Adenosine Receptors as Mediators of Both Cell Proliferation and Cell Death of Cultured Human Melanoma Cells

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Adenosine displays contradictory effects on cell growth: it improves cell proliferation, but it may also induce apoptosis and impair cell survival. Following the pharmacologic characterization of adenosine receptor expression on the human melanoma cell line A375, we chose A375 as our cellular model to define how the extracellular adenosine signals are conveyed from each receptor. By using selective adenosine receptor agonists or antagonists, we found that A_{2A} stimulation reduced cell viability and cell clone formation, whereas, at the same time, it improved cell proliferation. In support of this finding we demonstrated that the stimulation of A_{2A} adenosine receptors stably expressed in Chinese hamster ovary cell clone reproduced deleterious effects observed in human melanoma cells. A_3 stimulation counteracted A_{2A}-induced cell death but also reduced cell proliferation. Furthermore, we found that A_3 stimulation ensures cell survival. We demonstrated that adenosine triggers a survival signal via A_3 receptor activation and it kills the cell through A_{2A} receptor inducing a signaling pathway that involves protein kinase C and mitogen-activated protein kinases. ***Key words: adenosine receptors/apoptosis/cell proliferation/melanoma/molecular signaling. J Invest Dermatol 119:923–933, 2002***

Adenosine displays contradictory effects: it has been reported that adenosine improves and impairs cell proliferation (Ethier and Dobson, 1997; MacLaughlin *et al*, 1997; Fishman *et al*, 1998, 2000; Barry and Lind, 2000; Brown *et al*, 2000), induces apoptosis and cell death (Kohno *et al*, 1996; Barry and Lind, 2000; Fishman *et al*, 2000; Shneyvays *et al*, 2000), but also displays heart and brain cytoprotective functions during ischemia. Furthermore, adenosine affects the immune system by exerting immunosuppressive and anti-inflammatory activities (Ralevic and Burnstock, 1998).

Extracellular adenosine interacts with cells by two means: at nanomolar and micromolar concentrations adenosine exerts its effects as a consequence of the binding and activation of specific cell surface receptors; at higher doses (millimolar concentrations), it produces toxic effects probably by interfering with the homeostasis of the intracellular nucleotide pool (Schrier *et al*, 2001).

Four different adenosine receptors have been identified and pharmacologically characterized: A_1, A_{2A}, A_{2B}, and A_3 (Fredholm *et al*, 2001). These receptors are seven transmembrane glycoproteins coupled with G proteins and are widely distributed in human tissues and organs. A_{2A} and A_{2B} receptors are coupled to adenylate cyclase activity and their stimulation increases the intracellular cyclic adenosine monophosphate concentration. A_1 and A_3 receptor stimulation decreases cyclic adenosine monophosphate (cAMP) concentration and raises intracellular Ca^{2+} levels by a pathway involving phospholipase C (PLC) activation (Abbracchio *et al*, 1995; Fredholm *et al*, 2001).

The immunosuppressive and anti-inflammatory effects of adenosine together with the increased extracellular concentration found in tumor masses, have prompted us to hypothesize that adenosine receptors could play a part in tumor development, diffusion, and immunoescaping (Blay *et al*, 1997; Barry and Lind, 2000; Ohana *et al*, 2001).

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Abbreviations: PKA, protein kinase A; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; CHO, Chinese hamster ovary cells; CHO-hA_1, CHO cells transfected with the human recombinant A_1 adenosine receptors; CHO-hA_{2A}, CHO cells transfected with the human recombinant A_{2A} adenosine receptors; CHO-hA_3, CHO cells transfected with the human recombinant A_3 adenosine receptors; HENECA, 2-hexynyl-5′*N*–ethylcarboxamidoadenosine; Cl-IB-MECA, N^6(3-iodobenzyl)2-chloroadenosine-5′*N*–methyluronamide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; SCH 58261, 7-(2-phenylethyl)2-(2-furyl)pyrazolo[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine; MRE 3008F20, 5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3e]1,2,4-triazolo [1,5c] pyrimidine; MRE 3055F20, 5*N*-(4-phenylcarbamoyl)amino-8-propyl-2-(2-furyl) pyrazolo[4,3e]1,2,4-triazolo[1,5c]pyrimidine; MRE 3062F20, 5*N*-(4-phenyl-carbamoyl)amino-8-butyl-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5-c]pyrimidine; H89, *N*-[2-((p-bromocinnamyl) amino)ethyl]-5-isoquinolinesulfonamide, 2 XHEE; PD 98059, 2-amino-3-methoxyflavone; Z-VAD-FMK, Z-Val-Ala-Asp(OMe)-CH_2F; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; G418, geneticine; MEK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; pAb, polyclonal antibodies; DABCO, (1–4-diazibicyclo[2,2,2] octane.

The molecular mechanisms supporting adenosine effects on different normal and tumoral tissues have not yet been carefully explored. In particular, adenosine, acting at specific A_{2A} receptors, promotes wound healing in both normal animals and in animals with impaired wound healing (Montesinos *et al*, 1997). Furthermore, adenosine stimulates angiogenesis in the dermis, increases collagen and glycosaminoglycan production from fibroblasts (Ethier *et al*, 1993; Ethier and Dobson, 1997). More recently, it has been demonstrated that treatment of human keratinocytes with adenosine may arrest keratinocyte proliferation (Brown *et al*, 2000). Moreover, A_3 adenosine receptor blocks ultraviolet irradiation-induced apoptosis in mast-like cells (Gao *et al*, 2001).

It is well known that ultraviolet radiation is associated with cutaneous malignant melanoma (Landi *et al*, 2002). Excessive exposure to ultraviolet among Caucasians is the main etiologic factor implicated in the incidence of melanoma, a serious form of skin cancer (MacKie, 1998). In view of these hallmarks, clarification of the cellular and biochemical mechanisms of adenosine may be important for the understanding of its possible role in the pathogenesis of tumors of the skin, such as basal cell carcinoma, squamous cell carcinoma, and melanoma.

Because the contradictory effects of adenosine appear to be triggered by the simultaneous engagement of different adenosine receptor subtypes, a complete pharmacologic characterization of the type and the number of the expressed adenosine receptors is required for the understanding of adenosine's roles (Ohana *et al*, 2001). Recently, we have characterized the expression pattern of the adenosine receptors on the surface of human melanoma A375 cells and documented the ability of these receptors to signal after selective agonists binding (Merighi *et al*, 2001). A375 cells are an established human melanoma cell line that belongs to a relatively undifferentiated and highly metastatic class of melanocytic cells with epithelioid shape and absence of pigmentation (Okazawa *et al*, 1998). In these cells mRNA for all four adenosine receptor subtypes was detected. The A_{2A} and A_3 receptor proteins are present at high levels on the cell surface (220 ± 7 and 291 ± 50 fmol per mg of protein, respectively), whereas A_1 receptors are present in a lower amount (23 ± 7 fmol per mg of protein). The lack of commercially available selective radioligands for A_{2B} (Klotz *et al*, 1998) prevented direct labeling of this receptor subtype in A375 cells and similar systems containing other adenosine receptors; however, functional studies indicate that A375 cells express an A_{2B}-adenosine receptor coupled positively to adenylyl cyclase (Merighi *et al*, 2001).

In this study we investigated the effects of adenosine on cell proliferation, colony formation ability, and on the balance between cell survival and cell death in A375 cells. Furthermore, Chinese hamster ovary (CHO) cells stably transfected with human recombinant adenosine receptors were used to confirm the role of adenosine receptors.

MATERIALS AND METHODS

Chemicals and reagents A375 cells were obtained from American Tissue Culture Collection (ATCC). CHO cells transfected with the human recombinant A_1, A_{2A}, and A_3 adenosine receptors (CHO-hA_1, CHO-hA_{2A}, CHO-hA_3) were prepared as described earlier (Klotz *et al*, 1998). Tissue culture media and growth supplements were obtained from BioWhittaker (Bergamo, Italy). 2-hexynyl-5′*N*–ethylcarboxamidoadenosine (HENECA), was a kind gift of Prof. G. Cristalli, University of Camerino, Italy; N^6(3-iodobenzyl)2-chloroadenosine-5′*N*–methyluronamide (Cl-IB-MECA) and 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX), were from RBI (Milano, Italy; 7-(2-phenylethyl)2-(2-furyl)pyrazolo[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine (SCH 58261), 5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3e]1,2,4-triazolo[1,5-c]pyrimidine (MRE 3008F20), 5*N*-(4-phenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3e]1,2,4-triazolo[1,5c]pyrimidine (MRE 3055F20), and 5*N*-(4-phenyl-carbamoyl)amino-8-butyl-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5-c]pyrimidine (MRE 3062F20) were synthesized by Prof. P.G. Baraldi, University of Ferrara, Italy. Adenosine was dissolved in Dulbecco's modified Eagle's medium (DMEM) and stored at –80°C. HENECA, Cl-IB-MECA, SCH 58261, MRE 3008F20, MRE 3055F20, MRE 3062F20, and DPCPX were dissolved in dimethylsulfoxide (DMSO) and stored at –20°C. H89, *N*-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2XHCE [a selective inhibitor of protein kinase A (PKA)], chelerythrine-D [a selective inhibitor of protein kinase C (PKC)], staurosporine (a potent broad spectrum inhibitor of protein kinases A, C, and G), 2-amino-3-methoxyflavone [(PD 98059) a selective inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK)] and Z-Val-Ala-Asp(OMe)-CH_2F (Z-VAD-FMK) (a highly specific and irreversible inhibitor of caspases, including caspase-1, -3, -4, and -7) were obtained from Calbiochem (Milano, Italy). U0126 (an inhibitor of MEK-1 and MEK-2) and Anti-ACTIVE® MAPK polyclonal antibodies (pAb) and anti-ERK 1/2 pAb were from Promega (Milano, Italy). RNAse was purchased from Boehringer (Milano, Italy). [^{3}H]Thymidine was obtained from NEN. Mitochondrial dehydrogenase activity was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Unless otherwise noted, all other chemicals were purchased from Sigma (Milano, Italy).

