acts through an increase in tyrosine protein phosphatase activity to cause a decrease in the tyrosine phosphorylation of ERK1/2 mitogen-activated protein kinase (MAPK) that leads to the alteration in DPPIV mRNA and ultimately cell-surface protein. This novel action of adenosine has implications for our ability to manipulate adenosine-dependent events within the solid tumor microenvironment.

MATERIALS AND METHODS

Materials. HT-29 human colorectal carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Media were from ICN Biomedicals (Irvine, CA). Culture vessels (Nunc) and sera were purchased from Invitrogen Canada (Burlington, Ontario, Canada). All reagents for RNA isolation and RT-PCR were also from Invitrogen Canada. Adenosine, N¿(2-phenylisopropyl)adenosine (R-PIA), 8-(3-chlorostyryl)caffeine (CSC), 3-propylxanthine (enpro- fylline), 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-phenyl-1,4- (+/-)-dihydropyridine-3,5-dicarboxylate (MRS-1191), 9-chloro-2- (2-furanyl)-5-[phenylacetyl]laminol[1,2,4]-triazolo[1,5-c]quinazoline (MRS-1220), 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS-1523), N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6-hexahydro-1H-purin-8-yl)phenox]-aceta mine (MRS-1754), ephyto-9-(2-hydroxy-3-nonyl)-adene (EHNA), forskolin, 8-bromo-cAMP (8-Br-cAMP), RP diastereomer of adenosine cyclic 3',5'-phospho-ho (Rp-cAMPs), phorbol 12-myristate 13-acetate (PMA), ionomycin, 3,1-dimethylaminopropyl indol-3-yl-4[1-netyl-3-yl]maleimide hydrochloride (GF-109203X), genistein, herbimycin A, serine/threonine phosphatase inhibitor cocktail, protein tyrosine phosphatase inhibitor cocktail, sodium orthovanadate, and phenylarsine oxide were from Sigma (St. Louis, MO). 5'-N-ethylcarboxamidoadenosine (NECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), benzo[g]pteridine-2,4-[1H,3H]-dione (alloxazine), calf brain C, L-294002, and wortmannin were obtained from Research Biochemicals International (Natick, MA). Coformycin, PD-98059, SB-203580, 300125, bPv(phen), mpv PIC, and sodium stibogluconate were from Calbiochem (San Diego, CA). Mouse anti-human monoclonal antibody (mAb) against DPPIV/CDD6 (clone M-A261) and mouse IgG₁ (clone W3/25) isotype controls were from Cederlane Laboratories (Hornby, Ontario, Canada). ¹²⁵I-labeled sheep anti-mouse IgG as tracer. Data are corrected for background binding of isotype antibody, and for cell number. Where antagonists needed to be solubilized using a nonaqueous solvent, we used dimethyl sulfoxide such that the final concentration never exceeded 0.2% (vol/vol). Adenosine at the concentrations used in these studies does not induce apoptosis in HT-29 cells (48, 72). The figures show representative results from at least three separate experiments. Data were evaluated using two-tailed Student’s t-test for unpaired data, or when necessary, one-way ANOVA with Tukey-Kramer multiple-comparison posttest.

RT-PCR. Semiquantitative RT-PCR was used to evaluate DPPIV mRNA regulation by adenosine. The polymerase chain reaction was conducted in a PTC-100 thermal cycler (MJ Research, Watertown, MA) using standard protocols. The primer sequences used for the PCR were (the product size is given after the reverse primer): DPPIV (forward: 5'-CAAATTGAGCAAGGCACAGA-3'; reverse: 5'-CAGGGCTTTGAGAGATCTGAG-3') (354 bp) and GAPDH (forward: 5'-TGGAAATCCCATACCATCATT-3'; reverse: 5'-TAGTGACGGGTTGCTTCTG-3') (351 bp). The amplification protocols for DPPIV (25 cycles) and GAPDH (26 cycles) were chosen to yield PCR products in the linear range of amplification. The PCR products were visualized on a 1.5% agarose gel containing 0.2 µg/ml ethidium bromide. Band intensity was measured by densitometry and results were normalized to the steady-state expression of GAPDH. Real-time PCR amplification was performed using a Stratagene Mx3000P system (Cedar Creek, TX). DPPIV gene expression was analysed using the manufacturer’s software, standardized against GAPDH and normalized to control expression using the 2−ΔΔCt method. Similar results were obtained using different DPPIV primer sequences (data not shown).

