Identification of Soluble APO-1 in Supernatants of Human B- and T-Cell Lines and Increased Serum Levels in B- and T-Cell Leukemias

By Eckart Knipping, Klaus-Michael Debatin, Kirstin Stricker, Bernhard Heilig, Astrid Eder, and Peter H. Krammer

The cell-surface protein APO-1 is a member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily. APO-1 mediates apoptosis in susceptible cells upon stimulation with the monoclonal antibody anti-APO-1 or upon binding of its natural ligand. Soluble receptors had previously been identified for most members of the NGF/TNF receptor superfamily. Recently, a soluble form of APO-1 (sAPO-1) was described. We established a sandwich enzyme-linked immunosorbent assay to detect sAPO-1 in culture supernatants of human cell lines and in human sera.

MATERIALS AND METHODS

Cell cultures. The T-cell acute lymphoblastic leukemia (T-ALL)-derived leukemic cell lines Hut-78 and Jurkat and the B-lymphoblastoid cell line SKW6.4 were maintained in culture medium containing RPMI 1640, 10 mmol/L HEPES, pH 7.3, 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO, Karlsruhe, Germany), and 10% fetal calf serum (FCS; Seromex, Vilshofen, Germany) at 37°C with 5% CO₂.

Concentration of culture supernatants. The cell supernatants were filtered through 0.45-μm membranes (Sartorius, Göttingen, Germany) and centrifuged for 1 hour at 100,000 g at 4°C. The protein pellet was resuspended in a 1/1,000 of the initial volume of H₂O containing 2% sodium deoxycholate. 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 40 μg/mL bestatin, 2 μg/mL leupeptin, 1 μg/mL aprotonin, 2 μg/mL pepstatin A, 1 μg/mL trypsin inhibitor (Sigma, Deisenhofen, Germany), and 0.1% sodium azide.

Serum samples. Serum samples of patients with hairy cell leukemia (HCL); chronic lymphocytic leukemia (CLL); nodal B-cell

Fluids. Thus, the biologic effects of TNF were inhibited by soluble(s) TNF receptors in vitro. Although the physiologic functions of these soluble receptors are not completely clarified, it is conceivable that they play a role in regulating functions of the immune system. For many leukemic diseases and some solid tumors, eg, colon carcinoma, elevated levels of sTNF receptors or sCD30 have been found and, in some cases, correlated with disease progression.

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lymphomas of the centrocytic and centroblastic subtype (according to the Kiel classification\(^{8,9}\)) or of the small cleaved and large noncleaved subtype, respectively (according to the New Working Formulation of the World Health Organization\(^{14,15}\)); common acute lymphoblastic leukemia (cALL); and T-ALL were obtained at the time of diagnosis before antileukemic therapy from the Childrens Hospital and the Medizinische Klinik V, University of Heidelberg, Germany.

*baculovirus expression and purification of mAPO-1*. The cDNA encoding the complete human APO-1 molecule was recombined into *Autographa californica* baculovirus (AcNPV) using the transfer vector pVL 1393 (DiaNova, Hamburg, Germany). Recombinant viruses were isolated as previously described.\(^{9,10}\) S9 cells were infected with recombinant virus with a multiplicity of infection of 10 and harvested after 3 days. mAPO-1 was extracted as described\(^{9,10}\) with 2.5\% Tween-40 and solubilized with 2\% sodium deoxycholate. The protein solution obtained was further purified by affinity chromatography on an anti–APO-1 (IgG3)-coupled Sepharose 4B column (Pharmacia, Freiburg, Germany). Purified fractions were loaded on a reversed-phase high performance liquid chromatography (HPLC) column (PLRP-S, 300 Å, 8 μm, 250 × 4.6 mm; Polymer Laboratories, Shropshire, UK) and eluted in an acetone gradient from 5\% to 70\% in 0.1\% (vol/vol) trifluoro acid (TFA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10\%) showed two protein bands of 48 and 52 kD, reflecting different glycosylated forms of the recombinant mAPO-1. Dimerization and multimerization of the purified mAPO-1 molecule were also observed.

