Expression and Release of CD27 in Human B-Cell Malignancies


CD27, a transmembrane disulfide-linked 55-kD homodimer, belongs to the nerve growth factor-receptor family, a group of homologous molecules involved in lymphocyte differentiation and selection. It is expressed on mature thymocytes, peripheral blood T cells, and a subpopulation of B cells. We investigated the expression of CD27 on malignant B cells representative for a broad range of stages in physiologic antigen-independent and -dependent B-cell development. In normal lymphoid tissue CD27*B cells were only found in the peripheral blood (29.8% ± 10.8%, n = 13) and in germinal centers. With the exception of pro-B and the majority of pre-pre-B acute lymphocytic leukemias and of myelomas, CD27 expression of variable intensity was detected on almost all immature and mature malignant B cells tested. Moreover, using a sandwich enzyme-linked immunosorbent assay we could show the presence of sometimes very high (up to 6,000 U/mL; normal values <190 U/mL) amounts of the soluble 28- to 32-kD form of CD27 (sCD27) in the sera of patients with B-cell malignancies. The highest levels of sCD27 were observed in patients with chronic lymphocytic leukemia and low-grade non-Hodgkin's lymphomas. Most importantly, both in transversal and longitudinal studies, we found a strong correlation between sCD27 levels in the serum and tumor load, indicating that sCD27 can be used as a disease-marker in patients with acute and chronic B-cell malignancies.

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Material and Methods

Tissues. Normal and pathologic lymphoid tissues were selected from the files of the Department of Pathology, Academic Medical Center, University of Amsterdam, The Netherlands. Non-Hodgkin's lymphomas were classified according to the Working Formulation (WF). Preparation of T- and B-lineage markers was used for classification as T- or B-cell lymphomas.

Preparation and separation of cell suspensions. Normal peripheral blood mononuclear cells were obtained by Ficoll-Isoaque (Organon Teknika Corp, Durham, UK) density gradient centrifugation (density = 1.077 g/cm³) of EDTA blood from healthy volunteers. In some experiments we used purified normal tonsillar B cells. Freshly obtained normal tonsillar tissue was dissected free from surface epithelium and finely minced into a cell suspension. Mononuclear cells were isolated by Ficoll-Isoaque density gradient centrifugation. Monocytes were removed by plastic adherence (1 hour at 37°C in 10 cm² Petri dishes [Costar, Cambridge, MA]). T cells were depleted using 2-aminoethyl-isothiouronium bromide-modified sheep red blood cells (SRBCs). The purified B cells were then layered on a Percoll gradient (Pharmacia, Uppsala, Sweden) consisting of five density layers (1.085/1.077/1.067/1.056/1.043 g/cm³) and centrifuged for 15 minutes, 1,200g at 4°C. The cells at the 1.043/1.056 interface (low-density cells) and the 1.077/1.085 interface (high-density cells) were used in the experiments.

Similarly, suspensions of malignant mononuclear cells were prepared from peripheral blood, bone marrow (BM), or lymph node
Fig 1. (A) Coexpression of CD19 and CD27 on peripheral blood lymphocytes. (B) Lack of coexpression of CD5 and CD27 on gated CD19⁺ peripheral blood lymphocytes. Triple-labeling with biotinylated CD19/Cy-chrome labeled streptavidine, CD5-PE, and CD27-FITC was performed as described in Materials and Methods. CD27 expression on tonsil B cells: High (C) and low (D) density B cells were isolated from tonsil tissue and double-labeled with anti-CD27 and PNA-FITC as described in Materials and Methods.

Fig 2. Expression of CD27 on normal tonsil lymphocytes and B-cell NHLs. (A) Section of tonsil showing strong expression of CD27 in interfollicular T-cell areas (T). Mantle zone (M) B cells are CD27⁻ whereas Germinal center (GC) B cells show a variable CD27 expression. (B) Higher magnification of tonsillar GC showing variable expression of CD27 in GC cells. (C) B-CLL lymph node showing strong CD27 expression. (D) Burkitt's lymphoma showing weak CD27 expression. Counterstain hematoxylin. Bar: (A) 250 μM; (B through D) 100 μM.
biopsies of patients with B-cell malignancies. For the studies on the expression of CD27 on B-cell precursors we used normal BM aspirated from patients undergoing cardiac surgery or from patients with lymphoma whose BM was obtained for clinical staging and was found to be free from disease. BM mononuclear cells were isolated by Ficoll-Isoopaque density gradient centrifugation.