Radioantibody binding assay for DPPIV. Monolayer cultures of HT-29 cells in 48-well plates were assayed for cell-surface DPPIV protein, as previously described (72). Briefly, cultures were incubated with anti-DPPIV antibody or isotype control, washed, and the bound antibody was measured using ¹²⁵I-labeled sheep anti-mouse IgG as tracer. Data are corrected for background binding of isotype antibody, and for cell number. Where antagonists needed to be solubilized using a nonaqueous solvent, we used dimethyl sulfoxide such that the final concentration never exceeded 0.2% (vol/vol). Adenosine at the concentrations used in these studies does not induce apoptosis in HT-29 cells (48, 72). The figures show representative results from at least three separate experiments. Data were evaluated using two-tailed Student’s t-test for unpaired data, or when necessary, one-way ANOVA with Tukey-Kramer multiple-comparison posttest.

ERK1/2 immunoblot analysis. Total cellular protein was isolated from HT-29 cells grown to 60–75% confluence in 6-well plates. Cells were rinsed twice with ice-cold PBS and dissolved in lysis buffer composed of 50 mM Tris·HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, 1X protease inhibitor mix, for 45 min at 4°C. The cell lysates were clarified by centrifugation (10 min at 12,000 g) and quantified by Bradford protein assay with the manufacturer’s instructions. Twenty micrograms of protein extract per lane were separated by SDS-PAGE using 10% gels and electrophobed to nitrocellulose. Blots were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. Blots were then probed overnight at 4°C with anti-phospho-ERK1/2 antibody at a 1 µg/ml concentration followed by incubation with sheep anti-mouse IgG-HRP-conjugated secondary antibody for 1 h at room temperature. Protein expression was detected using an enhanced chemiluminescence detection system. To confirm equal sample loading, the blots were stripped and reprobed with anti-ERK1/2 (nonphosphorylated) mAb, followed by sheep anti-rabbit IgG-HRP secondary mAb.
RESULTS

Downregulation of cell-surface DPPIV protein is preceded by a decrease in DPPIV mRNA. We have previously shown that the abundance of DPPIV protein at the cell surface begins to decrease 12 h after a single dose of adenosine, continues to decline up to 48 h even though no further adenosine is added, and starts to recover by 72 h (72). Examination of DPPIV mRNA levels over the same period showed that the abundance of mRNA declines in advance of the protein downregulation. DPPIV mRNA fell substantially by 8 h after the adenosine dose, remained decreased over the next 24 h, but returned to control levels by 36 h (Fig. 1A). More careful quantitation of DPPIV mRNA using real-time PCR showed that the decline in DPPIV mRNA expression due to adenosine was ~50% at 12 h after exposure (Fig. 1B). The downregulation of DPPIV by adenosine therefore involves regulation at the mRNA level and is not simply a consequence of altered trafficking of mature protein to or from the cell surface.

Downregulation of DPPIV by adenosine occurs through a mechanism independent of adenosine receptors. We (72) previously showed that the ability of adenosine to downregulate DPPIV was not eliminated by 8(4-nitrobenzyl)-6-thiinoinsine, dilazep, or diprydamol, which are agents that block adenosine uptake. This shows that adenosine is acting at the cell surface. Indeed, the ability of adenosine to produce maximal downregulation of DPPIV at sustained concentrations in the low micromolar range (72) is also consistent with action at an extracellular site. We therefore anticipated that adenosine would be acting through a conventional cell-surface receptor. Adenosine acts through four receptor subtypes, designated A1, A2A, A2B, and A3, which are G protein-coupled receptors that signal primarily through coupling to adenylyl cyclase (25). The HT-29 human colorectal carcinoma cells used here express all four receptor subtypes (M. Mujoomdar and J. Blay, unpublished observations).

We used selective adenosine receptor antagonists in an attempt to dissect out the receptor pathway. Despite many efforts, we were unable to block DPPIV downregulation. Representative results are shown in Table 1. It should be noted that in these and other data, there is substantial variation in the apparent DPPIV expression in untreated cultures. This is due in part to the declining specific radioactivity with time of the 125I-labeled secondary antibody used to track the surface immunoreactive DPPIV, but also to different levels of DPPIV expression at different times in culture. DPPIV is a differentiation marker for intestinal epithelium, and increases in parallel with acquisition of a slightly more differentiated phenotype as the HT-29 cells approach confluence. Adenosine promptly downregulated DPPIV beginning ~12 h after exposure (72), irrespective of time in culture, showing that its action is not simply one of hindering this differentiation process.