*Production and purification of rabbit anti–APO-1 Ig*. Adult male New Zealand white rabbits were immunized subcutaneously with 50 μg recombinant purified mAPO-1, mixed 1:1 (vol/vol) with complete Freund's adjuvant (GIECO) in a final volume of 200 μL. Booster injections were performed with 50 μg purified mAPO-1 mixed 1:1 (vol/vol) with incomplete Freund's adjuvant every 4 weeks. Affinity purification of IgS from rabbit serum was performed on a protein A sepharose 4B column (Pharmacia). Serum samples were adjusted to pH 8 and loaded onto the column. After washing with 100 mmol/L Tris, pH 8, and 10 mmol/L Tris, pH 8, Ig was eluted with 100 mmol/L glycine-HCl (pH 3). Fractions collected were analyzed for purity on reducing 10\% SDS-PAGE gels and pure fractions were pooled for use in the sELISA.

To ensure that the sELISA detected the extracellular part of APO-1, we tested the rabbit anti–APO-1 serum in a functional assay. Hut-78 cells were incubated with the antiserum and apoptosis was observed on Kodak XAR-5 X-ray films (Kodak, Stuttgart, Germany).

ELISA plates (Falcon, Heidelberg, Germany) were coated with 100 μL/well of the murine MoAb anti–APO-1 (IgG3, 1 μg/mL)\(^{1}\) in NaHCO\(_3\), pH 9.6, at 4°C for 12 hours. Coated plates were washed three times with 100 μL phosphate-buffered saline (PBS) containing 0.05\% Tween-20 (PBS/Tween) and subsequently blocked with 2\% bovine serum albumin (BSA) in PBS/Tween for 30 minutes at room temperature. Undiluted culture supernatants or human sera were added in log 4 dilutions and incubated for 1.5 hours. The serum samples were centrifuged before addition at 17,300g for 10 minutes. The plates were washed as above and purified rabbit anti–APO-1 Ig, diluted in culture medium, was added and incubated for 1.5 hours. After three washes, peroxidase-conjugated mouse antirabbit Ig, absorbed against mouse, human, sheep, and goat serum proteins (Dianova, Hamburg, Germany), in PBS/Tween was added for 1.5 hours. Staining was developed after another wash cycle with 75 μL substrate solution (0.1\% orthophenylenediamine in 0.05 mol/L citric acid, 0.1 mol/L Na\(_2\)HPO\(_4\)) for 10 minutes and the reaction was stopped by the addition of 25 μL 1 N H\(_2\)SO\(_4\). The results were read with an ELISA reader (EAR 400AT LAB. Instruments, Grödig, Austria) at 492 nm. For standardization, all assays were controlled with standard sera from healthy donors and with purified recombinant mAPO-1. The amount of recombinant mAPO-1 was determined in a silver-stained 10\% SDS-PAGE by comparison with BSA. The detection limit for recombinant mAPO-1 in the sELISA was 2 ng/mL. Interassay variation was less than 9\% and intraassay variation less than 22\%. Within assay variations, the reproducibility of the sELISA was 100\%.

The validity of the sELISA was confirmed by the following tests. (1) Sera and culture supernatants from SKW6.4 cells were supplemented with increasing amounts of recombinant mAPO-1 similar to those present in positive patient sera and assayed as above. In all cases, mAPO-1 was quantitatively recovered. (2) Only background levels were seen in the sELISA after preabsorption of positive controls with 100 μg/mL of the coating antibody. (3) Precipitation of mAPO-1 in sELISA-containing sera with anti–APO-1–coupled sepharose beads resulted in an 85\% decrease of the signal in the sELISA. The same procedure performed with an isotype-matched control antibody did not affect the sELISA results. (4) Assay readings were not affected by clotting time (range, 1 to 24 hours), the use of plasma (collected with heparin) instead of serum, or by freezing and thawing the sera. Thus, the possibility that mAPO-1 released by leukocytes in response to clotting contributed to any extent to the measured serum levels of mAPO-1 was ruled out. (5) Multiple testing of the same samples always resulted, within assay variations, in the same ELISA readings.