Informed consent from patients and healthy volunteers was obtained according to the rules of our hospital.

Immunocytology. The phenotype of the cell suspensions was determined by fluorescence-activated cell sorter (FACS) analysis using the following MoAbs in an indirect immunofluorescence technique: CLB-CD3/3, CLB-CD4/1, CLB-CD5, CLB-CD8/4, CLB-CD10, CLB-CD19, CLB-CD20, CLB-Blast-2(CD23), CLB-CD24, CLB-HL2R/(CD25), CLB-CD27/(1, anti-IgM(MH1), anti-IgG(MH1)), all produced at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, and clustered in the international workshops on leukocyte differentiation antigens. Anti-α, anti-λ, anti-IgD, and CD22 were purchased from DAKO (Glostrup, Denmark). Binding of the antibodies was visualized with fluorescein isothiocyanate (FITC)-labeled polyclonal goat-antimouse IgG (GAM). In some double-labeling experiments we used CD19-phycocerythrine (PE) from Becton Dickinson (BD; Sunnyvale, CA). To block free binding sites on GAM antibodies, cells were incubated with normal mouse serum and washed before addition of CD19-PE. In studies with the purified tonsillar B cells we used goat-antimouse-PE (Southern Biotech. Assoc. Inc, Birmingham, AL) and PNA-FITC (Sigma, St Louis, MO). In the triple-labeling experiments peripheral blood mononuclear cells were incubated with CD27-FITC (CLB), CDS-PE (BD), and biotinylated CD19 (CLB). After washing, Cyochrome-labeled streptavidine (ITK Diagnostics, Uithoorn, The Netherlands) was added. In the triple-labeling experiments with BM mononuclear cells we used two different combinations of MoAbs during the first incubation step: (1) CD34-FITC (CLB), CD19-PE (BD), and biotinylated CD27 (CLB); (2) CD10-PE (BD), biotinylated CD19 (CLB), and CD27-FITC (CLB). PERCP-labeled streptavidine (BD) was added after washing.

Immunohistochemistry. Immunoperoxidase staining was performed as described previously. In brief, cryostat sections were fixed in acetone for 10 minutes, washed in phosphate-buffered saline (PBS), and preincubated with normal goat serum (10% in PBS). After preincubation the sections were incubated with the primary antibody for 1 hour. Before incubating with the secondary biotinylated antibody for 30 minutes (antimouse and antirabbit F(ab)2, DAKO Glostrup, Denmark), endogenous peroxidases were blocked by incubating with hydrogen peroxide (3%) for 10 minutes. Then biotinylated secondary antibody for 30 minutes [antimouse and antirabbit F(ab)2, DAKO Glostrup, Denmark] was added. In the double-labeling experiments with BM mononuclear cells we used two different combinations of MoAbs during the first incubation step: (1) CD34-PE (BD), CD19-PE (BD), and biotinylated CD27 (CLB); (2) CD10-PE (BD), biotinylated CD19 (CLB), and CD27-FITC (CLB). PERCP-labeled streptavidine (BD) was added after washing.

Table 1. Expression of CD27 in B-Lineage Leukemias

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<td>Myeloma</td>
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* Median fluorescence intensity (linear scale).

Table 2. Expression of CD27 in B-Lineage NHLs

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CD27 expression was analyzed by immunoperoxidase staining of cryostat sections. The intensity of the reaction was scored with CD27 expression on T cells (+) as a reference [see also Fig 2, C and D].

Abbreviations: —, negative; +/-, very weak/equivocal; +, weak; ++, moderate/strong.