There was no loss of adenosine-evoked DPPIV downregulation after pretreatment and in the continued presence of: the A1 receptor antagonist DPCPX; the A2A receptor antagonist CSC; any of the three A2B receptor antagonists alloxazine, enprofylline, or the highly potent MRS-1754; or any of three different A3 receptor antagonists, MRS-1191 and MRS-1220 (Table 1) or MRS-1523 (data not shown). This lack of antagonism by the adenosine receptor antagonists suggests that DPPIV downregulation is not simply a consequence of altered trafficking of mature protein to or from the cell surface.

Table 1. Lack of effect of adenosine receptor antagonists on adenosine-mediated downregulation of DPPIV

<table>
<thead>
<tr>
<th>Adenosine Receptor Antagonist</th>
<th>Control</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1,572±14</td>
<td>1,167±20*</td>
</tr>
<tr>
<td>DPCPX</td>
<td>1,377±26</td>
<td>1,059±31*</td>
</tr>
<tr>
<td>A2A</td>
<td>5,196±118</td>
<td>3,873±70*</td>
</tr>
<tr>
<td>Control</td>
<td>5,231±32</td>
<td>4,000±63*</td>
</tr>
<tr>
<td>CSC</td>
<td>2,068±18</td>
<td>1,507±50*</td>
</tr>
<tr>
<td>Alloxazine</td>
<td>1,945±31</td>
<td>1,536±38*</td>
</tr>
<tr>
<td>Control</td>
<td>1,551±18</td>
<td>1,189±19*</td>
</tr>
<tr>
<td>Enprofylline</td>
<td>1,370±37</td>
<td>944±28*</td>
</tr>
<tr>
<td>Control</td>
<td>1,354±23</td>
<td>1,157±15*</td>
</tr>
<tr>
<td>MRS-1754</td>
<td>1,359±12</td>
<td>1,229±27*</td>
</tr>
<tr>
<td>A3</td>
<td>3,565±51</td>
<td>2,855±49*</td>
</tr>
<tr>
<td>Control</td>
<td>3,503±46</td>
<td>2,924±67*</td>
</tr>
<tr>
<td>MRS-1191</td>
<td>4,053±93</td>
<td>2,920±56*</td>
</tr>
<tr>
<td>Control</td>
<td>4,163±87</td>
<td>3,240±38*</td>
</tr>
</tbody>
</table>

Values are means ± SE (cpm); *P < 0.01, significant reduction by adenosine.
onism was evident even though the agents were used at up to 30-fold the concentrations we find necessary to block other responses of HT-29 and other cells to adenosine (60, 83). It is not likely that the antagonists are being “out competed” by endogenous adenosine produced by the cells. Although HT-29 monolayer cultures do produce adenosine (~7.5 pmol/h in the culture format used here), the steady-state concentration (in the face of constant metabolism) of adenosine would reach only 0.21 ± 0.02 μM (M. Mujoomdar and J. Blay, unpublished observations). This is insufficient to perturb baseline DPPIV levels (72) or to interfere with antagonism.

To test whether simultaneous inhibition of more than one receptor subtype might abrogate adenosine-evoked DPPIV downregulation, we treated HT-29 cells with a combination of DPCPX, CSC, alloxazine, and MRS-1523 at concentrations reflecting their relative inhibitory potency in other systems and at the highest addition that could be used without incurring excessive cytotoxicity (cell death due to the antagonists ranged from 1.6 to 8.7% in these experiments). Figure 2A shows the result of this approach. There was absolutely no diminution of the effect of adenosine, even though this combination is sufficient to entirely block the effect of adenosine on CXCR4 chemokine receptor expression in these cells (60). Consistent with the failure of adenosine receptor antagonists to block the adenosine response, we found no effect of the broadly selective agonists NECA and R-PIA on DPPIV expression, at concentrations as high as 30 μM (Fig. 2B). Taken together, these data indicate that adenosine downregulates DPPIV by a mechanism not involving the conventional adenosine receptor subtypes.

Given these unexpected negative findings with adenosine receptor antagonists and receptor-selective analogs, we evaluated the effect of manipulating the cAMP signaling network, which is the principal transduction pathway that is triggered by conventional adenosine receptors (25). Strategies to activate cAMP-dependent targets (which would parallel signaling through adenosine A2A and A2B receptors) did indeed alter DPPIV levels, but in the opposite way to adenosine. Direct activation of adenyl cyclase with forskolin led to an increase in cell-surface DPPIV protein (Fig. 3A), as did treatment of the cells with the stable, cell-permeable cAMP analog 8-Br-cAMP (Fig. 3B). Furthermore, the protein kinase A (PKA) inhibitor Rp-cAMPs (62) had no effect on the adenosine-mediated downregulation of DPPIV on HT-29 cells (Fig. 3C). These experiments exclude changes in cAMP and modulation of PKA activity from the DPPIV downregulatory response; and furthermore argue strongly against the involvement of adenosine A2A and A2B receptors in mediating adenosine-induced downregulation. This supports our conclusion that the adenosine downregulation of DPPIV occurs through a mechanism that is independent of known adenosine receptors.

