Expression of P2X7 Receptor Increases In Vivo Tumor Growth

Elena Adinolfi, Lizza Raffaghello, Anna Lisa Giuliani, Luigi Cavazzini, Marina Capece, Paola Chiozzi, Giovanna Bianchi, Guido Kroemer, Vito Pistoia, and Francesco Di Virgilio

Abstract

The P2X7 receptor is an ATP-gated ion channel known for its cytotoxic activity. However, recent evidence suggests a role for P2X7 in cell proliferation. Here, we found that P2X7 exhibits significant growth-promoting effects in vivo. Human embryonic kidney cells expressing P2X7 exhibited a more tumorigenic and anaplastic phenotype than control cells in vivo, and the growth rate and size of these tumors were significantly reduced by intratumoral injection of the P2X7 inhibitor–oxidized ATP. The accelerated growth of P2X7-expressing tumors was characterized by increased proliferation, reduced apoptosis, and a high level of activated transcription factor NFATc1. These tumors also showed a more developed vascular network than control tumors and secreted elevated amounts of VEGF. The growth and neoangiogenesis of P2X7-expressing tumors was blocked by intratumoral injection of the VEGF-blocking antibody Avastin (bevacizumab), pharmacologic P2X7 blockade, or P2X7 silencing in vivo. Immunohistochemistry revealed strong P2X7 positivity in several human cancers. Together, our findings provide direct evidence that P2X7 promotes tumor growth in vivo. Cancer Res; 72(12); 2957–69. ©2012 AACR.

Introduction

Tumor microenvironment conditions antitumor immune response and strongly affects tumor progression and metastasis formation, including at the level of angiogenesis (1–3). Within solid tumors, immune cells are exposed to multiple different diffusible factors that enhance or decrease host antitumor response. Such soluble factors include cytokines, autacoids, enzymes, reactive oxygen and nitrogen species, nucleosides, and nucleotides. In this context, even more attention is being paid to purine metabolites in view of the fact that the ATP and adenosine concentration in the tumor interstitium is much higher than in healthy tissues (4, 5). ATP is the key energy currency as well as a ubiquitous extracellular messenger (6). Depending on its dose and the purinergic P2 receptor subtype engaged, ATP can trigger many different cell responses, including cell death or proliferation (7–10). Among the receptors engaged by extracellular ATP (P2 receptors), P2X7 is the one most consistently expressed (or even overexpressed) by tumor cells (see ref. 10 for a recent review).

P2X7 is an ATP-gated plasma membrane ion channel that has been consistently implicated in cytotoxicity. The functional P2X7 receptor is generated by the homotrimeric assembly of P2X7 subunits, but under certain conditions, homohexamers can also form (11, 12). Upon transient stimulation, P2X7 behaves like a cation-selective channel permeable to Na⁺, K⁺, and Ca²⁺. However, sustained stimulation drives, an as yet poorly understood, transition that leads to formation of a nonselective pore permeable to aqueous solutes of molecular mass up to 900 Da (13). P2X7 is ubiquitously expressed, although particularly abundant on immune cells, for example, macrophages, microglia, or proliferating lymphocytes (14). P2X7 is also overexpressed by several tumors (15–19). Over the last years, evidence has accumulated in support of an important role of P2X7 as an immunomodulatory receptor involved in interleukin (IL)-1β maturation and release, antigen presentation, graft-versus-host reaction, and contact hypersensitivity (20–24). In addition, several reports have highlighted the intriguing growth-promoting activity of this receptor (8, 25), which seems to be indispensable for some cell types, such as human T lymphocytes and primary mouse microglia cells (26–29). One of the most intriguing features of the trophic effect of P2X7 is its ability to support survival and growth in the absence of serum (8, 25). This has led several investigators to put forward the hypothesis that P2X7 overexpression might be a hallmark of cancers such as chronic lymphocytic leukemia, melanoma, neuroblastoma, prostate, breast, skin, and thyroid cancers (see ref. 10, for review).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

E. Adinolfi and L. Raffaghello contributed equally to this work.

Corresponding Author: Francesco Di Virgilio, Department of Experimental and Diagnostic Medicine, University of Ferrara, Via Borsari 46, Ferrara I-44121, Italy; Phone: 390532455353; Fax: 390532455351; E-mail: fdv@unife.it

doi: 10.1158/0008-5472.CAN-11-1947

©2012 American Association for Cancer Research.
The trophic signaling cascade activated by P2X7 involves subtle changes in intracellular Ca$^{2+}$ homeostasis, stimulation of mitochondrial metabolism, activation of NFATc1, and suppression of apoptosis (8, 9). NFATc1, a key transcription factor in lymphocyte division and cancer cell growth, exerts a central role in P2X7-mediated proliferation as it is strongly upregulated in HEK293 cells expressing different P2X7 isoforms (9, 12), and its blockade by selective inhibitors, such as cyclosporine or VIVIT, obliterates P2X7-dependent cell growth (9). P2X7 expression not only stimulates growth but also enhances invasiveness of HEK293 cells, as shown in vitro, in Matrigel infiltration experiments (12), and in vivo, in a zebrafish model of metastasis (30).