Fig 3. Levels of soluble CD27 (sCD27) in sera from healthy controls (normal, n = 43), ALL (n = 41), NHL (n = 65), and multiple myeloma (MM, n = 12). Dashed line indicates mean + 3 SD of sCD27 in control group.

Staining with irrelevant isotype-matched antibodies was always used as control.

Fig 4. Serum levels of soluble CD27 (sCD27) in B-cell NHLs of low- (n = 31), intermediate- (n = 24), and high- (n = 13) grade malignancy. Dashed line: mean + 3 SD of sCD27 serum levels in 43 healthy controls. *P < .0001; **P < .0035.
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lyzed for the simultaneous expression of CD19 and CD27. A double-positive population could easily be shown in all individuals tested. A representative example is shown in Fig 1A. The CD27+ B cells constituted 29.8% ± 10.8% (n = 13) of all peripheral blood B cells. The CD19+ and CD19- peripheral blood lymphocytes differed as to the amount of membrane-bound CD27 as measured by the median fluorescence intensities: 337 ± 26 versus 467 ± 8, respectively (P < .0001). In triple-labeling experiments CD27 was found to be confined to the CD19+CD5- B cells (n = 3) (Fig 1B). In histologic sections of normal tonsils CD27+ cells were abundantly present in the T-cell areas (Fig 2A). In contrast, significant numbers of CD27+ B cells were only found in the germinal center (Fig 2, A and B). Similar data were obtained in lymph nodes and spleen (not shown). Restriction of CD27 expression to the germinal center B cells in the secondary lymphoid organs was also demonstrated in experiments with suspensions of purified normal tonsil B cells. These cells were separated into high- and low-density B cells and analyzed for expression of CD27 and peanut agglutinin (PNA). As shown in Fig 1 (C and D) the vast majority of CD27+ B cells were found within the low-density PNA+ cells. These cells were also CD19+ and IgD−, a phenotype consistent with follicular center cells (data not shown).

RESULTS

Expression of CD27 on normal B cells and their precursors. To have a basis for comparison with the results obtained with malignant B cells, we studied the expression of CD27 on normal B cells and B-cell precursors. Peripheral blood lymphocytes from 13 healthy volunteers were ana-

![Fig 5. Serum levels of soluble CD27 (sCD27) in patients with CLL in different disease stages according to the Rai classification. Dashed line: mean ± 3 SD of serum sCD27 in 43 healthy controls. *Rai 0 versus Rai 1 + 2: P < .0029. **Rai 1 + 2 versus Rai 3 + 4: P < .085 (not significant).](image)

![Fig 6. Longitudinal study of serum soluble CD27 (sCD27) levels in two different patients with a B-cell malignancy. (A) Common ALL; (B) low-grade B-cell NHL. CR, complete remission; PR, partial remission; CT, chemotherapy.](image)
CD27 expression during early stages of B-cell development was studied by triple-labeling experiments on mononuclear cells prepared from aspirates of normal BM. In six separate experiments we found that in both the CD34+CD19+ cell fraction and in the CD19+CD10+ cell fraction CD27 expression did not exceed background levels (data not shown), indicating that normal B-cell precursors up to and including pre-B cells do not have membrane-bound CD27.

Expression of CD27 on malignant B cells. Malignant B cells representative for several differentiation stages were analyzed for the presence of membrane-bound CD27. The results are shown in Table 1. It can be seen that CD27 is already present on malignant B cells corresponding to the early stages of antigen-independent B-cell maturation. Although all four pro-B acute lymphocytic leukemia (ALL) (CD34+/CD19+/CD10~/TdT+) tested were CD27~, CD27 was present on 4 of 17 pre-pre-B ALL (CD19~/CD10+/cIgM~/TdT+) and 2 of 3 pre-B ALL (CD19+/CD10+/cIgM~/TdT~). The two B-ALLs tested (CD19+/CD10+/mIgM~/TdT~) had a high expression of membrane-bound CD27. Variable, but in general moderate to strong, expression was found on chronic lymphocytic leukemia (CLL) cells, prolymphocytic leukemia, and two of four hairy cell leukemias. Immunohistochemical analysis of non-Hodgkin’s lymphomas (NHLs) showed a moderate to high expression of CD27 in most low-grade diffuse and follicular lymphomas and a highly variable expression on intermediate- and high-grade lymphomas (see Fig 2, C and D, and Table 2). Myeloma cells were found to lack membrane expression of CD27 (n = 6).