Cell culture A375 cells were grown adherently and maintained in DMEM, containing 10% fetal bovine serum, penicillin (100 U per ml), streptomycin (100 μg per ml), and L-glutamine (2 mM) at 37°C in 5% CO_2/95% air.

The CHO-hA_1, CHO-hA_{2A}, and CHO-hA_3 cellular clones were grown adherently and maintained in DMEM with nutrient mixture F12, containing 10% fetal bovine serum, penicillin (100 U per ml), streptomycin (100 μg per ml), L-glutamine (2 mM), and geneticine (G418) 0.2 mg per ml at 37°C in 5% CO_2/95% air.

Cells were passaged two or three times weekly at a ratio between 1 : 5 and 1 : 10.

Metabolic inhibitors Cells labeled for the JAM test (Matzinger, 1991) were treated for 30 min with metabolic inhibitors or with drug vehicle (DMSO) prior to being challenged with adenosine or adenosine analogs. After 24 h, cells were harvested for DNA-lost quantification. H89 was used at 100 μM as a PKA inhibitor; Chelerytrine-D was used at 2 μM as a PKC inhibitor; Staurosporine was used at 100 nM as a PKA, PKC, and protein kinase G inhibitor. PD 98059 was used at 20 μM as an inhibitor of MEK to prevent MEK-1 activation. U0126 was used at 10 μM as inhibitor of MEK-1 and MEK-2 to prevent extracellular signal-regulated kinase (ERK)-1 and ERK-2 activation. Z-VAD-FMK was used at 1 μM as an irreversible inhibitor of caspase-1, -3, -4, and -7.

MTT assay The number of living cells was determined by evaluating the mitochondrial dehydrogenase activity by using MTT that is converted into a formazan product in living cells. Cells (10^5) were plated in 24-multiwell plates; 500 μl of complete medium were added to each well with different concentrations of adenosine receptor agonists and antagonists. The cells were then incubated for 24 h. At the end of the incubation period, 50 μl of MTT solution (5 mg per ml) were added to each well. The plates were incubated for 2 h at 37°C then 550 μl of an acid propanol solution (0.1 M HCl in isopropanol) were added to each well to dissolve the formazan. The optical density of each well was read on a spectrophotometer at 570 nm. For each experiment, four individual wells of each drug concentration were prepared. Each experiment was repeated three times.

JAM test This assay allows us to analyze cell death (apoptotic plus necrotic cells) by performing the quantification of the amount of fragmented DNA. Target cells were labeled with 1 μCi per ml of [^{3}H]thymidine for 20 h in DMEM containing 10% fetal bovine serum, penicillin (100 U per ml), streptomycin (100 μg per ml), and L-glutamine (2 mM). The cells were then washed and treated with new unlabeled medium containing adenosine or other analogs for 24 h. At the end of the incubation period the cells were trypsinized and dispensed in four wells of a 96 well plate, filtered through Whatman GF/C glass-fiber filters using a Micro-Mate 196 cell harvester (Packard Instrument Company, Pero, Italy). The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

The amount of apoptotic and necrotic cells, measured as the loss of radioactivity associated with the loss of fragmented and degraded DNA, was detected by filtration and subsequent washing with a Micro-Mate 196 cell harvester followed by quantification with a Top Count Microplate Scintillation Counter.

The percentage of cell death is expressed as $100 \times (dpm_{(U)} - dpm_{(T)})/dpm_{(U)}$ were $dpm_{(U)}$ is the radioactivity of untreated cells and $dpm_{(T)}$ is the radioactivity of treated cells (Secchiero *et al*, 2001).

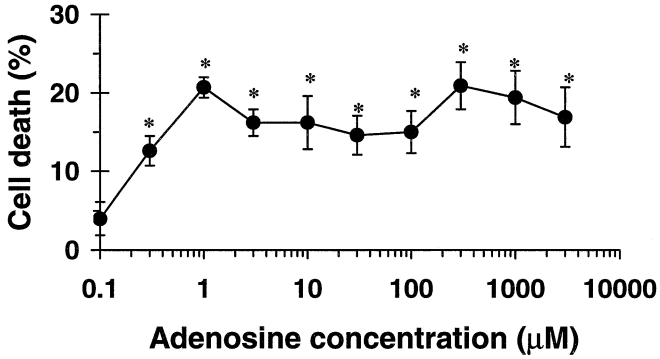

Figure 1. A375 cell death induction by adenosine. The dose–response curve of A375 cells sensitivity to adenosine is reported. A375 cells were treated with adenosine at indicated doses for 24 h and cell death was quantified by JAM test. Percentage of cell death ([^{3}H]thymidine-labeled DNA loss) is reported in ordinate with SD error (vertical bar). Values represent mean (± SEM) of four separate DNA-lost quantification in the same experiment. 100% indicates 100% loss of radioactivity incorporated by untreated cells. *$p < 0.05$ with respect to control ''untreated cells''; analysis was by ANOVA followed by Dunnett's test.

[^{3}H]thymidine incorporation: cell proliferation test Cells were seeded in fresh medium with 1 μCi of [^{3}H]thymidine per ml in DMEM containing 10% fetal bovine serum, penicillin (100 U per ml), streptomycin (100 μg per ml), and L-glutamine (2 mM), and simultaneously treated with adenosine or other analogs. After 24 h of labeling, cells were trypsinized, dispensed in four wells of a 96-well plate, and filtered through Whatman GF/C glass-fiber filters using a Micro-Mate 196 cell harvester (Packard Instrument Company). The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

Colony formation assay Exponentially growing cells were seeded at 300 cells per well in six-well plates with 2 ml of fresh medium [DMEM containing 10% fetal bovine serum, penicillin (100 U per ml), streptomycin (100 μg per ml), and L-glutamine (2 mM) for A375 cell line; DMEM-F12 containing 10% fetal bovine serum, penicillin (100 U per ml), streptomycin (100 μg per ml), L-glutamine (2 mM), G418 0.2 mg per ml for stable transfected CHO cell lines] and treated with adenosine or other analogs dissolved in DMSO solution. Control plates received the same volume of DMSO alone. After 7 d of growth at 37°C in humidified atmosphere containing 5% CO_2, the cells were fixed with absolute methanol for 5 minutes and stained with 1/10 Giemsa/phosphate-buffered saline (PBS) staining solution for 10 min. Staining solution was removed and colonies of greater than 30 cells were scored as survivors. For each treatment, six individual wells were scored.

Flow cytometry analysis A375 adherent cells were trypsinized, mixed with floating cells, washed with PBS and permeabilized in 70% (v/v) ethanol/PBS solution at 4°C for at least 24 h. The cells were washed with PBS and the DNA was stained with a PBS solution, containing 20 μg per ml of propidium iodide and 100 μg per ml of RNAse, at room temperature for 30 min point Cells were analyzed by FACScan (Becton-Dickinson, Milano, Italy) and the content of DNA was evaluated by the Cell-LISYS program (Becton -Dickinson). Cell distribution among cell cycle phases and the percentage of apoptotic cells were evaluated as previously described (Secchiero *et al*, 2001). Briefly, the cell cycle distribution is shown as the percentage of cells containing 2n (G_1 phase), 4n (G_2 and M phases), and $4n > \times > 2n$ DNA amount (S phase) judged by propidium iodide staining. The apoptotic population is the percentage of cells with DNA content lower than 2n.

Morphologic analysis To recover all seeded cells, the adherent culture fraction was trypsinized and mixed with the supernatant fraction. Then, the cell suspension was spun to a slide by cytospin 3 (Shandon, Cheshire, U.K.) at 250 r.p.m. (500 g) for 10 min. As previously described (Secchiero *et al*, 2001), cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in PBS solution containing 0.1% of Triton X-100, and the DNA was stained with 4′,6′,-diamino-2-phenyl-indole. Slides were mounted in (1–4-diazibicyclo[2,2,2]octane (DABCO) glycerol-PBS and observed on Zeiss Axiophot fluorescent microscope.