In this study, we show that 2 cell lines (HEK293 fibroblasts and CT26 colon carcinoma) transfected with either the human (hP2X7) or mouse (mP2X7) receptor are endowed with enhanced tumorigenesis when inoculated into immunodeficient or immunocompetent BALB/c mice, respectively. Moreover, tumor growth was also inhibited by endogenous P2X7 blockade in tumors derived from B16 mouse melanoma or ACN human neuroblastoma cell lines. Altogether, our data support an important growth-promoting activity of P2X7 and suggest a possible role of this receptor in cancer.

Materials and Methods

Reagents and antibodies

H$_2$O$_2$, xylene, and ethanol were from Carlo Erba whereas neutral-buffered formalin was from Diapath. Prediluted, ready-to-use anti-CD34 (clone QBEnd/10) antibody was purchased from Ventana. Epitope retrieval solution, pH 9, epitope retrieval solution citrate buffer, pH 6, wash buffer, 3.3'-diaminobenzidine (DAB) chromogen solution, EnVision system horseradish peroxidase (HRP), anti-mouse and anti-rabbit antibodies, anti-Ki67/Mib-1, and anti-SMA antibodies (clone 1A4) were purchased from Dako. Rabbit anti-P2X7 antibody, Mayer’s hematoxylin and DMEM-F12 were from Sigma-Aldrich; mouse anti-NFAT2/NFATc1 (clone 7A6) and VEGF antibodies (anti-P2X7 and anti-actin A2668; Sigma-Aldrich) were used at 1:200 dilution and incubated for 16 hours at 4°C (anti-P2X7) or 1 hour at room temperature (anti-actin). P2X7 activity was assessed by measuring changes in the cytosolic-free Ca$^{2+}$ concentration ([Ca$^{2+}$]) in a thermostat-controlled (37°C) and magnetically stirred Perkin Elmer fluorimeter with the fluorescent indicator fura-2/acetoxymethyl ester (fura-2/AM) as described by Adinolfi and colleagues (8). Briefly, cells were loaded with 2 μmol/L fura-2/AM for 30 minutes in the presence of 250 μmol/L sulfynpyrazone, rinsed, and resuspended at a final concentration of 10$^6$/mL in the following saline solution: 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO$_4$, 1 mmol/L NaH$_2$PO$_4$, 20 mmol/L HEPES, 5.5 mmol/L glucose, 5 mmol/L NaHCO$_3$, and 1 mmol/L CaCl$_2$. pH 7.4. Excitation ratio and emission wavelength were 340/380 and 505 nm, respectively.

Cell cultures and transfections

HEK293 were grown in Dulbecco’s Modified Eagle’s Media (DMEM)-F12, whereas CT26, B16, and ACN cells were grown in RPMI medium supplemented with nonessential amino acids (Sigma-Aldrich). Both media were also supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. HEK293 cells expressing hP2X7 (HEK293-hP2X7) and mock-transfected (HEK293-mock) HEK293 cells were obtained as previously described (8). CT26 cells overexpressing mouse P2X7 (CT26-mP2X7) were obtained by transfection of CT26 cells with an expression vector encoding full-length mP2X7 (Pro451Leu variant) kindly provided by Dr. Murrell-Lagnado (Department of Pharmacology, University of Cambridge, Cambridge, UK). ACNshRNA1, ACNshRNA2 silenced, or ACN scrambled cell clones were obtained by transfection of predesigned HuSH-29 shRNA obtained from OriGene targeting the following sequences: ACGTTTGCTTCTCTGGTAAGTGACAG (shRNA1, c.n. T202483); CATTAGGAGTTGAAACAGCAGTACTAG (shRNA2, c.n. T202486), or scrambled shRNA cassette (c.n. TR30012). Stably transfected cell lines were obtained from HEK293-hP2X7 or CT26-mP2X7 cells by selection with G418 sulphate (0.2–0.8 mg/mL; Calbiochem). Stably transfected ACNshRNA1 and ACNshRNA2, and scrambled cells were obtained by selection with puromycin (0.5–1 μmol/L). Single-cell–derived clones were obtained by limiting dilution. To exclude possible artifacts because of clonal selection, 3 different HEK293-hP2X7 cell clones (HEK293-hP2X7 A, B, and C), 3 CT26-mP2X7 cell clones (CT26-mP2X7 c1, c2, and c3), and 2 ACNshRNA cell clones (ACNshRNA1 and ACNshRNA2) were used for the in vivo experiments.

Characterization of B16, CT26-mP2X7, and ACNshRNA clones

Expression of P2X7 by B16, CT26, and ACNshRNA clones was verified by semiquantitative PCR and Western blot analysis as previously described (9). For Western blot analysis, primary antibodies (anti-P2X7 and anti-actin A2668; Sigma-Aldrich) were used at 1:200 dilution and incubated for 16 hours at 4°C (anti-P2X7) or 1 hour at room temperature (anti-actin). P2X7 activity was assessed by measuring changes in the cytosolic-free Ca$^{2+}$ concentration ([Ca$^{2+}$]) in a thermostat-controlled (37°C) and magnetically stirred Perkin Elmer fluorimeter with the fluorescent indicator fura-2/acetoxymethyl ester (fura-2/AM) as described by Adinolfi and colleagues (8). Briefly, cells were loaded with 2 μmol/L fura-2/AM for 30 minutes in the presence of 250 μmol/L sulfynpyrazone, rinsed, and resuspended at a final concentration of 10$^6$/mL in the following saline solution: 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO$_4$, 1 mmol/L NaH$_2$PO$_4$, 20 mmol/L HEPES, 5.5 mmol/L glucose, 5 mmol/L NaHCO$_3$, and 1 mmol/L CaCl$_2$. pH 7.4. Excitation ratio and emission wavelength were 340/380 and 505 nm, respectively.