Serum levels of soluble CD27 in patients with B-cell malignancies. By means of an ELISA sCD27 levels were measured in sera from groups of untreated patients with different types of B-cell malignancies. In sera from 43 healthy controls the mean (±SD) level of sCD27 was 103.3 ± 28.8 U/mL. With the exception of multiple myeloma (n = 12), in 98 of the 110 B-cell leukemia and lymphoma patients tested, sCD27 was above the mean + 3 SD (190 U/mL) of the control group (Fig 3). However, striking differences were observed between different groups. Very high levels (up to 6,000 U/mL) of sCD27 were found in sera from patients with CLL. In patients with NHLs serum sCD27 concentrations in those with low-grade lymphomas were significantly higher than in those with intermediate and high-grade lymphomas (P < .0001 and P < .0035, respectively) (Fig 4). sCD27 levels in low-grade NHL were not significantly different from those in CLL (P = .42). In the few T-cell malignancies tested (3 ALL and 2 NHL), sCD27 levels were only moderately increased: range 278 to 684 U/mL.

sCD27 is related to tumor load. In CLL a correlation was found between stage (Raj classification) and serum sCD27 (Fig 5). Similarly, within this group of patients there was a correlation between sCD27 and the absolute number of circulating lymphocytes (r = .79; data not shown). The possible correlation between clinical stage and sCD27 could not be investigated in NHL because the vast majority of patients in this series had NHL stage 3 or 4 (Ann Arbor). In five patients we had the opportunity to study sCD27 levels longitudinally. In all these patients (four with NHL and one with pre-pre B-ALL) CD27 serum levels matched clinical disease activity or tumor load: increased levels at diagnosis and relapse and normal levels during remission. Two examples are shown in Fig 6. A dramatic change in the levels of sCD27 could be observed in a patient with a villous splenic lymphoma who, only partially responding to chemotherapy, underwent a splenectomy. After removal of the very
large spleen (weight approximately 4 kg) sCD27 almost normalized within 24 hours (Fig 7), reflecting not only the amount of tumour reduction obtained but also indicating that sCD27 has a plasma half-life time of less than 8 hours. We studied only one patient with a T-cell malignancy longitudinally. In this T-ALL patient the sCD27 normalized rapidly during chemotherapy and remained so during complete remission (1.5 years) (data not shown).

DISCUSSION

The two key observations in this study are: (1) membrane-bound CD27 is present on malignant B cells representative of almost all stages of B-cell ontogeny, and (2) soluble CD27 can be detected in the serum of many patients with B-cell malignancies and can be used as a marker for tumor load.

Initial studies suggested that the expression of CD27 was confined to a (major) subset of T lymphocytes and thymocytes. More recently CD27 was also shown to be present on a subpopulation of mature B cells and natural killer cells. Our finding that about 30% of the normal peripheral blood B cells are CD27+ is in close agreement with the data published by Maurer et al. In comparison with the peripheral blood T lymphocytes, B cells have a somewhat weaker CD27 expression. In normal tonsils, lymph nodes, and spleen we could only show CD27+ B lymphocytes in the more apical zones of the germinal centers. In 1992, Maurer et al reported that only CD27+ B cells are able to secrete Ig after stimulation with mitogens, suggesting that in B cells CD27 expression is related to maturation stage. This would be analogous to the situation for the T-cell lineage, where CD27 expression is acquired at the stage of the mature thymocyte. Recent evidence indicates that CD27- T cells represent a subset of memory cells arising after prolonged antigenic stimulation. In view of the putative roles of other members of the NGF receptor family (CD40 and FAS/APO-1) in lymphocyte selection and maturation, the observation that membrane-bound CD27 expression is restricted to germinal center B cells and a subpopulation of peripheral blood B cells makes it tempting to speculate that CD27 is involved in the selection of B lymphocytes. The recent identification and cloning of the CD27 ligand will enable studies on the role of CD27 in proliferation, differentiation, and/or selection of normal and malignant B cells.