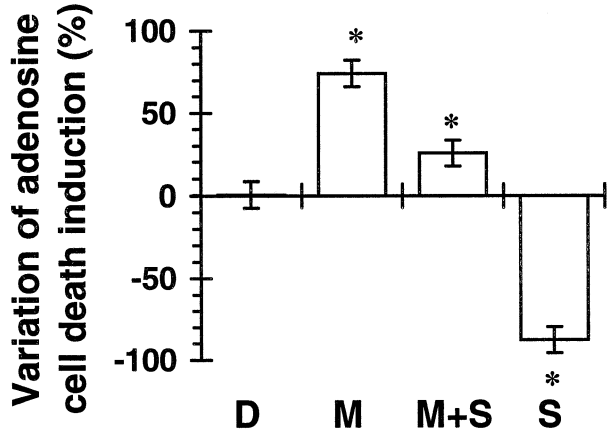

Figure 2. Role of A_{2A} and A_3 in adenosine-induced cell death on A375 cells. A375 cells were treated with 1 μM of adenosine to induce cell death quantified by JAM test. Cells were pretreated with drug vehicle (DMSO), with MRE 3008F20 1 μM, with SCH 58261 1 μM or with MRE 3008F20 1 μM plus SCH 58261 1 μM. Vertical axis reports percentage of cell death variation with respect to adenosine-induced cell death: positive/negative values represent increase/decrease of adenosine-induced cell death. Horizontal axis: ''D'' DMSO control, ''M'' MRE 3008F20, ''S'' SCH 58261, ''M + S'' MRE 3008F20 + SCH 58261. Negative 100% (– 100%) indicates restored vitality. *$p < 0.05$ with respect to control ''DMSO''; analysis was by ANOVA followed by Dunnett's test.

Immunofluorescence detection of activated ERK-1 and ERK-2 ERK-1 and ERK-2 were detected by Anti-ACTIVE®MAPK pAb. Anti-ACTIVE®MAPK pAb is an affinity-purified rabbit serum that specifically recognizes the dually phosphorylated active forms of MAP (also known as p44/ERK-1 and p42/ERK-2) enzymes.

A375 cells were seeded on glass coverslips in 24-well plates by overnight growth at 37°C in humidified atmosphere containing 5% CO_2. A375 cells were treated with 100 μM of adenosine or with 10 μM of Cl-IB-MECA, MRE 3008F20, and SCH 58261, or with 100 nM of HENECA. After 15 or 30 min the medium was removed, cells were washed two times with PBS, fixed in 10% paraformaldehyde for 30 min, permeabilized in a PBS solution containing 0.1% of Triton X-100 and incubated for 30 min with PBS plus 5% goat serum and 0.5% bovine serum albumin. The cells were then stored for 24 h at 4°C in a humidified chamber with 30 μl of a 1 : 200 dilution of Anti-ACTIVE®MAPK pAb solution containing 0.5% of goat serum and 0.5% of bovine serum albumin in PBS. Excessive antibody was washed away with PBS and rabbit antibodies were detected with fluorescein isothiocyanate-labeled goat anti-rabbit IgG. Coverslips were stained with 4′,6′,-diamino-2-phenyl-indole, mounted in DABCO glycerol-PBS and observed on Zeiss Axiophot fluorescent microscope.

Western blotting The phosphorylation and activation of ERK-1 and ERK-2 was detected by western blotting using the Anti-ACTIVE®MAPK pAb, according to the manufacturer's protocol. Briefly, A375 cells were starved for 5 d and treated with 100 μM of adenosine or with 10 μM of Cl-IB-MECA, MRE 3008F20, SCH 58261, or with 100 nM of HENECA for 2 h. Cells were harvested and washed with ice-cold PBS containing 1 mM sodium orthovanadate. Cells were then lyzed in Triton lysis buffer. Equivalent amounts of protein (40 μg) were subjected to electrophoresis on 10% sodium dodecyl sulfate–acrylamide gel. The gel was then electroblotted on to a nitrocellulose membrane. The membranes were probed with Anti-ACTIVE®MAPK pAb (1 : 5000) in TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20)/0.5% bovine serum albumin for 1 h at room temperature. Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin G (1 : 5000). Specific reactions were revealed with the Enhanced Chemiluminescence western blotting detection reagent (Amersham Corp., Arlington Heights, IL). The membranes were then stripped and reprobed with Anti-ERK 1/2 pAb, rabbit serum that cross-react with total ERK to ensure equal protein loading.

Statistical analysis All values in the figures and text are expressed as mean ± SD of n observations (with n ⩾ 3). Data sets were examined by analysis of variance (ANOVA) and Dunnett's test (when required). $P < 0.05$ was considered statistically significant. Representative images obtained by fluorescent microscope or by FACScan are reported, with similar results having been obtained in at least three different experiments.

RESULTS

Adenosine kills A375 via the A_2A receptor A375 cells were treated for 24 h with A_{2A} or A_3 selective agonists (HENECA and Cl-IB-MECA) or antagonists (SCH 58261 and MRE 3008F20), respectively (Baraldi *et al*, 2000; Klotz, 2000; Varani *et al*, 2000). By using trypan blue exclusion, cell counts and metabolically induced formazan production, we observed that MRE 3008F20, Cl-IB-MECA, and HENECA produced small but reproducible toxic and anti-proliferative effects (less than 10%) (data not shown). To evaluate if the reduced number of viable cells quantified by MTT was due to a massive cell death or to a proliferative block, we analyzed the effect of adenosine receptor stimulation on cell survival by the JAM test. A375 cells, previously labeled with [^{3}H]thymidine, were treated for 24 h with increasing amounts of adenosine. Adenosine induced cellular death (apoptosis and necrosis) in a concentration-dependent manner, starting at 0.1 μM and reaching the maximal effect at 1 μM. **Figure 1** shows a typical result with about 20% of A375 cells killed by adenosine (1–3000 μM).

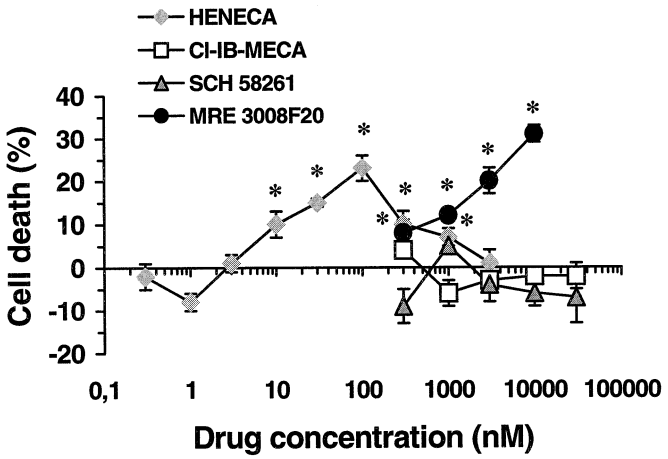

Figure 3. Cell death induction by HENECA, Cl-IB-MECA, MRE 3008F20, and SCH 58261 of A375 cell line evaluated 24 h after drug treatment by JAM test. Cell death is expressed in ordinate as percentage of DNA lost *vs* untreated cells. Cells treated with 0.3–3000 nM of HENECA (◆), with 0.3–30 μM of Cl-IB-MECA (□), 0.3–10 μM of MRE 3008F20 (●) and 0.3–30 μM of SCH 58261 (▲) are reported. 100% indicates 100% loss of radioactivity incorporated by untreated cells. *$p < 0.05$ with respect to untreated cells; analysis was by ANOVA followed by Dunnett's test.

To establish if the A_{2A} or A_3 receptor translated the adenosine cell death signal, we attempted to block single receptors by the pretreatment of A375 cells with SCH 58261 (1 μM), MRE 3008F20 (1 μM), or DMSO (as negative control) for 30 min before addition of adenosine at 1 μM. **Figure 2** shows that SCH 58261 was able to inhibit up to 95 ± 10% of adenosine-induced deleterious effects (bar labeled with "S") thereby promoting cell survival with respect to control cells (bar labeled with "D"). In contrast, MRE 3008F20 exerted the opposite effect, increasing adenosine-mediated cell death up to 75 ± 11% (bar labeled with "M"). This prompted us to hypothesize that adenosine stimulation via A_{2A} receptors induces cell death (apoptosis and/or necrosis), whereas A_3 signaling has a protective effect on cell survival in the presence of 1 μM extracellular adenosine. When MRE 3008F20 and SCH 58261 were tested simultaneously on adenosine-treated cells (**Fig 2**; bar labeled with "M + S"), we found a small increase of adenosine-induced cell death (25 ± 7%), suggesting that A_3 protective signaling also counteracted A_{2A}-independent deleterious signals (autogenous cell death, cell aging). Similar results were obtained with 0.3 μM and 10 μM of adenosine (data not shown). To illustrate the role of the A_1 and A_{2B} receptors on adenosine-induced cell death, A375 cells were preincubated for 30 min with 1, 10, 100, and 500 nM of the antagonist DPCPX (Klotz *et al*, 1998) and then exposed to 1, 10, and 100 μM of adenosine for 24 h. DPCPX did not prevent cell death, supporting the notion that A_1 and A_{2B} receptors do not play a part in adenosine-induced necrotic and/or apoptotic cell death signaling (data not shown).