*Immunoprecipitation and Western blotting*. Serum samples were preabsorbed for 12 hours at 4°C by incubation with 15 μg control antibody coupled to protein A sepharose beads. mAPO-1 was subsequently precipitated for 12 hours at 4°C with 15 μg anti–APO-1 (IgG3) coupled to protein A sepharose beads. After washing in a solution containing 0.1\% NP-40, 1 mmol/L vanadate, 1 mmol/L PMSF, 30 mmol/L Tris, pH 7.5, 10 mmol/L NaF, and 150 mmol/L NaCl, beads were resuspended in 60 μL 5× SDS-PAGE sample buffer and denatured for 2 minutes at 95°C. Samples were run on a 12\% SDS-PAGE gel under nonreducing conditions. Proteins were transferred onto nitrocellulose membranes by electroblotting (1.5 hours at 0.8 mA/cm\(^2\)). Subsequently, the membranes were saturated with 5\% nonfat dry milk powder in PBS. After 30 minutes, the blocking solution was removed and biotinylated monoclonal anti–APO-1 (IgG3, 1 μg/mL) diluted in PBS/Tween was added for 1.5 hours. Membranes were washed with PBS/Tween and subsequently incubated for 1 hour with biotinylated streptavidin-horseradish peroxidase (HRPO) conjugate (Amersham, Frankfurt, Germany) diluted in PBS/Tween, followed by another washing step. Protein bands were developed using the enhanced chemiluminescence (ECL) method (Amersham, Frankfurt, Germany) and light emission was detected on Kodak XAR-5 X-ray films (Kodak, Stuttgart, Germany).

*Statistics*. Data sets of the groups studied were not symmetrically distributed. Thus, observed serum concentrations did not show a normal distribution. Because underlying distributions were the same for all groups, the Wilcoxon rank sum test was used for individual comparison of control and experimental groups. Exact P values were computed for all comparisons. For an explicit protection of false rejecting the null hypothesis, the computed P values were compared with a Bonferoni-corrected value of the significance level \(\alpha = .05^{16}\).

**RESULTS**

*Validity of sELISA for detection of APO-1*. To obtain a rapid reliable system for detection of mAPO-1 in culture...
supernatants and human sera, we established a sensitive sELISA. Using purified recombinant mAPO-1, the sensitivity of the sELISA was found to be 2 ng/mL (OD$_{0.02}$ = 0.3 = 30 U/mL; dashed line in Fig 1A). Detection was not affected by using recombinant mAPO-1 in either culture medium or human serum. Even undiluted human serum could be used, indicating that human serum proteins did not interfere with the sELISA. All sELISAs were performed with recombinant mAPO-1 as a standard. Interassay variation was less than 6% and intra-assay variation was less than 22% (Fig 1A). sELISA values reflected the APO-1 content of the samples because immunoprecipitation with an anti-APO-1 antibody resulted in a strong reduction of the sELISA signal by depletion of APO-1 from the sample (Fig 1B). Specificity of the sELISA was further confirmed by the addition of recombinant mAPO-1 to sAPO-1-containing serum or culture supernatants. In all tested samples complete recovery of added purified recombinant mAPO-1 was seen (data not shown). Thus, our sELISA detected APO-1 in a quantitative way in cell culture supernatants and in human sera.

Increased serum levels of sAPO-1 in B- and T-cell leukemias. The first set of experiments was designed to identify sAPO-1 in culture supernatants of various mAPO-1 expressing B- and T-cell leukemia cell lines. Unconcentrated supernatants did not show the presence of sAPO-1. However, in 100-fold concentrated supernatants, sAPO-1 was clearly detected. The concentrated supernatants of the T-cell lines Hut-78 and Jurkat contained 280 and 300 U/mL of sAPO-1, respectively. Similar concentrations were detected in the concentrated supernatant of the B-lymphoblastoid cell line SKW6.4 (320 U/mL).

The finding of sAPO-1 in SKW6.4 cell culture supernatants prompted us to analyze the presence of sAPO-1 in serum from patients with various leukemic diseases. The sAPO-1 was determined by sELISA in sera from patients (n = 143) with B- and T-cell leukemias. These leukemias included progenitor cell-derived T-ALL, cALL, and leukemias of a more advanced maturation stage such as HCL, CLL, and centroblastic and centrocytic B-cell lymphomas (Fig 2). Sera from T-ALL and cALL were obtained from pediatric patients; all other sera were from adult patients. The corresponding control sera were obtained from age-matched children for the pediatric group and from healthy adults, respectively. The sera analyzed were taken at time of diagnosis before antileukemic therapy. Control sera contained negligible quantities of sAPO-1, whereas leukemic sera showed sAPO-1 levels up to 210 U/mL. Patients with cALL, T-ALL, HCL, CLL, and centroblastic and centrocytic B-cell lymphomas showed increased levels of sAPO-1 in the serum. Using recombinant mAPO-1 as a standard, we found approximately 2 ng/mL (30 U/mL) sAPO-1 in the sera of healthy donors. The difference of levels of sAPO-1 in our control sera compared with the control sera in the report by Cheng et al might be attributed to technical differences in the ELISA.