An important question is whether the observed CD27 expression on malignant B cells representing almost all of the stages of the antigen-independent and -dependent B-cell development is a reflection of its expression during normal B-cell ontogeny. This is probably not the case because we were unable to show CD27 expression on normal BM B-cell precursors up to and including the pre-B cell stage. Aberrant CD27 expression on malignant B cells is also suggested by our data on CLL cells: these pathologic B cells are CD27+, whereas their supposed normal counterparts in the follicle mantle zone are CD27-. Likewise, in triple-labeling experiments we found that CD5+ peripheral blood B cells are CD27-. Furthermore, cord blood B cells, containing a large proportion of CD5+ cells, have been reported to be CD27 negative. The same argument holds for the observed (weak to moderate) CD27 expression on Burkitt's lymphoma cells and the absence of CD27 on their putative normal counterparts in the germinal center basal dark zone. The mechanism and functional significance of aberrant CD27 expression on malignant B cells remains to be elucidated.

Our findings with respect to the soluble form of CD27 indicate that in patients with B-cell malignancies, serum levels of sCD27 are correlated to disease activity or tumor load. The lower sCD27 levels in intermediate-/high-grade NHL in comparison with CLL/low-grade NHL can be explained both by the in general lower membrane expression of CD27 and the lower absolute tumor load. Although suggestive, the limited number of observations as well as the (in)frequency of serum sampling in our study do not yet allow for conclusions as to predictive value of sCD27 determinations. Our data strongly suggest that the sCD27 is derived from the malignant B cells. However, we could not prove this because immunoprecipitation studies did not show differences between the sCD27 from the serum of the two patients tested (CLL and low-grade NHL) and a culture supernatant of CD3-stimulated T lymphocytes (data not shown). Hence, the increased sCD27 levels could reflect T-cell activation as has been shown during acute cytomegalovirus and human immunodeficiency virus infections, in synovial fluid of RA patients and in the cerebrospinal fluid of multiple sclerosis patients. There are several arguments against this possibility. First, the sCD27 levels in patients with B-cell malignancies are not only much higher than those reported in the patients with acute or chronic T-cell activation, but they also correlated with disease stage, as illustrated for CLL in Fig 5. Secondly, the splenectomy of a patient with a splenic NHL resulted in a rapid and permanent decrease in sCD27. Finally, production of sCD27 could be shown in the culture supernatant of purified CLL-B cells (<1% CD3+ cells; data not shown).

The mechanism of the generation of sCD27 by the malignant B cells is not yet known. Studies in T cells have indicated that membrane-bound and soluble CD27 have a common mRNA and protein precursor, and that sCD27 is generated from the membrane-bound CD27 by proteolysis. This process is strongly enhanced by T-cell activation. In vitro studies with B cells have shown that activation of CD27+ B cells by staphylococcus aureus Cowan (SAC) and interleukin-2 can induce expression of membrane bound CD27. Under these conditions these B cells release soluble CD27 (R.Q.H., unpublished observations). However, in malignant B cells regulation of membrane expression and release of CD27 might be different from that in normal T and B cells. This is suggested by our unpublished observations that purified CLL-B cells, cultured for 4 days without any stimulation, release sCD27 into the supernatant. Neither this release nor membrane expression of CD27 were influenced by addition of PMA and TNF, a combination shown to induce strong proliferation of CLL cells. If membrane-bound CD27 is involved in the functional activity of the CD27+ T and B lymphocytes, the presence of high amounts of soluble CD27 in the plasma might interfere with these functions. It would be interesting to investigate whether sCD27 contributes to the impaired cellular and humoral immunity and autoimmune phenomena frequently observed within this group of patients.
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