A375 cells were treated for 24 h with increasing amounts of adenosine analogs to characterize the capability of A_{2A} and A_3 receptor activation to induce or prevent A375 cell death, respectively. HENECA induced cell death in a concentration-dependent manner reaching a maximum of activity at about 100 nM, although it was less active at higher doses (**Fig 3**, diamonds). SCH 58261 (**Fig 3**, filled triangles) did not exert any effect as did Cl-IB-MECA (**Fig 3**, open squares). MRE 3008F20 promoted a concentration-dependent cell death response reaching 30 ± 4% at 10 μM (**Fig 3**, filled circles). MRE 3008F20 was tested only up to 10 μM due to limited solubility. We also tested two different antagonists with a high affinity for A_3 adenosine receptors (Merighi *et al*, 2001): MRE 3055F20 and MRE 3062F20.

Table I. Quantification of A375 DNA content by propidium-iodide staining and flow analysis

		Cell cycle analysis[b]			Apoptosis detection
Treatment	Dose	G_1	S	G_2/M	Subdiploid cells[a]
DMSO		44 ± 2	13 ± 2	43 ± 1	4 ± 1
Cl-IB-MECA	10 μM	58 ± 2[d]	11 ± 2	31 ± 2[d]	3 ± 1
HENECA	0.001–10 μM[c]	44 ± 2	13 ± 1	43 ± 2	3 ± 1
MRE 3008F20	10 μM	42 ± 1	11 ± 1	47 ± 1	8 ± 1[d]
SCH 58261	10 μM	40 ± 2	14 ± 2	46 ± 2	4 ± 1
Untreated		44 ± 2	13 ± 2	43 ± 2	4 ± 1
Adenosine	1–300 μM[c]	43 ± 3	14 ± 1	43 ± 2	5 ± 1
Adenosine	1 mM	30 ± 1[e]	46 ± 2[e]	24 ± 1[e]	11 ± 2[e]
Adenosine	3 mM	32 ± 2[e]	36 ± 1[e]	32 ± 2[e]	20 ± 2[e]

[a]Values are % of total culture showing sub-diploid DNA content (< 2n).
[b]Values are % of living cells with normal DNA content (⩾2n).
[c]HENECA was used at 1, 3, 10, 30, 100, 300, 1000, 3000, and 10000 nM and adenosine at 1, 3, 10, 30, 100, and 300 μM and the results obtained with these concentrations were reported as mean ± SD of at least three different experiments.
[d]$p < 0.05$ *vs* DMSO.
[e]$p < 0.05$ *vs* untreated.Statistical analysis was by ANOVA followed by Dunnett's test.

Likewise, these drugs, tested in the range 0.3–10 μM for 24 h, induced A375 necrotic and/or apoptotic cell death in a concentration-dependent manner reaching maximum activity between 1 and 10 μM (20 ± 2%) (data not shown).

To define if the cell death signal triggered by A_{2A} receptor stimulation or by A_3 silencing was an apoptosis program, we investigated cellular morphology of A375 drug-treated cells. Typical apoptotic features, characterized by nuclear condensation and fragmentation, were detected after 3 mM adenosine treatment but not with lower doses (data not shown). Apoptotic cells were occasionally detected also with 10 μM MRE 3008F20. Notably, necrotic cells, characterized by extensive membrane damage (irregular swelling). Cells treated with 10 μM SCH 58261, 100 nM HENECA, and 10 μM Cl-IB-MECA were similar to control test (DMSO-treated cells, data not shown).

To quantify the number of apoptotic cells we analyzed the cellular DNA content by flow cytometry after propidium-iodide nuclear staining. Any significant difference in the amount of apoptotic DNA detected in cultures treated with HENECA (from 1 to 10,000 nM), 10 μM Cl-IB-MECA or 10 μM SCH 58261 with respect to DMSO-treated cells is listed in **Table I**. Only MRE 3008F20 was able to induce apoptosis slightly (8% *vs* 4%). Adenosine did not induce apoptosis when added at concentrations less than 1 mM, whereas massive apoptosis was noted at 1 and 3 mM (up to 20%). These data agree with those obtained observing cell morphology: A375 cells preferentially die from a nonapoptotic program when treated with MRE 3008F20, HENECA, and adenosine (from 1 to 300 μM).

Analysis of A375 distribution among the different phases of the cell cycle was also performed. Only Cl-IB-MECA was able to alter the amount of cells in the different phases of the cell cycle. The accumulation of cells in the G_1 phase was increased by 32% with a corresponding decrease of cells in G_2/M (by 28%; $p < 0.05$). HENECA (1–10 000 nM), MRE 3008F20, and SCH 58261 (at 10 μM) did not exert any effect compared with DMSO-treated cells. With adenosine in concentrations up to 300 μM no effect on the cell cycle was noted. Interestingly, millimolar doses of adenosine, that induced apoptosis, produced a decrease in the amount of cells in G_1 and a strong accumulation in S phase with respect to untreated cells.

Adenosine receptors in A375 proliferation To evaluate better if adenosine could interfere with cell proliferation, A375 cells were treated with increasing amounts of adenosine and the proliferation rate was determined by [^{3}H]thymidine incorporation assay (**Fig 4**, open circle), which is more sensitive than flow cytometry for the evaluation of cell proliferation. Only small increases in cell growth were observed with the addition of 100 or 300 μM extracellular adenosine (10 ± 2%). In agreement with flow cytometry analysis, adenosine at millimolar concentrations impaired cell proliferation up to 60 ± 5%. To establish which receptor induces the adenosine modulation of cell proliferation, A375 cells were treated with increasing concentrations of adenosine plus A_{2A} (1 μM SCH 58261) or A_3 (1 μM MRE 3008F20) selective antagonists followed by cell

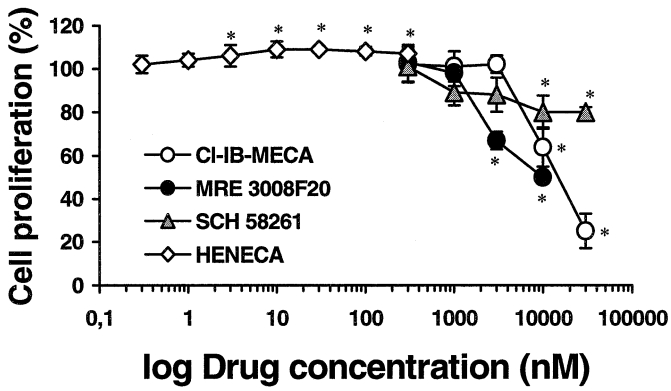

Figure 5. A_{2A} and A_3 role on A375 cell-proliferation by [^{3}H]thymidine incorporation test. A375 cells were treated with HENECA (◇), with Cl-IB-MECA (○), with MRE 3008F20 (●), and with SCH 58261 (▲) at the indicated concentrations. [^{3}H]thymidine incorporation is reported as percentage of DNA-labeled recovered on drug vehicle-treated cells. Ordinate reports means of four different [^{3}H]thymidine incorporation quantifications with SD (vertical bar). 100% indicates the thymidine incorporation of untreated cells. *$p < 0.05$ with respect to untreated cells; analysis was by ANOVA followed by Dunnett's test.

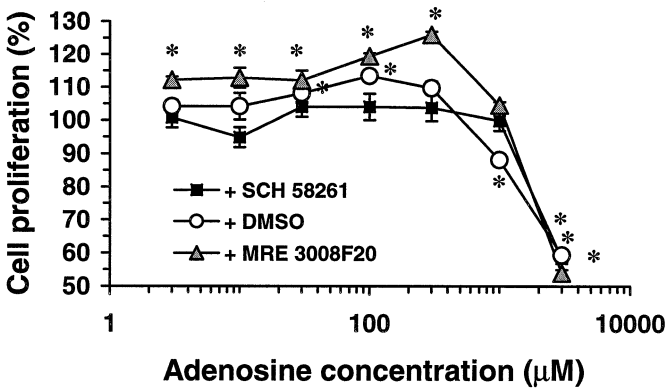

Figure 4. Effect of MRE 3008F20 (1 μM) and SCH 58261 (1 μM) on adenosine-mediated proliferation. A375 proliferation was monitored by [^{3}H]thymidine incorporation for 24 h. Proliferation rate at different adenosine concentrations (from 3 to 3000 μM) is reported in ordinate as percentage of [^{3}H]thymidine incorporation of mock untreated cells. Reported values represent the mean of four [^{3}H]thymidine incorporation quantifications with SD (vertical bar). The graph reports A375 adenosine-induced proliferation curves under treatment with MRE 3008F20 (▲), with SCH 58261 (■) or DMSO (○). 100% indicates the thymidine incorporation of untreated cells. *$p < 0.05$ with respect to untreated cells; analysis was by ANOVA followed by Dunnett's test.