Adenosine downregulation of DPPIV occurs independently of PKC, PI3K, and PTK signaling pathways. Given the lack of involvement of classic adenosine signaling pathways, we investigated other transduction pathways that have either been shown to be additional routes of adenosine signaling, or might be anticipated to be involved in the regulation of a surface adhesion molecule and peptidase such as DPPIV.

Phospholipase C (PLC) activation, which has been observed in a minority of responses to adenosine (61) leads to the generation of diacylglycerol and inositol triphosphate, which signal through PKC and the elevation of cytosolic Ca2+ levels, respectively (25). Direct activation of PKC using the phorbol ester PMA, or elevation of intracellular Ca2+ with the Ca2+ ionophore ionomycin, did not however, mimic the adenosine effect, but in contrast caused an elevation of cellular DPPIV (Fig. 4A). In addition, neither of two agents that are inhibitory for PKC, GF-109203X (75), or calphostin C (39) interfered with adenosine-evoked suppression of DPPIV (Fig. 4B). These observations exclude the involvement of the phosphoinositide cycle and PKC from the DPPIV downregulatory response caused by adenosine.

Phosphatidylinositol 3-kinase (PI3K) is a lipid/serine kinase that has been implicated in certain adenosine effects (71, 84). We used wortmannin and LY-294002, two potent and selective cell-impermeable inhibitors of PI3K (51, 78), to investigate the possible role of this pathway in the adenosine-mediated downregulation of DPPIV. However, neither LY-294002 nor wortmannin impeded the adenosine response (Fig. 4C), excluding PI3K as the proximal step in adenosine signaling in this context.

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![Fig. 2. The downregulation of DPPIV cell-surface protein due to adenosine is not due to activation of A1, A2A, A2B, or A3 adenosine receptors. A: HT-29 cells were pretreated for 30 min with control vehicle or a combination of 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 1 μM 8-(3-chlorostyryl)caffeine (CSC), 5 μM benzo[g]pteridine-2,4(1H,3H)-dione (alloxazine), and 1 μM 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS-1523). The cells were then incubated with medium alone (open bars) or adenosine at 10 μM (solid bars) plus 2.5 μM coformycin. B: HT-29 cells were treated with control vehicle or with the nonselective agonists 5′-N-ethylcarboxamidoadenosine (NECA; ●) or N′-(L-2-phenylisopropyl)adenosine (R-PIA; ○) at the concentrations indicated. Forty-eight hours later, surface expression of DPPIV was measured by radioantibody binding assay. The data are means ± SE (n = 4). **P < 0.01, significant reduction by adenosine.](image-url)

AJP-Cell Physiol • VOL 291 • SEPTEMBER 2006 • www.ajpcell.org
We next explored whether adenosine might be activating protein tyrosine kinase (PTK) pathways. We used two broad-spectrum PTK inhibitors to assess the role of PTKs in DPPIV downregulation in response to adenosine. Genistein is a potent inhibitor of most cellular PTKs (1). At concentrations of 20 μM (Fig. 4D) or 50 μM (data not shown) genistein failed to block the adenosine downregulation of DPPIV on HT-29 cells. We also used herbimycin A, a PTK inhibitor that is relatively

![Graph A](image1.png)

**Fig. 3.** cAMP/PKA-dependent signaling pathways are not required for the adenosine-mediated downregulation of DPPIV. A and B: cell-surface DPPIV levels were measured after 48-h treatment with control vehicle or forskolin (50 μM; A) or 8-bromo-cAMP (8-Br-cAMP; 1 mM) (B). C: HT-29 cells were pretreated with the PKA inhibitor Rp-cAMPS (50 μM), followed by vehicle alone (open bars) or 30 μM adenosine with 2.5 μM coformycin (solid bars). Cell-surface DPPIV levels were measured 48 h later. The data are means ± SE (n = 4). **P < 0.01, significant change.