Tumor generation and in vivo drug administration

Female, 5- to 7-week-old, nude/nude, BALB/c or C57Bl/6 mice, weighing 15 to 18 g, were purchased from Harlan Laboratories and kept in sterile cages throughout the experiment when required. To induce tumors, 2 × 10$^6$ HEK293-mock or HEK293-hP2X7 cells were inoculated into subcutaneous fat of the right limb. Total number of mice injected was 65, of which 24 received HEK293-mock cells, 5 HEK293-hP2X7 clone A, 5 HEK293-hP2X7 clone B, and 31 HEK293-hP2X7 clone C. Unless otherwise stated, experiments shown were conducted with specimens from HEK293-hP2X7 clone C tumor, but similar results were obtained with tumors produced by HEK293-hP2X7 clone A or clone B. Similarly, 3 × 10$^6$ ACNshRNA1, 2, or ACNshRNA scrambled were injected subcutaneously in the right limb of 15 nude/nude mice (5 per cell type). Mouse tumor models were obtained by inoculating CT26 colon carcinoma or B16 melanoma cells in syngeneic mice, BALB/c, and C57Bl/6 for CT26 and B16, respectively. A total of 2.5 × 10$^6$ CT26-wt and
CT26-mP2X7 or 10^6 B16 cells were subcutaneously injected into the right limb of shaved mice. Total number of mice injected was 75, of which 30 received CT26-wt cells, 10 CT26-mP2X7c1, 20 CT26-mP2X7c2, 10 CT26-mP2X7c3, and 15 received B16 cells. Animals were examined every 2 days to assess general health conditions and evaluate tumor growth. Within 5 to 20 days from the inoculation (depending on the animal model and the injected clone), tumor masses became detectable by palpation and visual inspection, with a volume range from 1 to 50 mm^3. Tumor size was measured with a manual caliper, and tumor volume was calculated, approximating the tumor mass to a sphere, according to the following equation: volume = \( \pi/6(\text{w}_1 \times (\text{w}_2)^2) \) where \( \text{w}_1 \) is the major diameter and \( \text{w}_2 \) is the minor diameter. Tumor growth was monitored with daily (CT26, B16, ACN) or biweekly (HEK293) measurements for up to 18 or 31 days, respectively. Periodate-monitored with daily (CT26, B16, ACN) or biweekly (HEK293) solution pH 9.0 (Dako) for P2X7 or NFATc1 staining, respectively. After several rinses in wash buffer (Dako), tissue sections were incubated overnight at 4°C with rabbit polyclonal anti-P2X7 antibody (1:20), or 1 hour at room temperature with mouse monoclonal anti-NFATc1 antibody (1:100). For SMA and VEGF staining, tissue slides were boiled for 15 minutes in 1 mmol/L EDTA (pH 8.0) in a microwave oven. For SMA and VEGF staining, tissue sections were rinsed twice in PBS, and incubated for 30 minutes at room temperature with Dako Envision System HRP-conjugated rabbit (for P2X7 and VEGF staining) or mouse (for NFATc1 and SMA staining) antibodies. Tissue sections were then washed in PBS, and peroxidase activity detected by 6 to 10 minutes incubation at room temperature with Dako Liquid diaminobenzidine (DAB) Substrate Chromogen System (Dako). Counterstaining was conducted with Mayer’s hematoxylin (Sigma-Aldrich). For immunofluorescence analysis, tissue sections were incubated for 1 hour at room temperature with optimal concentrations of rat anti-mouse CD34 (ImmunoTools), rat anti-mouse CD31 (clone MEC 13.3; BD Pharmingen), and mouse-anti human CD31 (clone JC70A; Dako) antibodies. Isotype-matched mAbs were used as control to exclude nonspecific reactivity. Binding of the primary antibodies was detected with tetramethyl rhodamine–conjugated goat anti-rat IgG (Molecular Probe) or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen). After washing, slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield mounting; Vector Laboratories).

**Histology**

Excised tumor masses were processed as follows. Five-micrometer thick sections were cut from formalin-fixed, paraffin-embedded blocks, stained with hematoxylin–eosin (HE) solution, and analyzed with a Nikon eclipse 90i digital microscope (Nikon Instruments Europe). Vascular network was evaluated by counting the number of blood vessels per microscope field with a × 20 objective. Data shown are the result of counts from 3 different sections from each tumor. Fifteen fields, in the center and in the periphery of the mass, were analyzed for each tumor. For HEK293-derived tumors, histologic analysis was done on masses excised 21 days from inoculation.