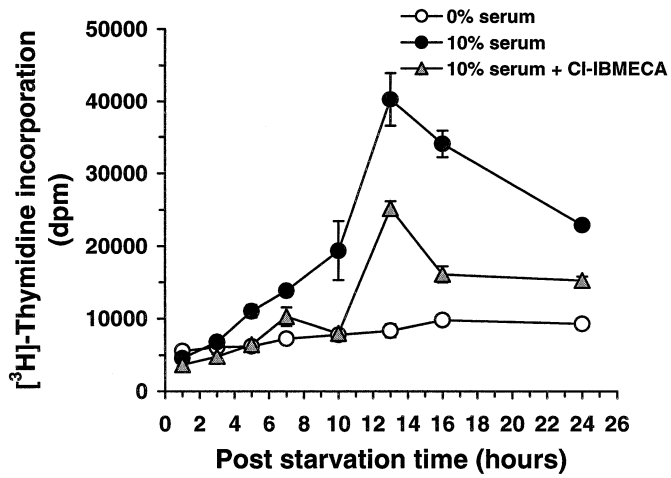

Figure 6. Kinetic analysis of A375 starved cells progression through S cell cycle phase and evaluation of Cl-IB-MECA interference. Starved A375 cells were challenged with new medium containing no serum and vehicle DMSO (○); with 10% of serum and Cl-IB-MECA vehicle DMSO (●); with 10% of serum and 10 μM of Cl-IB-MECA (▲). At the indicated time A375 cells were pulsed with [^{3}H]thymidine for 2 h and mean of four separate [^{3}H]thymidine incorporations was reported with SD (vertical bar) as ordinate.

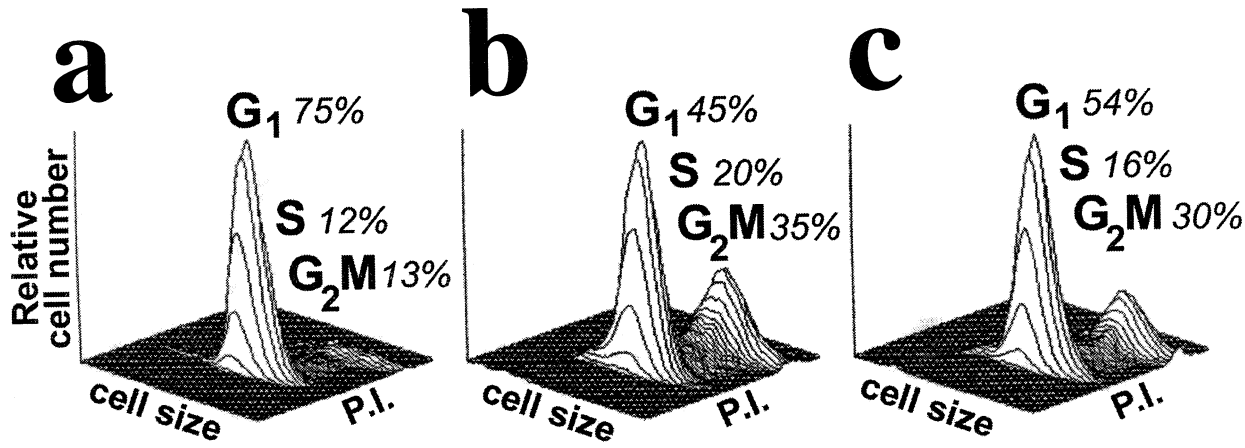

Figure 7. DNA content analysis of A375 starved cells by flow cytometry. Density curves show relative cell number (vertical axis) *vs* propidium-iodide fluorescence (PI = right horizontal axis) and cell size (left horizontal axis). Cells in different cycle phases (G_1, S, and G_2/M) and relative percentages of distribution are reported in each panel. (*A*) Starved A375 cells treated for 18 h with new medium containing no serum. (*B*) Starved A375 cells treated for 18 h with new medium containing 10% of serum. (*C*) Starved A375 cells treated for 18 h with new medium containing 10% of serum and 10 μM of Cl-IB-MECA. (*A,B*) A375 cells were challenged with Cl-IB-MECA vehicle (DMSO). A representative of four separate experiments is shown.

proliferation analysis by [^{3}H]thymidine incorporation assay. MRE 3008F20 (**Fig 4**, filled triangle) enhanced adenosine-induced proliferation in contrast to SCH 58261, which annihilated adenosine's proliferative effect (**Fig 4**, filled square). Remarkably, adenosine at millimolar concentrations arrested cell proliferation even in the presence of MRE 3008F20 and SCH 58261. When using the A_1 and A_{2B} antagonist DPCPX at 10, 100, and 500 nM, no alteration in adenosine-induced proliferation was observed, leading us to exclude that the A_1 and A_{2B} receptors could be the mediators of adenosine-induced proliferation (data not shown). To verify if adenosine promotes cell proliferation via A_{2A} and impairs cell proliferation via A_3, we performed a dose–response growth curve by treating A375 cells with increasing concentrations of A_{2A} (HENECA, SCH 58261), and A_3 (Cl-IB-MECA, MRE 3008F20) agonists and antagonists, respectively. As shown in **Fig 5**, HENECA (diamonds) induced a small but significant increase in cell proliferation (up to 8%), whereas a decrease in cell growth was observed after SCH 58261 treatment (**Fig 5**, filled triangles). These data support the role of A_{2A} as a positive modulator of A375 cell proliferation, also under tonic stimulation by basal adenosine levels.

Specific stimulation of the A_3 receptor by Cl-IB-MECA caused a reduction of A375 proliferation with a 75 ± 6% of inhibition at 30 μM (**Fig 5**, open circles). We observed that MRE 3008F20 had negligible effects on A375 cell growth (**Fig 5**, filled circles) because we hypothesize that the loss of [^{3}H]thymidine incorporation observed in **Fig 5** is probably due, to a major extent, to the induction of cell death and not a block of proliferation (see **Fig 3**, filled circles).

We have performed further studies to consider the role of A_3 on cell proliferation. To evaluate if A_3 stimulation delays the progression to S cell cycle phase or blocks a subset of A375 population in G_1, A375 cells were synchronized in G_0–G_1 phase by starvation in serum-free medium for 72 h. Starved cells were then resuspended in fresh medium with 10% serum and labeled with [^{3}H]thymidine for 2 h at different time points. **Figure 6** shows that A375 cells entered into S phase 13 h after serum administration (filled circle), whereas starved cells cultured in medium without serum (open circle) incorporated an insignificant amount of [^{3}H]thymidine. When 10 μM of Cl-IB-MECA was added to A375 cells after starvation (**Fig 6**, filled triangle), the cells continued to enter into S phase at 13 h postserum addition, but the maximum amount of [^{3}H]thymidine incorporation was only 64% of the control. During the same experiment, cells were analyzed by FACScan to quantify cell distribution among the cell cycle phases. **Figure 7**(***A***) shows that starved cells were blocked in G_1 (75%), whereas 18 h postserum addition (**Fig 7*B***) A375 cells were forced into S (20%) and G_2/M (35%) phases. Cl-IB-MECA (**Fig 7*C***) significantly impaired the serum-induced cell transition from G_0/G_1 to S phase (16 ± 1% instead of 20 ± 1%, $p < 0.05$) and to G_2/M phase (30 ± 1% instead of 35 ± 1%, $p < 0.05$), thereby supporting the hypothesis that A_3 stimulation blocks the transition into G_1 without affecting DNA synthesis. Finally, we emphasize that Cl-IB-MECA reduces [^{3}H]thymidine incorporation in the presence of 1 μM DPCPX and SCH 58261 (as A_1 and A_{2A} antagonist, respectively) proving that Cl-IB-MECA acts through A_3 receptor interaction (data not shown).

The A_3 receptor: protective effects We also investigated the role of the simultaneous stimulation of A_{2A} and A_3 adenosine receptors on cell survival. In a new set of experiments HENECA-treated cells (30, 100, and 300 nM) were incubated with increasing amounts of Cl-IB-MECA (1, 3, and 10 μM) and the residual necrotic and/or apoptotic cell death was quantified by the JAM test. We observed that A_3 stimulation reduced HENECA-induced cell death and promoted cell survival in a concentration-dependent fashion. **Figure 8**(***A***) summarizes the data obtained with 100 nM HENECA; Cl-IB-MECA abolished not only HENECA-induced cell death, but also increased cell survival up to 26 ± 2% with respect to control cells (DMSO treated). Similar results were obtained with 30 and 300 nM HENECA (data not shown), suggesting distinct roles for the A_{2A} and A_3 receptors.

To verify if tonic stimulation (mediated by basal extracellular adenosine) of A_{2A} receptors would have a deleterious effect on cell survival impaired during A_3 blockage, A375 cells were treated with A_{2A} or A_3 antagonists (1 μM SCH 58261 and 1 μM MRE 3008F20, respectively), alone or in combination. **Figure 8**(***B***) illustrates that cell death promoted by MRE 3008F20 (bar labeled with "M") was abolished by SCH 58261 (bar labeled with "M + S"), supporting the hypothesis that the A_{2A} receptor triggers cell death also under tonic stimulation.