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 4.** Adenosine regulation of DPPIV is independent of PKC, phosphatidylinositol 3-kinase (PI3K), and protein tyrosine kinase (PTK) signaling pathways. A: HT-29 cells were treated with control vehicle, the PKC activator phorbol 12-myristate 13-acetate (PMA; 5 nM) or the calcium ionophore ionomycin (1 μM) for 48 h, after which cell-surface DPPIV levels were measured by radioantibody binding assay. The effect of 30 μM adenosine with 2.5 μM coformycin is shown for comparison (solid bars). B–D: inhibition of PKC, PI3K, or PTK signaling pathways does not block the adenosine effect. HT-29 cells were pretreated with either (B) PKC inhibitors GF109203X (1 μM) or calphostin C (100 nM), (C) PI3K inhibitors LY294002 (2 μM) or wortmannin (100 nM), or (D) PTK inhibitors genistein (20 μM) or herbimycin A (1 μM) for 30 min, followed by control vehicle (open bars) or 30 μM adenosine with 2.5 μM coformycin (solid bars). Forty-eight hours later, surface expression of DPPIV was measured by radioantibody binding assay. The data are means ± SE (n = 4). **P < 0.01, significant change.
selective for src-like PTK (37). Herbimycin A at 1 μM also did not block the adenosine effect (Fig. 4D).

Adenosine downregulation of DPPIV is dependent on increased protein tyrosine phosphatase activity. Certain effects of adenosine have been shown to involve serine/threonine protein phosphatases (41, 52); while we and others have recently found that adenosine action may also be linked to the activation of protein tyrosine phosphatase (PTP) activity (30, 50, 83). We therefore examined whether the downregulatory response of DPPIV that we see here might occur through the adenosine activation of phosphatase(s). We first tested two commercially available broadly inhibitory cocktails of agents against serine/threonine and tyrosine protein phosphatases. Inhibition of serine/threonine phosphatases did not abrogate the adenosine effect at up to 0.2% vol/vol cocktail (Fig. 5A), or at a higher concentration (0.5% vol/vol, data not shown) that caused substantial (>60%) loss of cell viability. However, the tyrosine phosphatase inhibitor cocktail caused a progressive dampening of the adenosine response up to 0.2% vol/vol cocktail (Fig. 5B) and complete abrogation of the adenosine-induced downregulation of DPPIV at 0.5% vol/vol (data not shown), although that high concentration again led to a substantial (~50%) loss of cell viability.

Having found that the adenosine downregulation of DPPIV was blocked with a broad mixture of PTP inhibitors, we focused in on the possible mechanism that was initiated by adenosine. Of the constituents of the mix, the adenosine response was inhibited by sodium orthovanadate and not by molybdate, tartrate, or imidazole; all at concentrations equivalent to their presence in the cocktail (data not shown). We therefore treated HT-29 cells with adenosine in the presence and absence of either sodium orthovanadate or its peroxy derivative, bpV(phen). Orthovanadate is an inhibitor of tyrosine and dual-specificity phosphatases (35), whereas bpV(phen) is a potent and more selective PTP inhibitor (58). Figure 6, A and B, show that both of these inhibitors completely abrogated the inhibition due to adenosine. Although the blocks imposed by orthovanadate and bpV(phen) at concentrations of 200 and 20 μM, respectively, were highly reproducible, other agents with PTP inhibitory activity were not as effective. The inhibitor mpV(pic) at concentrations up to 50 μM did not block, or at best gave a partial block, in multiple experiments (data not shown). The ability of phenylarsine oxide to block the adenosine effect was difficult to assess because this inhibitor was highly cytotoxic (EC50 = 1 μM, reaching complete cell death at 5 μM) over the 48-h treatment period. However, no inhibition of the adenosine response was evident in experiments with phenylarsine oxide at 0.5 μM or 1 μM (data not shown). Taken together, these data demonstrate the involvement of a PTP(s) in downregulation of cell-surface DPPIV on HT-29 cells exposed to adenosine. The selectivity to certain
PTP inhibitors and not others should provide clues as to the particular PTP that is activated by adenosine.

Adenosine downregulation of DPPIV is dependent upon ERK1/2, but not p38 or JNK MAP kinases. We next considered the possible role of MAPK signaling pathways in mediating the adenosine effect. MAPK pathways are subject to regulation by PTPs (10, 22, 49, 59). Furthermore, adenosine has been reported to have the capacity for signaling through the p38, extracellular signal-regulated kinase (ERK), and c-Jun NH2-terminal kinase (JNK) MAPK signaling pathways (30, 42, 65; reviewed in Ref. 66). It seemed possible that adenosine-activated PTP(s) might act through MAPK pathways to cause the changes in DPPIV.