**Immunohistochemistry and immunofluorescence**

Five-micrometer thick tumor sections were stained with anti-human CD34 (ready to use) and anti-Ki67 (1:25 dilution) antibodies in an automated immunohistochemistry processor (Immunostainer Benchmark XT; Ventana and Tuckson). For the detection of P2X7, NFATc1, SMA, VEGF, human and mouse CD31, and mouse CD34, sections were cut from formalin-fixed, paraffin-embedded blocks, deparaffinized with xylene, and rehydrated by sequential passages through decreasing (from 100% to 80%) concentrations of ethanol. Endogenous peroxidase activity was blocked by a 30-minute incubation at room temperature with methanol containing 3% H2O2. Tissue sections were then incubated at 98°C for 40 minutes in Target retrieval solution citrate buffer, pH 6.0, or target retrieval solution pH 9.0 (Dako) for P2X7 or NFATc1 staining, respectively. After several rinses in wash buffer (Dako), tissue sections were incubated overnight at 4°C with rabbit polyclonal anti-P2X7 antibody (1:20), or 1 hour at room temperature with mouse monoclonal anti-NFATc1 antibody (1:100). For SMA and VEGF staining, tissue slides were boiled for 15 minutes in 1 mmol/L EDTA (pH 8.0) in a microwave oven. Sections were then washed in PBS and incubated 1 hour at room temperature with monoclonal mouse anti-human SMA (1:50) or polyclonal VEGF (1:20) antibody. After the incubation with the primary anti-antibody, tissue sections were rinsed twice in PBS, and incubated for 30 minutes at room temperature with Dako Envision System HRP-conjugated rabbit (for P2X7 and VEGF staining) or mouse (for NFATc1 and SMA staining) antibodies. Tissue sections were then washed in PBS, and peroxidase activity detected by 6 to 10 minutes incubation at room temperature with Dako Liquid diaminobenzidine (DAB) Substrate Chromogen System (Dako). Counterstaining was conducted with Mayer’s hematoxylin (Sigma-Aldrich). For immunofluorescence analysis, tissue sections were incubated for 1 hour at room temperature with optimal concentrations of rat anti-mouse CD34 (ImmunoTools), rat anti-mouse CD31 (clone MEC 13.3; BD Pharmingen), and mouse-anti human CD31 (clone JC70A; Dako) antibodies. Isotype-matched mAbs were used as control to exclude nonspecific reactivity. Binding of the primary antibodies was detected with tetramethyl rhodamine–conjugated goat anti-rat IgG (Molecular Probe) or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen). After washing, slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield mounting; Vector Laboratories).

**VEGF ELISA assay**

A total of 2 × 10^5 HEK293 or 5 × 10^5 ACN cells were plated into 6-well plates (Falcon) in complete medium and allowed to adhere for 24 hours. After this time, cells were transferred to fresh medium and incubated for further 24 hours in the absence or presence of ATP (Sigma-Aldrich). VEGF release into the supernatants was measured with Quantikine Immunoassay for human VEGF (R&D Systems) as described by the manufacturer.

**TUNEL assay**

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay on tumor sections was done with TumorTACS In Situ Apoptosis Detection Kit (Trevena Inc.) according to manufacturers’ instructions. Briefly, sections were heated at 57°C for 5 minutes, deparaffinized with xylene, rehydrated by passages through decreasing ethanol...
concentrations (from 100% to 70%), and treated for 15 minutes at 37°C with proteinase K (1:50 dilution). Endogenous peroxidase activity was blocked by a 5-minute incubation at room temperature in 10% H₂O₂ in methanol. 3'-OH double-stranded DNA fragments generated during apoptosis (but not single-stranded necrosis-generated DNA fragments) were end-labeled with biotinylated nucleotides by a terminal deoxynucleotidyl transferase enzyme in a labeling buffer developed for apoptosis-specific labeling. The incorporated nucleotides were subsequently detected using a streptavidin-HPF followed by reaction with DAB to generate a dark brown precipitate. Sections were finally counterstained with methyl green.

**Image acquisition, cell counts, and statistics**

Images were acquired either with a Nikon eclipse 90i digital or an E-1000F Nikon microscope (Nikon) equipped with the NIS-elements or Genikon imaging system software (Nikon), respectively. Cell count was done with Image J1.42i software (Wayne Rasband, NIH, Bethesda, MD). Images of human cancers were acquired with an Olympus BX61 microscope equipped with Color View Soft Imaging System (Olympus Europe GmbH). Unless otherwise stated, data are shown as mean ± SE of the mean. Test of significance was done with Student t test using Graphpad InStat (GraphPad Software Inc.). Coding: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Results**