To evaluate further the effects of adenosine on the clonogenic ability of A375 cells, we performed colony formation assays. Cl-IB-MECA promoted cell survival, increasing the number of colonies to as much as 185 ± 20% (**Fig 9*A***), whereas HENECA impaired the clonogenic potential of A375 cells (**Fig 9*B***). As expected, A_{2A} receptor blockage by SCH 58261 increased colony number (**Fig 9*D***). A_3 silencing produced a strong negative effect, deleting

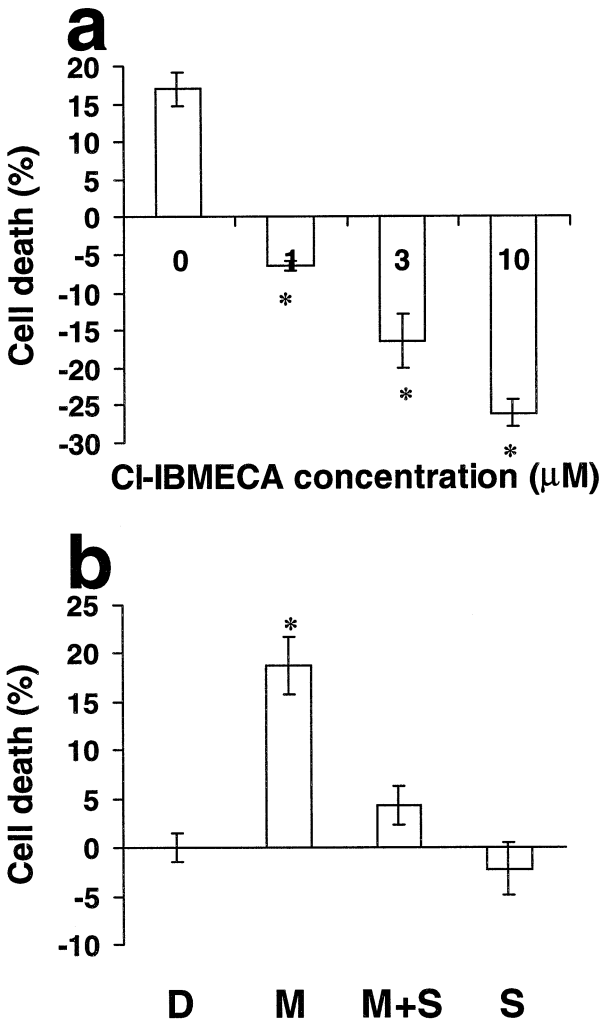

Figure 8. Study of the effect on cell survival of simultaneous stimulation of A_{2A} and A_3 receptors. After 24 h of treatment, cell death was quantified by JAM test. Percentage of cell death calculated as mean of four different DNA-loss quantifications is reported in ordinate with SD (vertical bar). Negative values indicate increase in cell survival respect to A375 cells treated with drug vehicle (DMSO). 100% indicates 100% loss of DNA. (*A*) Variation of A_{2A}-induced cell death by A_3 stimulation. A375 cells treated with HENECA (100 nM) were challenged with Cl-IB-MECA at the indicated concentrations reported in abscissa (1, 3, and 10 μM). *$p < 0.05$ with respect to control "0"; analysis was by ANOVA followed by Dunnett's test. (*B*) Induction of cell death by 1 μM of MRE 3008F20 (bar labeled with M), by 1 μM of SCH 58261 (bar labeled with S) or by a combination of MRE 3008F20 and SCH 58261 (bar labeled with M + S). *$p < 0.05$ with respect to control "DMSO"; analysis was by ANOVA followed by Dunnett's test.

by 48 ± 2% the number of colonies when MRE 3008F20 was applied at 10 μM (**Fig 9*C***).

This assay provides additional evidence that A_{2A} is deleterious, whereas A_3 is necessary and useful for A375 cell culture growth.

The human A_{2A} receptor induces cell death in CHO cell line To verify whether the A_{2A} receptor is able to induce necrotic and/or apoptotic cell death when expressed in a nonhuman cell line, CHO-hA_{2A} cells were treated with HENECA (A_{2A}–A_3 agonist) or Cl-IB-MECA (A_3 selective agonist) and the amount of cell death was detected by the JAM test. **Figure 10** shows that HENECA (filled triangles), but not Cl-IB-MECA (open circles), caused the death of CHO-hA_{2A} cells, according to their different affinity for the A_{2A} receptor. To ascertain that the HENECA killer effect was the consequence of A_{2A} receptor stimulation and not of CHO sensitivity to this compound, CHO-hA_1 and CHO-hA_3 clones were treated with HENECA and no cell death was observed by the JAM test (data not shown). Of note, CHO-hA_3 cells were not killed by MRE 3008F20 treatment, suggesting that the deleterious effects of MRE 3008F20 on A375 cells were a consequence of the abolishment of the antagonistic effect of A_3 on the A_{2A} death signal pathway, and not to a generic toxic effect.

To establish whether the A_{2A} receptors expressed in CHO-hA_{2A} cells were able to maintain their ability to impair colony formation, CHO-hA_1, CHO-hA_{2A}, and CHO-hA_3 cells were treated with increasing amounts of HENECA (ranging from 3 to 10,000 nM) and residual clonogenic ability was monitored. HENECA impaired clonogenicity of CHO-A_{2A} clones, reducing the number of residual colonies up to 60 ± 5% when compared with untreated cells. In contrast, HENECA was not effective on CHO-hA_1 and CHO-hA_3 clones (mean of 110 ± 3% and 92 ± 6% of DMSO control cells, respectively), further supporting the idea that A_1 and A_3 human receptors were not able to kill CHO cells.

The A_{2A}-induced death signaling pathway To investigate the molecular mediators of the death signaling initiated by the surface A_{2A} adenosine receptor, A375 cells were treated with adenosine (10 μM) in the presence of a panel of metabolic inhibitors, and cell death was monitored by the JAM test, quantifying necrosis and/or apoptosis.

Treatment with PD98059 or with U0126 (MEK-1 and MEK-2 inhibitors) abolished the adenosine effect (cell death was 3.3 ± 2.2% and 4.5 ± 2.3% *vs* 18.3 ± 2.5%, respectively). PKC inhibitors chelerythrine-D and staurosporine deleted the negative effects of adenosine (cell death was –0.6 ± 1.7% and –0.9 ± 2.5% *vs* 18.3 ± 2.5%, respectively), whereas H89 (PKA inhibitor) and Z-VAD-FMK (caspases inhibitor) did not prevent cell death (**Table II**).

A375 cells were treated with 100 nM HENECA to induce cell death (22.0 ± 4.7%) specifically via A_{2A}: as observed with adenosine, the inhibitors of ERK kinases and of PKC prevented cell death (–30.0 ± 2.6%, –32.1 ± 3.2% and –11.8 ± 4.7%, 0.9 ± 1.9%, respectively), whereas PKA and caspases inhibitors did not affect HENECA killer effect (**Table II**). As expected, also MRE 3008F20-induced cell death (31.0 ± 4.3%) was sensitive to MEK and PKC inhibitors (–16.8 ± 1.0, 14.5 ± 4.2, and 1.3 ± 3.1, 12.7 ± 0.9, respectively) because, during A_3 blockage, A_{2A} tonic stimulation induces cell death (**Table II**).

ERK-1 and ERK-2 activation under adenosine, A_{2A} stimulation and A_3 block The treatment of A375 cells with HENECA 100 nM (**Fig 11*A***, lane 2), MRE 3008F20 10 μM (**Fig 11*A***, lane 5), and adenosine 100 μM (**Fig 11*A***, lane 6) stimulated ERK-1 and ERK-2 activation. On the other hand, SCH 58261 10 μM and Cl-IB-MECA 10 μM (**Fig 11*A***, lanes 3 and 4, respectively) had no effect on the expression level of ERK-1 and ERK-2 phosphorylated isoforms.

By using specific antibody targeting phosphorylated ERK-1 and ERK-2, we have also investigated the intracellular distribution of activated ERK-1 and ERK-2 MAP kinases during adenosine (10 μM), HENECA (100 nM), or MRE 3008F20 (10 μM) induced cell death. **Figure 11*B*** shows that faint fluorescence of anti-ERK-1 and anti-ERK-2 activated isoforms was detected in cytoplasm of drug-vehicle cells (panel 3) but a strong fluorescence was present in the nuclei of HENECA and MRE 3008F20 treated cells (panels 1 and 4, respectively). Activated ERK were detected also in nuclei of A375 cells after 30 min of treatment with adenosine (data not shown) but not after Cl-IB-MECA (panel 2) and SCH 58261 challenge (data not shown).

Lastly, we report that A375 cell proliferation is strongly dependent on ERK activation: U0126 blocked [^{3}H]thymidine incorporation up to 63% and reduced the percentage of cells into S phase up to 80% (data not shown).