Inhibition of p38 with SB-203580 (12, 36) did not block or enhance the adenosine effect (Fig. 7A). Similarly, the JNK inhibitor SP-600125 (7, 27) failed to block or enhance the adenosine downregulation of DPPIV (Fig. 7B). Although inhibition of JNK led to a dose-dependent increase in baseline DPPIV expression (mean increase, 70.5% at 20 μM SP-600125), there was no change in the extent of the adenosine depression. However, the effect of PD-98059, a MAPK kinase (MEK1) inhibitor (2), was more informative (Fig. 7C). First, treatment of HT-29 cells with PD-98059 by itself caused a downregulation of DPPIV cell-surface protein comparable to adenosine. This is consistent with the notion that a decrease in the active tyrosine-phosphorylated form of ERK, caused either through adenosine-stimulated PTP activity, or through PD-98059-mediated inhibition of MAPKK (MEK), is linked to a decline in DPPIV. In addition, PD-98059 plus adenosine caused a greater but not additive decrease in DPPIV, consistent with these two interventions acting through the same process (Fig. 7C). These findings provide indirect evidence that the adenosine-mediated downregulation of DPPIV involves the ERK1/2 pathway and is associated with a decrease in ERK1/2 activation.

Adenosine causes a decrease in phospho-ERK1/2 that is dependent upon a vanadate- and bpV(phen)-dependent PTP. Given the evidence of an adenosine-triggered PTP activity and involvement of ERK1/2 in the downregulation of DPPIV, we investigated directly the effect of adenosine on ERK1/2 tyrosine phosphorylation. As shown in Fig. 8, both ERK1 and ERK2 were constitutively activated in HT-29 cells. Exposure to a single dose of adenosine markedly decreased the level of tyrosine-phosphorylated ERK1 and ERK2. The effect of adenosine was evident within 10 min, maximal by 20 min, and sustained for 60 min after stimulation (Fig. 8A). Adenosine (single dose, no coformycin present) caused an observable decrease in ERK1/2 phosphorylation at 10 μM and a maximum effect at a concentration of ~100 μM (Fig. 8B). This dose-response relationship exactly parallels the concentration dependence of the adenosine downregulation of DPPIV following a single adenosine dose (72). A reduction in overall tyrosine protein phosphorylation in the 40–50 kDa range of proteins from adenosine-treated HT-29 cells was also observed after Western blot analysis of total cellular lysates with anti-phospho-tyrosine mAb (data not shown), consistent with a reduction in ERK1/2 phosphorylation. These findings, Figs. 6, A and B, and 7C, strongly argue that the adenosine reduction, together with those of cell-surface DPPIV in HT-29 cells occurs in close association with negative regulation of the ERK1/2 signaling pathway.

A corollary of the above is that activation of the ERK1/2 pathway should lead to increased expression of DPPIV. We
therefore examined whether PMA treatment, which increases DPPIV on HT-29 cells (Fig. 4A), was associated with an increased level of ERK1/2 phosphorylation over the same time course as the decrease in phosphorylation produced by adenosine. PMA indeed caused a substantial elevation in ERK phosphorylation (Fig. 9), producing a ~45% increase in ERK2 phosphorylation at the same concentration (5 nM) that led to a ~40% increase in the amount of DPPIV protein at the cell surface (Fig. 4A).

Finally, HT-29 cells were treated with sodium orthovanadate to inhibit PTPs, and then exposed to adenosine for 40 min before examining the consequences for ERK1/2 phosphorylation. Figure 10 shows that the dose-dependent inhibitory effect of adenosine on ERK1/2 tyrosine phosphorylation was abrogated in the presence of sodium orthovanadate. The same result was observed using bpV(phen) (20 μM; data not shown). These findings show that reduced phosphorylation and therefore activity of ERK1/2 due to adenosine is dependent on PTP(s). The inhibition of ERK1/2 phosphorylation due to adenosine is sensitive to inhibition by either vanadate or bpV(phen), which abrogate the adenosine-induced downregulation of DPPIV.

DISCUSSION

The ability of high concentrations of adenosine in the tumor microenvironment (8) to modulate DPPIV (72) has substantial implications for the evolution of the tumor and the fate of the cancer cells. We have shown that the ability of adenosine to downregulate DPPIV from the surface of HT-29 colorectal carcinoma cells is accompanied by functional sequelae that should favor tumorigenesis (72). It is therefore important to understand how extracellular adenosine leads to a decrease in cell-surface DPPIV, because if we were able to interfere with this process we might to some extent restore DPPIV levels and thereby reverse the negative consequences of the loss of DPPIV functional activities.