**Accelerated tumorigenesis by P2X7-expressing HEK293 and CT26 cells**

Female athymic (nu/nu) mice were subcutaneously inoculated with either HEK293-mock cells or each of 3 different stably transfected HEK293-hP2X7 clones. Twelve of 24 mice inoculated with HEK293-mock cells, 4 of 5 mice inoculated with HEK293-hP2X7 clone A, 5 of 5 mice inoculated with HEK293-hP2X7 clone B and 28 of 31 mice inoculated with HEK293-hP2X7 clone C developed palpable cancers within 31 days after inoculation (Fig. 1A). Already at day 17 postinoculation, tumor masses were unequivocally detectable and measurable in mice injected with HEK293-hP2X7 cells (Fig. 1B). In contrast, no tumors were detected in mice inoculated with HEK293-mock cells up to day 21. Size of HEK293-hP2X7 tumors was significantly larger than that of HEK293-mock tumors until the very late stages of growth (Fig. 1B). At day 31, shortly before mice were compassionately euthanized, differences in size were no longer significant. These data indicate that HEK293-hP2X7 tumors engrafted with higher efficiency and proliferated at a significantly faster pace than those derived from HEK293-mock cells. As expected, HEK293-hP2X7 tumors showed a homogenous expression of the P2X7 receptor, whereas HEK293-mock were consistently negative (Supplementary Fig. S1A–S1F). Histologic analysis revealed substantial differences in cell shape, size, and distribution between HEK293-mock and HEK293-hP2X7 tumors (Supplementary Fig. S1C and S1D). HEK293-mock cells proliferated in a rather ordered fashion, generating well-organized anastomized trabecules, or a "palisade-like" pattern, and were elongated or spindle-like in shape, reminiscent of the typical flattened fibroblast-like morphology of wild-type HEK293 cells in culture (Supplementary Fig. S1E). HEK293-hP2X7 cells had large, nonhomogeneous nuclei, generated a disordered, random, and were rounded or polygonal in shape, reminiscent of the rounded morphology assumed by these cells in vitro (Supplementary Fig. S1F). Mitotic figures were highly frequent in HEK293-hP2X7 tumors (Supplementary Fig. S1D, arrows) but were only occasionally observed in HEK293-mock tumors (Supplementary Fig. S1C, arrows). Cell density was significantly higher in HEK293-hP2X7 than in HEK293-mock tumor specimens (number of HEK293-hP2X7 cells was 388 ± 10 per field vs. 310 ± 9 HEK293-mock cell per field, P < 0.001, number of fields analyzed for each clone = 16). To confirm the growth-promoting activity of the P2X7 receptor in a different cell model and in an immunocompetent host, we took advantage of mouse CT26 colon carcinoma cells that exhibit a low level of endogenous P2X7 expression (Supplementary Fig. S2A). Three different stable cell clones overexpressing mP2X7 were obtained and named CT26-mP2X7 c1, c2, and c3 (Supplementary Fig. S2; Fig. 1C). When inoculated into BALB/c mice, CT26-mP2X7 clones generated tumors characterized by faster growth than those generated from parental CT26-wt (Fig. 1D–F). Figure 1D shows measurement of in vivo tumor size, whereas Fig. 1F shows average size of excised tumor. One of the most widely used blockers of P2X7 is oxidized oATP (31), thus we tested the effect of this inhibitor on growth of tumors generated by inoculation of CT26-mP2X7c2 clone. Intratumor injection of this inhibitor caused marked reduction of tumor growth and size in CT26-mP2X7 (Fig. 1E and F). In contrast, oATP failed to affect CT26-wt tumor size or growth rate (Fig. 1E and F).

**Accelerated growth of HEK293-hP2X7 tumor depends on an increased vascular network and VEGF secretion**

Growth of solid tumors depends on adequate vascularization and blood supply. HE staining showed a thicker vascular network in HEK293-hP2X7 and in CT26-mP2X7 than in HEK293-mock and CT26-wt tumors, respectively (Fig. 2A–D). Number of vessels was 4- and 3-fold higher in HEK293-hP2X7 and CT26-mP2X7 than in HEK293-mock and CT26-wt tumors, respectively (Fig. 2E). This observation was confirmed by smooth muscle actin (SMA) staining in HEK293 cells (Fig. 2F and G). In HEK293 tumors, we investigated the origin of the neovascularized vessels by staining with species-specific endothelial markers. Blood vessels stained negatively for human CD31 and CD34 (Fig. 3A, B, and E–G). CD34 staining showed a uniform expression of the P2X7 receptor, whereas HEK293-mock were consistently negative (Supplementary Fig. S3A–S3F). Histologic analysis revealed substantial differences in cell shape, size, and distribution between HEK293-mock and HEK293-hP2X7 tumors (Supplementary Fig. S3C and S3D). HEK293-hP2X7 tumors contained an about 4-fold higher number of CD34+ cells compared with HEK293-mock (Fig. 3I). These findings indicated that vascular mimicry, that is, the differentiation of tumor cells into endothelial cells lining tumor microvessels (32), did not occur in our experimental model. Interestingly, P2X7 blockade by oATP caused a large decrease in angiogenesis in CT26-mP2X7 but not in CT26-wt tumors (Fig. 2F).

Tumor angiogenesis is known to depend on the release of angiogenic factors either from tumor or from infiltrating inflammatory cells or both, the main inducer of angiogenesis being VEGF. P2X7 has been recently reported to promote...
VEGF release from rat glioma cells and human monocytes (33, 34). We thus measured the ability of HEK293-hP2X7 cells to release VEGF in vivo and in vitro. HEK293-hP2X7 tumors exhibited diffuse staining for human VEGF (Fig. 4B), whereas HEK293-mock tumors were virtually negative (Fig. 4A). This result was confirmed by an in vitro test showing that HEK293-hP2X7 cells produced about twice as much VEGF (140 ± 8.6 pg/ml; Fig. 4C) under basal conditions compared with HEK293-mock (77.7 ± 5.3 pg/ml; Fig. 4C). Stimulation with ATP for 24 hours caused a further increase in VEGF secretion from HEK293-hP2X7 (194 ± 4.6 pg/ml) but not from HEK293-mock (63.5 ± 6 pg/ml; Fig. 4C). The importance of neovascularization in P2X7-dependent tumor growth was further supported by the effect of Avastin (bevacizumab; ref. 35). Intratumor injection of Avastin caused a statistically significant decrease in HEK293-hP2X7 tumor size (Fig. 4D and E). Accordingly, Avastin-treated tumors were much paler than controls (Fig. 4E) and had a reduced vascular network (not shown). VEGF secretion was also investigated in CT26-mP2X7 tumors. As shown in Fig. 4F–I, CT26-mP2X7, but not CT26-wt, tumors stained positive for VEGF. VEGF staining was abrogated by intratumor injection of oATP.