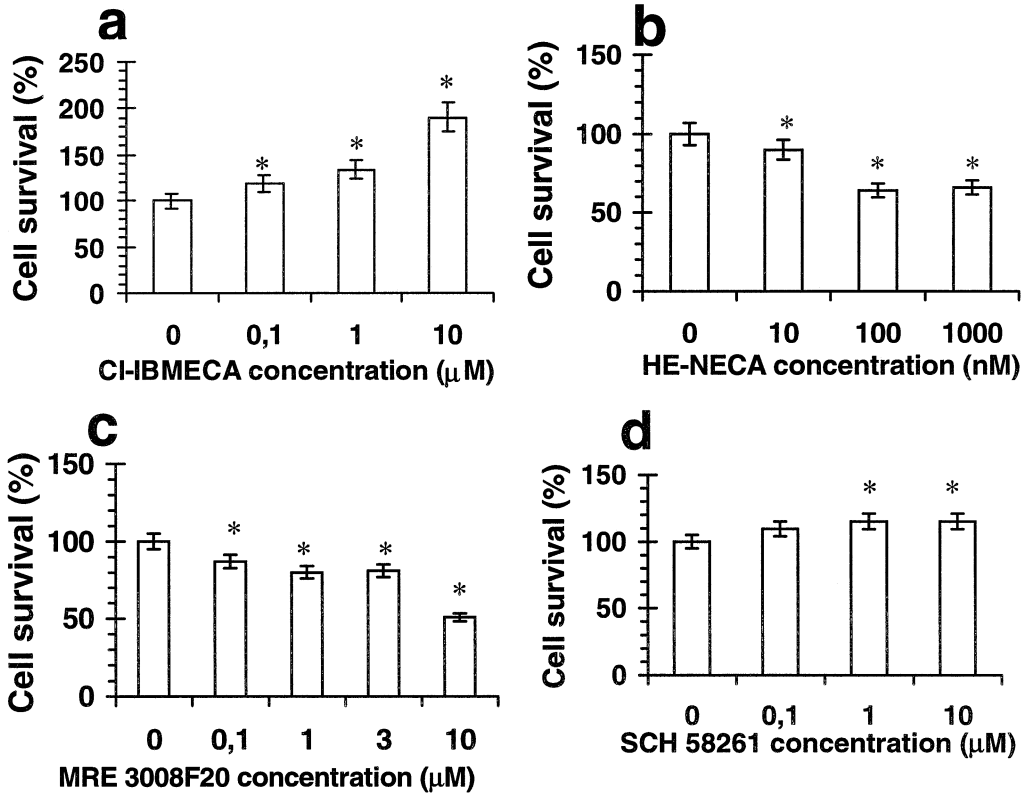

Figure 9. Modulation of A375 colony formation ability by adenosine receptors engagement. A375 cells were treated with Cl-IB-MECA (*A*), HENECA (*B*), MRE 3008F20 (*C*), and SCH 58261 (*D*) at the indicated concentrations. Number of scored colonies is reported in ordinate as percentage of DMSO-treated cells. Cell survival percentages reported are expressed as mean of six independent plating experiments with relative SD (vertical bar). 100% indicates the 100% of the number of the colonies obtained with untreated cultures. *p < 0.05 with respect to control "0"; analysis was by ANOVA followed by Dunnett's test.

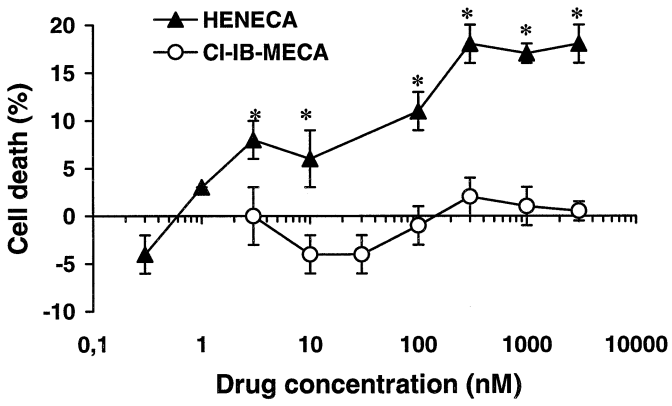

Figure 10. Cell death induction of CHO-hA_{2A} clone by HENECA and Cl-IB-MECA. CHO-hA_{2A} cells were treated with indicated concentrations of HENECA (▲) or with Cl-IB-MECA (○) for 24 h and cell death induction was quantified by JAM test. Percentage of cell death calculated as mean of four different DNA-loss quantifications is reported in ordinate with SD (vertical bar). Drug concentrations are reported in abscissa. 100% indicates 100% lost of thymidine incorporation of untreated cells. *p < 0.05 with respect to DMSO-treated cells; analysis was by ANOVA followed by Dunnett's test.

DISCUSSION

In agreement with published reports showing that adenosine could promote the growth of the tumor masses and cell proliferation (Ethier and Dobson, 1997; MacLaughlin *et al*, 1997) and also impair cell viability (Ongini and Schubert, 1998; Fishman *et al*, 2000; Shneyvays *et al*, 2000), we have found that adenosine affected cell proliferation (**Fig 4**) and also induced cell death (both apoptosis and necrosis) of A375 human melanoma cell line (**Fig 1**). At the same time numerous reports document that adenosine is able to impair cell proliferation as well as to improve cell survival (Brown *et al*, 2000; Ohana *et al*, 2001). The underlying basis of these apparently contradictory effects, however, is not yet clearly understood.

Role of adenosine receptors in A375 culture growth Although the presence of all four adenosine receptor subtypes has been documented in A375 melanoma cells (Merighi *et al*, 2001), only A_{2A} and A_3 receptors seem to be involved in the regulation of cell proliferation and survival. This is very surprising as the A_{2B} and A_1 subtypes are signaling through the same pathways, respectively. One explanation may be that their low abundance in these cells is not sufficient for productive effector coupling.

In spite of a large body of literature, the role of A_{2A} and A_3 in apoptosis and cell death induction remains incompletely understood. Evidences from *in vivo* studies suggest that A_{2A} block achieved neuroprotection and support the view that A_{2A} stimulation is detrimental in neurons and also in thymocytes (Ongini and Schubert, 1998; Apasov *et al*, 2000). Conversely, A_{2A} receptor activation appeared to reduce ischemia-reperfusion injury in the kidney (Okusa *et al*, 2000). Different authors showed that at the same time low concentrations of A_3 receptor agonists have protective effects and, in contrast, high concentrations of agonists for A_3 receptor can induce apoptosis. Recent studies suggested the involvement of the A_3 receptor in adenosine-induced apoptosis using Cl-IB-MECA (in rat astrocytes, in human peripheral blood mononuclear cells, in both myeloid and lymphoid cells, in cardiac

Table II. Neutralization of adenosine, HE-NECA, and MRE 3008F20 cell death signaling by metabolic inhibitors

	Adenosine	HE-NECA	MRE 3008F20
DMSO	18.3 ± 2.5	22.0 ± 4.7	31.0 ± 4.3
PD98059	3.3 ± 2.2[a]	–30.0 ± 2.6[a]	–16.8 ± 1.0[a]
U0126	4.5 ± 2.3[a]	–32.1 ± 3.2[a]	14.5 ± 4.2[a]
Chelerythrine-D	–0.6 ± 1.7[a]	–11.8 ± 4.7[a]	1.3 ± 3.1[a]
Staurosporine	–0.9 ± 2.5[a]	0.9 ± 1.9[a]	12.7 ± 0.9[a]
H89	17.1 ± 1.6	27.6 ± 2.4	28.6 ± 2.9
Z-VAD-FMK	19.0 ± 5.1	22.8 ± 3.5	28.0 ± 2.9

Table expresses cell death by pretreatment of A375 cells with metabolic inhibitors respect to mock (cells treated with drug vehicle = DMSO). JAM test was performed to quantify cell death. Values are reported as mean ± SD of at least three different experiments.

[a] $p < 0.05$ *vs* DMSO. Analysis was by ANOVA followed by Dunnett's test. Negative values indicate an increased vitality of treated cells respect to DMSO alone treated culture. Adenosine and MRE 3008F20 were challenged at 10 μM, and HE-NECA at 100 nM.

myocytes) (Kohno *et al*, 1996; Yao *et al*, 1997; Abbracchio *et al*, 1998; Barbieri *et al*, 1998; Baraldi *et al*, 2000; Shneyvays *et al*, 2000); however, further investigations on CHO-hA_3 and on mast and lymphoma cell lines (Brambilla *et al*, 2000; Fishman *et al*, 2000; Gao *et al*, 2001) revealed no involvement of A_3 receptors on cell death induction, suggesting a role for A_3 receptor signaling, which is highly dependent on the cell- or tissue-specific context. We believe that important new inroads in the research on A_{2A} and A_3 receptor functions will come from understanding cells as complex systems in which different receptors are coexpressed to serve as active modulators in signal transduction. The interactions between them will prove to be a critical mechanism determining cellular survival. The rationale for such a concept comes from our experiments showing that adenosine acts on A375 culture growth through two independent mechanisms. One pathway seems to involve the activation of A_{2A} receptors playing a key role in cell death (**Figs 2 and 3**). A second pathway involves the A_3 receptor that protects A375 cell line from A_{2A}-induced cell death (**Fig 8*A*,*B***). Analogously, A_{2A} stimulation impaired plating efficiency of A375 cells (**Fig 9*B***), whereas A_3 exerted positive effects (**Fig 9*A***). Irrespective of the cytostatic effect promoted by A_3 stimulation, the A_3 agonist improves clonal expansion. In this regard, we underline that the colonies obtained under A_3 stimulation are smaller and formed by less cells than the untreated one. These observations seem to support the simultaneous presence of the following opposite effects after A_3 receptor stimulation: an high clonogenic ability and a low rate of proliferation. The ability of adenosine receptors in modulating clonal potential of A375 cells (evaluated by colony formation assay) may be the consequence of the interaction with at least four biologic properties: (i) resistance to detachment stress that induces apoptosis of adherent cells when cell adhesion is impeded (anoikis); (ii) interference with cell adhesion mechanism; (iii) inhibition of spontaneous cell cycle progression (G_1–G_0 transition) or recruitment of cell from G_0; and (iv) survival of cells committed to clonal expansion. Further support for the opposite role of A_{2A} and A_3 receptors stems from experiments with CHO cells expressing human A_1, A_{2A}, and A_3 receptors. Human recombinant adenosine receptors stimulation by agonists showed that only the A_{2A} subtype has the intrinsic ability to induce cell death and to impair clonogenic potential of CHO cells. This ability was not found for A_1 and A_3 receptors.