We confirmed here that the downregulation of DPPIV by adenosine involved regulation of mRNA levels, and was not purely a perturbation of cellular trafficking of mature DPPIV protein to or from the cell surface, although such changes in trafficking may occur. The decline in mRNA expression (~50%) is at first sight somewhat small compared with such changes in many other model systems. However, the decrease was maintained over a period of some 36 h and was similar in magnitude to the decrease in DPPIV protein. It is therefore
consistent with the ensuing protein response. The ~50% decrease in the abundance of cell-surface DPPIV is likely important because its impact is to alter enzyme activities (dipeptidase and deaminase) and cell-extracellular matrix interactions that collectively provide a multiple and magnified response. This is shown by observations that adenosine-treated cells, due to downregulated DPPIV, migrate at less than half the rate of untreated cells on cellular fibronectin (72) and that the decline in ecto-ADA bound to DPPIV leads to an increase in the half-life of adenosine in HT-29 cell cultures from ~2 h (48) to >24 h (60). Given that adenosine is immunosuppressive, mitogenic for tumor cells, and also angiogenic (4, 33, 34, 43, 44, 47, 48) the latter event would have major consequences in the context of an intact tumor.

Because we had excluded any possible action of adenosine on intracellular targets such as the adenyl cyclase “P” site (72), our expectation was that one of the existing well-characterized adenosine receptor subtypes (A1, A2A, A2B, or A3) would be involved in triggering this process. However, numerous experiments using eight different established antagonists (and with particular attention paid to the A2B and A3 receptors that are most robustly expressed on the HT-29 cells; M. Mujoomdar and J. Blay, unpublished observations) failed to block the adenosine response. Most convincingly, when a combination of antagonists against all four subtypes (at the highest feasible concentrations) was used, there was absolutely no reduction in the adenosine-evoked downregulation of DPPIV. Furthermore, the broadly selective agonists NECA (A1, A2, A3) and R-PIA (A1, A2) failed to mimic the action of adenosine even at concentrations as high as 30 μM. Finally, the failure of the PKA inhibitor Rp-cAMPs to alter the adenosine-mediated downregulation of DPPIV shows that the major signaling route employed by all four of the adenosine receptor subtypes, the cAMP signaling pathway, is not involved. We are therefore compelled toward the conclusion that the adenosine downregulation of DPPIV does not occur through the conventional adenosine receptors so far known.

Other researchers (21, 23, 28, 81) have also found evidence for the existence of atypical, or nonclassic, adenosine receptor subtypes. We have already established that high levels of adenosine are sensed at the cell surface, rather than evoking a change in DPPIV after entry into the cell through nucleoside transporters (72). The downregulation is not simply a cell-surface perturbation triggered by extracellular adenosine that leads to intracellular sequestration of DPPIV (as for example occurs with Caco-2 cells treated with forskolin; Ref. 5). In addition to the alteration in mRNA, the time course is relatively slow (maximum reached at 48 h; Ref. 72), and we show here that there is clearly an intermediary intracellular signaling pathway that leads to the decline in DPPIV protein.

Our extensive studies using inhibitors of signaling pathways lead us to the conclusion that the adenosine effect on DPPIV is exerted through intermediate steps that involve 1) activation of a protein tyrosine phosphatase that is sensitive to inhibition by orthovanadate and bpV(phen), and 2) reduction in the activation of ERK1/2 MAPK. Our conclusion that ERK1/2 is involved in regulation of DPPIV levels is further supported by our demonstration that PMA, which in contrast to adenosine, increases the expression of cell-surface DPPIV, also acts oppositely to increase the activation of ERK1/2 over the same timeframe.

Regulation of ERK1/2 (p42/p44) MAPK in response to adenosine has been reported in other cellular systems. Human A1, A2A, A2B, and A3 adenosine receptors expressed in Chinese hamster ovary cells [and A2A receptors expressed in human embryonic kidney-293 (HEK-293) cells] all couple to the ERK1/2 MAPK pathway, but in each case adenosine or an appropriate synthetic agonist increase the phosphorylation of ERK1/2 (16, 64, 67, 76). Such expression models may risk revealing pathways that normally do not play a major role (26,
32). However, stimulation of ERK1/2 through endogenous adenosine receptors (mainly A2) has also been observed, in primary human endothelial cells (A2A; Ref. 68), untransfected HEK-293 human embryonic kidney cells (A2B; Ref. 26), HMC-1 human mast cells (A2B; Ref. 24), BR canine mastocytes (A2B; Ref. 26), PC12 rat pheochromocytoma cells (A2A; Ref. 3) and XS-106 mouse dendritic cells (A2B and A3; Ref. 17). There are data showing that adenosine may inhibit the stimulation of ERK1/2 phosphorylation by other factors, including thrombin (32) and NGF (3). However, this report is the first observation, to our knowledge, of adenosine itself causing negative regulation of the ERK1/2 signaling pathway. It is notable that most studies of ERK1/2 activation have used stable ligands such as NECA or agents selective for the appropriate receptor subtype, whereas we would argue, based on earlier observations (48), that adenosine itself may elicit different cellular responses to its analogs.