Figure 1. hP2X7 and mP2X7 expression accelerates tumor engraftment and growth in nude/nude and BALB/c mice, respectively. A, percentage of mice showing palpable tumors at 31 days after inoculation. B, increase of tumor size over time. Tumor volumes were measured as described in Materials and Methods and expressed in mm$^3$. Data are averages ± SE of multiple determinations ($n > 20$) of HEK293-mock and HEK293-hP2X7 tumors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus HEK293-mock.

C, P2X7-dependent $[Ca^{2+}]_i$ increases in CT26-wt (green), CT26-mP2X7c1 (black), CT26-mP2X7c2 (red), and CT26-mP2X7c3 (blue). Cells were loaded with fura-2/AM and incubated in a thermostated fluorometer as described in Materials and Methods. BzATP was used at a concentration of 100 μmol/L. D, kinetics of CT26 tumor growth: CT26-wt (green), CT26-mP2X7c1 (black), CT26-mP2X7c2 (red), and CT26-mP2X7c3 (blue). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus CT26-wt.

E, effect of 3 intratumoral injections of oATP (600 μmol/L) administered at days 5, 9, and 13 postinoculum of CT26-mP2X7c2 clone or CT26-wt. CT26-wt (green); CT26-mP2X7c2 (red); CT26-wt plus oATP (green broken line); CT26-mP2X7c2 plus oATP (red broken line). *, $P < 0.05$; **, $P < 0.01$. F, representative tumor masses excised at day 15 postinoculum, corresponding to day 10 after first oATP injection.
Pharmacologic P2X7 blockade or silencing decrease tumor growth

The mouse melanoma B16 cells express high levels of the P2X7 receptor (Supplementary Fig. S3A), whose activity was stimulated by benzoyl ATP (BzATP; Fig. 5A) and inhibited by the selective blocker AZ10606120 (Fig. 5A). Intratumor injection of AZ10606120 caused a strong inhibition of tumor growth in B16-inoculated C57Bl/6 mice (Fig. 5B and C) and caused in parallel a large reduction in VEGF staining (Fig. 5D and E) and vessel formation (Fig. 5F). We then tested the tumorigenic ability of the human neuroblastoma ACN line silenced with hP2X7-specific short hairpin RNA (shRNA). As shown in Fig. 5G, silencing abolished almost fully P2X7-dependent responses and significantly reduced tumor growth and in vitro VEGF secretion (Fig. 5H–J).

HEK293-hP2X7 overexpress proliferation markers

The increased size of HEK293-hP2X7 tumors could be because of enhanced proliferation rate, reduced apoptosis, or both. Staining for Ki67, a marker of cell-cycle progression and proliferation, showed a stronger positivity in HEK293-hP2X7 than in HEK293-mock tumors (Fig. 6A–C). We had previously shown that expression of P2X7 does not only promote proliferation but also inhibit apoptosis in vitro (9). This was also the case for HEK293-hP2X7 tumors in which the frequency of TUNEL-positive cells was less than half that of HEK293-mock (Fig. 6D–F). Another key inducer of cell proliferation is the transcription factor NFATc1, which is strongly activated in HEK293-hP2X7 cells in vitro (9). Figure 5G–I shows that NFATc1 was activated to a higher level in HEK293-hP2X7 than in HEK293-mock tumors.

Different human cancers stain positively for P2X7

Finally, we investigated P2X7 expression in 8 specimens from human tumors: gastric cancer, neuroendocrine gut carcinoma, colon mucoid cancer, grade II colon cancer, high-grade kidney urothelial carcinoma, kidney clear cell carcinoma, undifferentiated ovarian carcinoma, and breast intracystic papillary carcinoma. As shown in Fig. 7, all specimens stained positively with the rabbit anti-P2X7 polyclonal antibody extensively used to investigate P2X7 expression in this study (see Supplementary Figs. S1, S3, and S4). Although the sample size is...
obviously small, these data confirm and strengthen published data showing P2X7 overexpression by human cancers (15, 16, 19, 36).

Discussion

The P2X7 receptor has been long known for its cytotoxic activity, but this receptor has also gained an undisputed role as potent inducer of NALRP3 inflammasome activation and IL-1 release (37). Most studies aimed at investigating P2X7 function under pathophysiologic conditions have focused on receptor stimulation with high doses (pharmacologic doses) of ATP or ATP-mimetic agonists, mostly BzATP. There is no doubt that pharmacologic stimulation of P2X7 may perturb cellular homeostasis to a level incompatible with survival. However, little attention has been paid to the effect of basal expression of P2X7. P2X7 is characterized by high plasticity, as its expression is modulated in a cell type- or differentiation-dependent fashion (38, 39). Some cell types, for example, human lymphocytes, express this receptor at a low level under resting conditions and increase P2X7 expression several fold upon mitogenic activation. In contrast, P2X7 expression is