We underline that adenosine-induced apoptosis observed with doses $\geqslant 1$ mM is receptor independent, as previously reported (Schrier *et al*, 2001). In addition, we have observed a biphasic behavior of HENECA (**Fig 3**), probably due to the lack of selectivity. Consequently, we hypothesize that at low HENECA concentrations (from 0.3 to 100 nM) A_{2A} is the principal subtype activated and exerts its deleterious role, but at higher HENECA

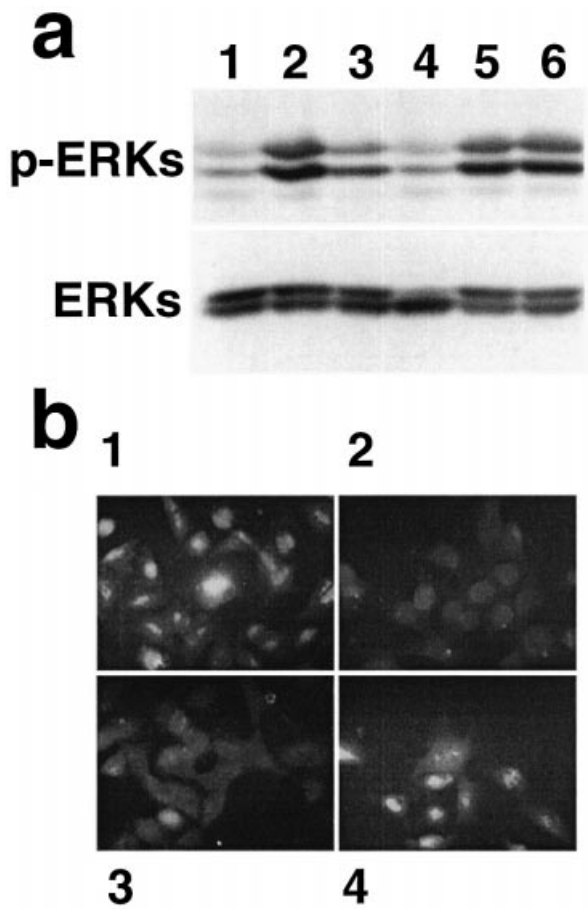

Figure 11. ERK-1 and ERK-2 activated isoforms detection. (*a*) A375 cells were starved for 5 d (untreated cells, *lane 1*) and treated for 2 h with HENECA 100 nM (*lane 2*), SCH 58261 10 μM (*lane 3*), Cl-IB-MECA 10 μM (*lane 4*), MRE 3008F20 10 μM (*lane 5*), and adenosine 100 μM (*lane 6*). The detection of ERK-1 and ERK-2 phosphorylated isoforms (*top panel*) and the total ERK isoforms (*bottom panel*) is shown. (*b*) A375 cells treated with HENECA 100 nM (*panel 1*), Cl-IB-MECA 10 μM (*panel 2*), drug vehicle DMSO (*panel 3*), and with MRE 3008F20 10 μM (*panel 4*) were stained with antibody to ERK-1 and ERK-2 phosphorylated isoforms and detected by goat fluorescein isothiocyanate anti-rabbit IgG. Faint and diffuse cytoplasmatic staining was present in *panels 2 and 3*, whereas strong nuclear fluorescence was concentrated in nuclei (*panels 1 and 4*) after 30 min of treatment. Original magnification × 400.

doses ($\geqslant$ 300 nM) A_3 stimulation becomes sufficiently strong to antagonize A_{2A} killer effect, as obtained with Cl-IB-MECA (**Fig 8*A***).

We point out that these opposite roles of A_{2A} and A_3, discovered under overstimulation, are also present during tonic adenosine stimulation exerted by basal levels of this autacoid nucleoside. We prove that the tonic stimulation of A_3 receptors counter-balanced the tonic killer effects mediated by A_{2A} receptors (**Fig 8*B***).

Molecular signaling of A_{2A} activation Whereas A_{2A} activation plays a key part in cell death, adenosine also improves A375 cell proliferation by A_{2A} activation (**Fig 4**); on the other hand, if A_3 receptors protect the cells from A_{2A}-induced cell death, their stimulation blocks A375 proliferation (**Fig 5**), according to the anti-apoptotic and anti-proliferative role of A_3 previously reported (Brambilla *et al*, 2000; Gao *et al*, 2001). Interestingly, we observed that adenosine-induced cell proliferation followed by A_{2A} stimulation seems to involve MEK-1 and MEK-2 by increasing the concentration of phosphorylated ERK-1 and ERK-2 isoforms with marked nuclear localization (**Fig 11*B***, panel 1). Our observations

agree with previous results showing that A_{2A} stimulation induces phosphorylation of mitogen-activated protein kinase (Sexl *et al*, 1997; Arslan and Fredholm, 2000; Schulte and Fredholm, 2000). Therefore, we attempted to determine in our model the origin of the proliferative block induced by A_3 stimulation. This anti-growth signal blocks cells into G_1-late cell cycle phase. We have found that A_3 blockage enhances adenosine function promoting cell proliferation (**Fig 4**) and that A_3 antagonists are able to improve MEK activity (**Fig 11*A***, lane 5; **Fig 11*B***, panel 4): these results emphasize the role of A_3 as inhibitor of ERK activation. To sustain this hypothesis we have found that Cl-IB-MECA was unable to activate ERK phosphorylation (**Fig 11*A***, lane 4; **Fig 11*B***, panel 2). In conclusion, A_3 stimulation arrests proliferation of A375 cells: how this dysregulation of cell cycle progression affects homeostasis of the cells has yet to be established.

More intriguing is the discovered signaling pathway that we have depicted during adenosine-induced cell death. The molecular mechanism promoted by A_{2A} receptor engagement is not mediated by caspase activation: these data agree with the absence of apoptosis noted by flow analysis. Furthermore, despite the ability of A_{2A} to increase cAMP levels (Merighi *et al*, 2001), we have noted that PKA (cAMP dependent kinase, protein kinase A) is not involved in killer signaling, which appeared to be mediated by PKC, ERK-1, and ERK-2 phosphorylation. It has been reported that in melanocytes, cAMP-dependent activation of ERK is mediated by B-Raf (kinases of Raf superfamily kinases), but not by PKA (Buscà *et al*, 2000). This model could explain the absence of PKA involvement on A_{2A} cell-death signaling: A_{2A} improves adenylyl cyclase activity that in turn promotes PKA-independent ERK activation. It has been reported that PKC could induce and activate MAP/ERK signaling pathway through the activation of Ras (Miranti *et al*, 1999) and PKC has been shown to stimulate adenylyl cyclases (Marjamaki *et al*, 1997). Prompted by these data we hypothesized the existence of cross-talk between PKC and cAMP-Ras-ERK pathways in two ways: (i) PKC may activate directly Ras, and (ii) PKC may improve adenylyl cyclase activity resulting in increased cAMP levels that in turn activate Ras.

Despite the consolidated role of MEK as anti-apoptotic agents, recent evidence proved that hyperoxia, nitric oxide, some anti-cancer drugs (taxol and resveratrol), and *de novo*-synthesized ceramide induced cell death by ERK-1 and ERK-2 activation (Petrache *et al*, 1999; Blazquez *et al*, 2000; Bacus *et al*, 2001; She *et al*, 2001). Further studies are necessary to investigate how ERK activation (mediated by adenosine or by other factors) could modulate cell death of melanocytic cells.

In conclusion, this study explains the conflicting effects of adenosine on malignant melanoma cells resulting from simultaneous activation of A_{2A} and A_3 adenosine receptors. We hypothesize that, in the presence of raised extracellular concentration of adenosine (during hypoxia, inflammation and inside tumor masses), a cell expressing high levels of A_3 adenosine receptor and low levels of A_{2A} adenosine receptor could be an advantage and positively selected. These findings persuade us to assert that the antagonists of A_3 adenosine receptors and the agonists of A_{2A} adenosine receptors may represent a potential new class of pharmacologic drugs in the fight against cancer, improving conventional anti-cancer therapies by impairing clonal expansion and cell survival of malignant skin-derived cells.

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