The dose-response relationship for adenosine inhibition of ERK1/2 phosphorylation in HT-29 cells exactly parallels that of the reduction in cell-surface protein, indicating a single affinity of ligand interaction for the two adenosine effects, which is consistent with linkage to a common receptor that initiates the response. However, our results differ from other studies of ERK1/2 activation not only in that we see an inhibition, rather than a stimulation, of activity by adenosine (or analogs), but that we fail to attribute the sensing of the initial signal to any of the four well-characterized receptor subtypes. This is not an artifact of inappropriate adenosine concentration, as our data show sensitivity to adenosine down to the 10^{-3} M range without inhibiting its metabolism; and we routinely treat with 10–30 μM adenosine in the presence of 2.5 μM coformycin or 20 μM EHNA to inhibit ADA-mediated breakdown. This contrasts, for example, with the millimolar dosing used by Harrington and colleagues (30) in their studies of adenosine p38α modulation, which leads to apoptosis.

The lack of evidence for the involvement of cAMP pathways in adenosine-induced DPP IV downregulation is consistent with data showing that adenosine modulates ERK1/2 signaling irrespective of whether increases (A2A, A2B) or decreases (A1, A3) in cAMP would be expected to occur (16, 17, 16, 67, 76); and that stable cAMP analogs do not produce changes in ERK1/2 (67, 68). Furthermore, the dose-response relationships for ERK1/2 phosphorylation vs. cAMP accumulation in sensitive cells differ by two orders of magnitude (64). The situation differs in adenosine receptor-transfected Chinese hamster ovary and HEK-293 cell models, in which cAMP seems to play a major role (see Refs. 26, 32).

In contrast, we have found striking evidence for adenosine stimulation of a protein phosphatase activity that is able to produce the dephosphorylation of ERK1/2 that we have observed. The individual PTP inhibitors orthovanadate and bpV-(phen) completely abrogated both the adenosine suppression of ERK1/2 activation and the downregulation of cell-surface DPP IV. This observation parallels our findings in a recent exploration of adenosine inhibition of IL-2 signaling in T cells (83). In that work, we found that adenosine activates Src homology PTP 2 (SHIP-2) to reduce the tyrosine phosphorylation of STAT5α/b, and that this is blocked by orthovanadate and bpV(phen).

The PTP target that is the focus of adenosine action and which is inhibited by orthovanadate and bpV(phen) is currently under investigation. Our preliminary studies have excluded the involvement of SHP-2, which in any case is usually coupled to an increase in ERK activation (13, 69), as well as SHP-1 and PTP1B: sodium stibogluconate, at a concentration of 100 μM (105 μg/ml), which is sufficient to inhibit these three PTPs (54), produced no change in the decrease in DPP IV levels due to adenosine (data not shown). We are currently using other approaches to identify which of the many other potential ERK-specific phosphatases (56, 59, 85) is/are activated by adenosine to reduce ERK1/2 phosphorylation and lead to DPP IV downregulation.

In summary, we have identified a pathway leading from the encounter of the cancer cell with adenosine at concentrations that exist in the tumor extracellular fluid, to the downregulation of DPP IV and its associated functions. This pathway involves the activation of a PTP by adenosine through a route that is independent of the existing adenosine receptor subtypes, and does not require participation of the PKA, PLC, PKC, PI3K, or PTK pathways. Activation of the PTP by adenosine is associated with the reduced tyrosine phosphorylation and activity of ERK1/2 MAPK, which is required for the downregulation of DPP IV to occur. The steps that are involved in initial sensing of the adenosine signal, and the precise phosphatase(s) involved in ERK1/2 suppression, remain to be elucidated. Protein-tyrosine phosphatases play a substantial role in regulation of solid cancers, particularly in colorectal carcinoma (53, 79).

Understanding this particular pathway should allow us to be able to block the downregulation of DPP IV due to adenosine and its adverse consequences, leading to new ways of interfering with tumor expansion and spread. The independence of this DPP IV-downregulatory pathway from known adenosine receptors is an advantage, as the conventional signaling pathways for adenosine are involved in numerous aspects of physiologic regulation (such as blood flow), which we would prefer to spare in any pharmacological intervention focusing on adenosine’s tumor-enhancing actions.

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