Figure 3. HEK293-hP2X7 tumor contain a higher number of murine CD31\(^+\) and CD34\(^+\) cells. Tissue sections stained with anti-mouse CD31 (A and B), anti-human CD31 (C and D), or anti-mouse CD34 (E-H). Images in A–D and G and H were acquired with a \(\times 100\) oil-immersion objective, whereas those in E and F with a \(\times 40\) objective. I, number of mouse CD34\(^+\) cells per field. Fifteen fields were analyzed for each histotype. **, \(P < 0.01\).
downmodulated by inflammatory stimuli in other cell types, for example, primary microglia (27). There is general consensus that P2X7 stimulation by exogenous ATP does not mimic physiologic conditions. First, it seems unlikely that cells are exposed in vivo to high and homogeneous ATP concentration at the same time over the whole cell surface. Rather, it is more likely that patchy areas of the cell membrane are exposed to ATP in a pulsatile fashion. Second, in parenchymatous tissues, secluded areas of the plasma membrane may not be accessible to exogenous ATP. Third, different tissues harbor cell types with different ATP-scavenging ability, and this will generate a complex array of local ATP concentrations that will have a composite effect on cell responses. Finally, it is well established that ATP is continuously secreted by all cell types, and this generates an autocrine loop that can be mimicked by exogenous addition of ATP only to a very limited extent. The best evidence that P2X7 is in a state of tonic activation in the absence of added ligands stems from the finding that addition of ATP-degrading enzymes, selective pharmacologic blockers or anti-P2X7 monoclonal antibodies reverts the cell phenotype conferred by P2X7 transfection (8, 9, 25, 40).

The most striking property conferred by P2X7 transfection is the ability to survive in the absence of serum (8, 25). Faster growth of P2X7-transfected cultures depends on both a higher

Figure 4. HEK293-hP2X7 and CT26-mP2X7 tumors produce large amounts of VEGF. A–B and F–I, anti-VEGF staining. C, measurement of VEGF in cell supernatants in the absence or presence of 1 mmol/L ATP. Data are averages ± SE of sextuplicate determinations from 3 separate experiments. ***, P < 0.001 versus HEK293-mock. Cells were plated and VEGF assayed as described in Materials and Methods. D, HEK293-mock and HEK293-hP2X7 clone C–derived tumors received 3 intratumor injections of Avastin (10 mg/kg) at days 14, 18, and 22 postinoculum. Control HEK293-mock and HEK293-hP2X7 clone C–derived tumors received sterile saline. ***, P < 0.01; ***, P < 0.001 versus control HEK293-hP2X7. E, representative tumor masses excised at day 27 postinoculum, that is, day 13 after first treatment with Avastin; top row, control samples; bottom row, samples treated with Avastin. oATP treatment in I was done as described in Fig. 1.
potential. However, all these cells infected with BzATP (100 μmol/L) in the absence or presence of AZ10606120 (300 nmol/L) showed a higher capacity to invade Matrigel, whereas with the different hP2X7-transfected clones, the rate of success of tumor formation ranged from 80% to 100%. Under the experimental conditions used here, wt or HEK293-derived tumors had a higher level of anaplasia, strong cellular dishomogeneity, and distorted cytoarchitecture as compared with HEK293-mock-derived tumors. Striking morphologic differences could also be observed between HEK293-mock and HEK293-hP2X7 cultures. In fact, although HEK293-mock or wt cells in vitro were flattened, proliferated to cover the entire surface of the flask, and rarely if ever overgrew one on top of the other, HEK293-hP2X7 in vitro were rounded, tended to grow in clusters and to pile up, suggesting a total suppression of contact inhibition.

Moreover, tumors generated by hP2X7-transfected cells showed a faster growth rate and a lower occurrence of apoptosis, in agreement with our in vitro observations (8, 9). Furthermore, HEK293-hP2X7–derived tumors had a higher level of anaplasia, strong cellular dishomogeneity, and distorted cytoarchitecture as compared with HEK293-mock–derived tumors. Striking morphologic differences could also be observed between HEK293-mock and HEK293-hP2X7 cultures. In fact, although HEK293-mock or wt cells in vitro were flattened, proliferated to cover the entire surface of the flask, and rarely if ever overgrew one on top of the other, HEK293-hP2X7 in vitro were rounded, tended to grow in clusters and to pile up, suggesting a total suppression of contact inhibition.

In this study, we have addressed some of the issues relevant to the comprehension of the possible role of P2X7 in tumorigenesis in vivo. We took advantage of the HEK293 cellular model that is characterized by a P2X-null background and has been extensively studied by our group (8, 9, 12). HEK293 cells are known to be weakly tumorigenic in athymic mice (41). Under the experimental conditions used here, wt or HEK293-mock cells produced tumors in about 50% of inoculated mice, whereas with the different hP2X7-transfected clones, the rate of success of tumor formation ranged from 80% to 100%.

