Human leukocytes express a receptor for extracellular nucleotides, named P2X7-R, that in lymphocytes can either mediate cell death or proliferation, depending on the level of activation. The authors have investigated P2X7-R expression and function in 21 patients affected by B-cell chronic lymphocytic leukemia, 13 with an evolutive and 8 with an indolent variant of the disease. Resting cytoplasmic Ca^{++} concentration was significantly higher in lymphocytes from patients with the evolutive compared with indolent variant. Furthermore, in the former, P2X7-R stimulation triggered a Ca^{++} influx significantly larger. Higher Ca^{++} influx correlated with an increased P2X7-R expression in the lymphocytes from patients with the evolutive form. Finally, incubation in the presence of extracellular adenosine triphosphate decreased spontaneous proliferation of lymphocytes from patients affected with the evolutive variant but had no effects on lymphocytes from patients with the indolent form. These results suggest that expression and function of P2X7-R may correlate with the severity of B-cell chronic lymphocytic leukemia. (Blood. 2002;99:706-708)
rabbit polyclonal anti-P2X 7 receptor serum was kindly provided by Dr Gary...zymase, and 0.2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM
mM and 1 mM MgSO 4 , 5.5 mM glucose, 20 mM HEPES (pH 7.4), 1 mM
ions were resuspended in RPMI supplemented or Ca 2+ -free solution at a concentration of 2 × 10 6 /mL. The Ca 2+ -free solution also contained 500 μM EGTA. ATP and ionomycin (iono) were 1 mM and 1 μM, respectively.

Reverse-transcriptase–polymerase chain reaction

Total cytoplasmic RNA was extracted as described by Chomczynski and Sacchi, 16 reverse transcribed, and amplified with P2X 7 -specific primers. 14 After hybridization with a digoxigenin-labeled P2X 7 -specific internal oligoprobe, P2X 7 complementary DNA was visualized by chemiluminescent detection after incubation with a dilution of antidigoxigenin Fab fragments conjugated to alkaline phosphatase. As a control for the mRNA content of the samples, β-actin was used. The β-actin amplification products are shown as ethidium bromide–stained agarose gel electrophoresis bands.

Western blotting

Cells were lysed in lysis buffer containing 300 mM sucrose, 1 mM K 2 HPO 4 , 1 mM MgSO 4 , 5.5 mM glucose, 20 mM HEPES (pH 7.4), 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.2 μg deoxyribonuclease, and 0.2 μg ribonuclease by repeated freeze/thawing (3 cycles). Proteins were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel. The rabbit polyclonal anti-P2X 7 receptor serum was kindly provided by Dr Gary Buell (Serono Pharmaceutical Research Institute, Geneva, Switzerland). The primary antibody was used at a dilution of 1:100 in triethanolamine-buffered saline buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0). The secondary antibody was a goat antirabbit antibody conjugated to alkaline phosphatase. Intensity of the bands was measured with a Multimage Light Cabinet Alpha Image 1220 densitometer (Alpha Innotech, San Leandro, CA) and expressed as integrated density value.

Proliferation

For [ 3 H]thymidine incorporation, cells were resuspended in RPMI supplemented with 10% fetal calf serum and plated overnight at a concentration of 10 6 /mL. In the presence of radiolabeled thymidine (1 μCi [3.7 × 10 4 Bq] per well). In parallel, cells were also counted with a phase contrast light microscope.

Statistics

Statistical significance was assessed by the Student t test.