Figure 5. Effect of P2X7 blockade or silencing on tumor growth. A, changes in $[\text{Ca}^{2+}]_i$, in B16 melanoma cells challenged with BzATP (100 μmol/L) at days 5, 7, 9, and 13 postinoculum. B, size of tumors derived from ACN shRNA 1, 2, and scrambled shRNA was measured every 2 days until day 18 postinoculum. C, basal release of VEGF from ACN shRNA1, 2, and scrambled shRNA was measured 10 days after addition of AZ10606120 (300 nmol/L). D and E, specimens were stained with the anti-VEGF antibody as described in Materials and Methods, in the absence and presence of AZ10606120 treatment. F, vessels were counted in HE-stained sections. G, ACN shRNA1, 2, and scrambled shRNA were loaded with fura-2/AM and challenged with BzATP (100 μmol/L). H, size of tumors derived from ACN cells stably transfected with P2X7-specific shRNA or with scrambled shRNA was measured every 2 days until day 18, I, basal in vitro release of VEGF from ACN cells transfected with P2X7-specific shRNAs or with scrambled shRNA. J, representative tumor masses excised at day 15 postinoculum, that is, day 10 after first treatment with AZ10606120. D and E, specimens were stained with the anti-VEGF antibody as described in Materials and Methods, in the absence and presence of AZ10606120 treatment. F, vessels were counted in HE-stained sections. At least 15 fields were counted from 3 separate sections. * P < 0.001, ** P < 0.001 versus untreated B16 tumors. C, representative tumor masses excised at day 15 postinoculum, that is, day 10 after first treatment with AZ10606120. D and E, specimens were stained with the anti-VEGF antibody as described in Materials and Methods, in the absence and presence of AZ10606120 treatment. F, vessels were counted in HE-stained sections. At least 15 fields were counted from 3 separate sections. * * P < 0.001, ** P < 0.001 versus untreated B16 tumors.
NFATc1 is another marker that has been associated with cancer progression (44) and proliferation of cells as different as lymphocytes and osteoblasts (45). In keeping with our in vitro observations (9), NFATc1 was overexpressed in HEK293-hP2X7. Faster growth rate of HEK293-hP2X7 tumors was also showed by the high mitotic index. Finally, apoptosis was much less frequent in HEK293-hP2X7 than in HEK293-mock tumors, providing yet another sign of the more aggressive phenotype conferred by P2X7 transfection. Overall, these data are in keeping with previous reports linking increased P2X7 expression with poor prognosis in different leukemias (15, 46) and solid tumors (18, 19, 36).

Our findings were further strengthened by transfecting mP2X7 into the mouse colon cancer cell line CT26, and by generating CT26-mP2X7–derived tumors in syngeneic, immunocompetent BALB/c mice. Also in this model, P2X7-expressing tumors showed faster growth rate and reached larger size. Interestingly, postinoculum treatment with local injections of the P2X7 blocker oATP caused a substantial decrease of CT26-mP2X7 but not CT26-wt tumor growth.

An additional novel observation of great potential impact stemming from our study is that both HEK293-hP2X7 and CT26-mP2X7 tumors possess a thicker vascular network and stain positive for VEGF. It is known that stimulation of monocyte P2X7 triggers VEGF release, but there was until now no previous demonstration that P2X7 expression in vivo might cause release of this angiogenic factor from tumor cells. This is obviously another feature that converges to emphasize the possible participation of P2X7 in cancer progression.

Stimulation of VEGF release by ATP is of interest also in view of the high ATP level present within tumor microenvironment: we have calculated that tumor interstitium may contain several hundred micromolar ATP (5), an amount sufficient to drive VEGF secretion, as shown by the in vitro data. This suggests that extracellular ATP might be an important factor contributing to tumor angiogenesis. A further proof of the importance of VEGF in P2X7-dependent tumorigenesis was provided by the significant reduction in size of HEK293-hP2X7–derived tumors treated with Avastin. This humanized anti-VEGF antibody was the first antiangiogenic drug to be approved by the U.S. Food and Drug Administration for clinical use and is currently used for the treatment of selected human cancers. Of further interest is the observation that HEK293-derived VEGF induced angiogenesis from host (mouse) endothelia, underlining the strong ability of this growth factor to mould host responses to the benefit of the tumor.

Of further interest, pharmacologic blockade or silencing of P2X7 caused a large growth decrease of 2 tumors, the mouse melanoma B16 and the human neuroblastoma ACN, expressing high endogenous levels of this receptor, and accordingly VEGF release and vessel formation were reduced. Increased neovascularization of P2X7-expressing tumors was also recently reported by others (47). The recent demonstration that P2X7 expression enhances cancer cell invasiveness in vivo (30) is in keeping with its angiogenetic activity and further stresses the possible role of this receptor in cancer.
Finally, P2X7 is overexpressed by several human cancers (see Fig. 7 and refs. 10, 15–19). These observations raise the obvious issue of whether this receptor might be a useful marker as well as a novel target for therapy.

In conclusion, in this study, we show that expression of P2X7 confers several key features of cancer cells, namely, enhanced engraftment ability and \textit{in vivo} growth rate, increased expression of proliferation markers, reduced apoptosis, and enhanced VEGF release and angiogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

The authors thank Drs. Simonetta Falzoni, Francesca Amoroso, Giovanna Donvito, and Eros Magri for technical assistance.

**Grant Support**

F. Di Virgilio is supported by grants from the Italian Association for Cancer Research (no. IG 5334), Telethon of Italy (no. GGP06070), the Italian Space
References


Cancéroplie Île-de-France, and Fondation Bettencourt-Schueller. L. Rafaghello is a recipient of a My First AIRC Grant (MFAG). G. Bianchi is a recipient of an FIRC fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 8, 2011; revised March 27, 2012; accepted March 29, 2012; published OnlineFirst April 13, 2012.
Expression of P2X7 Receptor Increases In Vivo Tumor Growth

Elena Adinolfi, Lizzia Raffaghello, Anna Lisa Giuliani, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1947

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/04/13/0008-5472.CAN-11-1947.DC1

Cited articles
This article cites 47 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/12/2957.full.html#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at:
/content/72/12/2957.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.