Results and discussion

It was previously reported by Wiley and Dubyak 3 that ATP increases cation permeability of lymphocytes from patients affected by B-CLL but not from healthy patients. We have reevaluated their findings in 2 groups of patients—1 with an evolutive and 1 with an indolent course of the disease. Exemplificative traces of resting and ATP-stimulated levels of intracellular Ca 2+ ([Ca 2+ ] i ) are shown in Figure 1. Lymphocytes from evolutive B-CLL showed an elevated resting [Ca 2+ ] i and a higher ATP-stimulated peak compared with the patient with the indolent variant. We screened 13 evolutive B-CLLs and 8 indolent B-CLLs, finding a mean resting [Ca 2+ ] i of 88 nm ± 27 and 52 nm ± 23 (P < .01), respectively. The stimulated [Ca 2+ ] i increase above resting level was 100 nm ± 30 and 32 nm ± 16 (P < .001) in evolutive B-CLL and indolent B-CLL, respectively. There was a good correlation...
between an elevated basal \([Ca^{2+}]\), and a higher ATP-stimulated rise \((r = 0.63)\). We confirmed, as previously shown by Wiley and Dubyak,\textsuperscript{5} that B-CLL cells do not express functional P2Y receptors and that the whole \([Ca^{2+}]\) increase is due to influx across the plasma membrane following the opening of an ATP-gated ion channel (Figure 1). Because previous studies showed that the main ATP-activated channel of lymphoblastoid cells is P2X-R,\textsuperscript{5,14} we investigated the expression of this receptor by reverse-transcriptase–polymerase chain reaction (RT-PCR) in peripheral lymphocytes from all the patients recruited in the 2 groups. Figure 2A reports P2X-R expression in 2 evolutive B-CLL and 2 indolent B-CLL patients representative of the respective groups; human macrophages are shown for comparison. Control β-actin amplification fragments are shown in Figure 2B. Lymphocytes from patients with evolutive B-CLL express P2X-R to a level higher than those affected by the indolent variant, and comparable to that of macrophages, a cell type well known for its high expression of this receptor subtype. Patients with a higher ATP-stimulated \([Ca^{2+}]\), showed a higher P2X-R expression (Figure 2A). Figure 2C shows a Western blot of peripheral lymphocytes from the same patients to confirm higher expression of the P2X-R protein in evolutive compared with indolent B-CLL. In our screening of all the 21 patients recruited in this study, we found a good positive correlation \((r = 0.924, P < 0.01)\) between increases in \([Ca^{2+}]\), stimulated by ATP and P2X-R protein expression measured by densitometry of Western blots. We and others have demonstrated in the past that expression of P2X-R confers susceptibility to ATP-mediated cytotoxicity.\textsuperscript{7,10,19} Thus, we selected 8 patients—4 B-CLL indolent (low P2X-R) and 4 B-CLL evolutive (high P2X-R)—and tested their sensitivity to ATP. In the experiment reported in Figure 2D, we show that extracellular ATP inhibited in vitro proliferation of peripheral lymphocytes isolated from patients affected by the evolutive but not the indolent form of the disease. Data on \([\text{H}]\)thymidine incorporation were corroborated by cell number count (not shown).

Receptors for extracellular nucleotides have recently become a focus of interest in immunology and hematology due to their high level of expression in blood cells and the vast potential of therapeutic applications afforded by their modulation.\textsuperscript{6} It was originally put forward that P2X-R might participate in shedding of CD23 and CD62L or killing by apoptosis.\textsuperscript{15,20,21} We have previously observed that transfection of P2X-R, the most widely diffused P2X receptor subtype in immune cells, confers a substantial proliferative advantage when transfected into human B lymphoblastoid cells that lack it constitutively.\textsuperscript{14} The P2X-R transfectants become able to grow in the absence of serum, show an elevated resting \([Ca^{2+}]\), respond to ATP, and secrete large amounts of this nucleotide into the culture supernatant. Thus, we speculated that P2X-R expression might also confer an advantage in vivo and anticipated that patients with the worse prognosis (ie, evolutive B-CLL) might be characterized by a B-cell population with a higher P2X-R expression. Such a cell population might be less affected than normal B lymphocytes, or B-CLLs that express low levels of P2X-R (ie, indolent B-CLL cells), by antimitabolites or might recover more quickly after chemotherapy. It is of interest that while P2X-R expression confers to evolutive B-CLL cells a proliferation advantage, it also makes these cells more susceptible to the cytotoxic effect of high concentrations of extracellular ATP. It may appear paradoxical that stimulation of a single receptor may bring about such utterly different effects, but this might be explained by the different pattern of intracellular signals generated by a tonic, low-level activation of the P2X-R, such as that presumably responsible for growth stimulation, as opposed to the massive receptor activation that is known to trigger cytotoxicity. In any case, this bifunctional capacity points to P2X-R as a novel and useful target for antitumor therapy.

References

P2X<sub>7</sub> receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia

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