To assess the relation between standard disease parameters and sAPO-1 levels, we compared factors indicating tumor cell burden such as white blood cell count or risk factors as defined by Berlin-Frankfurt-Münster (BFM) leukemia protocols (blast count or extent of hepatosplenomegaly) with sAPO-1 levels in all patients. No correlation was found indicating that sAPO-1 levels are not determined by tumor load but rather by the biology of the tumor.

Comparison of sAPO-1 levels with karyotype and bcl-2
expression did not show any correlations, e.g., T-ALL have low bcl-2 expression, whereas c-ALL showed high bcl-2 expression. Both of these leukemias had elevated levels of sAPO-1. Therefore, sAPO-1 levels seem not to be directly related with standard disease parameters of the leukemias analyzed.

In addition to leukemic disorders of the immune system, we also analyzed a cohort of human immunodeficiency virus (HIV)-infected children for sAPO-1 (n = 367). These sera contained no detectable levels of sAPO-1, despite the fact that sAPO-1 expression on T cells in HIV-1-infected children is greatly increased. This finding shows that the presence of high numbers of mAPO-1–positive cells is not always correlated with elevated sAPO-1 levels.40

To determine whether sAPO-1 in culture supernatants and in human sera represents the complete APO-1 molecule, a proteolytic cleavage, or a splice product, its molecular weight was analyzed. sAPO-1 was purified from the supernatant of SKW6.4 B-lymphoblastoid cells by immunoprecipitation with anti–APO-1–coupled protein A beads. The precipitates were analyzed by SDS-PAGE and Western blotting. Figure 3A shows that sAPO-1 in two sELISA-positive sera appeared as a band of 96 kD on the Western blot (Fig 3A, lanes 1 and 2). Occasional preparations also showed a minor band at 48 to 52 kD (data not shown). No sAPO-1 was detectable in a control serum (Fig 3A, lane 3). sAPO-1 in sera, therefore, showed a tendency to dimerize like sAPO-1 in SKW6.4 cell culture supernatants (Fig 3A), mAPO-1 purified from SKW6.4 membranes,1 and recombinant baculovirus-expressed mAPO-1. In two cases in which CLL patients have been completely resistant to therapy, we observed constantly elevated sAPO-1 levels. In contrast, other patients with normal response to therapy showed variable sAPO-1 levels. Sera obtained from patients 1 to 3 years after successful antileukemic therapy showed normal levels of sAPO-1 (data not shown).

The specificity of our test system to detect sAPO-1 in human sera was further evident from experiments in which purified recombinant mAPO-1 was added to control sera and quantitatively recovered by sELISA (data not shown). In addition, binding of dimerized or multimerized sAPO-1 from human sera in the sELISA (Fig 4, □) was competed by soluble anti–APO-1 antibody (100 µg/mL IgG3), and only
a background sELISA signal was detected (Fig 4, a). Specificity was again shown by the fact that the addition of isotype-matched control antibody did not change the sELISA results significantly (Fig 4, b).

DISCUSSION

We have identified sAPO-1 in culture supernatants of T- and B-cell leukemias using an sELISA. In addition, we compared sAPO-1 levels in normal human sera with sAPO-1 levels in sera from patients with CALL, T-ALL, HCL, CLL, and B-cell lymphomas. Increased sAPO-1 levels were found in sera from all leukemias examined. sAPO-1 from the culture supernatant of SKW6.4 cells and from sera of leukemic patients was further analyzed. The molecular weight of sAPO-1 was found to be similar to that of the purified recombinant baculovirus-expressed mAPO-1 and to that of mAPO-1 purified from the lymphoblastoid B-cell line SKW6.4.1 A common feature of all APO-1 preparations was the tendency to form dimers and multimers. This phenomenon is also known for other soluble receptors, eg, sCD58.42

Evidence is accumulating that many cell surface receptors also occur as soluble receptors. Soluble receptors for the following cytokines have been identified: interleukin-1β (IL-1β),43 IL-2,44 IL-4,45 IL-6,46 IL-7,47 and interferon-γ.48 In addition, soluble receptors have been described for members of the TNF/NGF receptor family. Three different types of soluble NGF receptors identified by molecular weight have been described. Recent experiments suggested various mechanisms for the generation of soluble receptors. One mechanism, as shown for the NGF receptor and the TNF receptor type II, is cleavage from the cell surface by proteolysis.49,50 Likewise, a 32-kD sCD27 molecule was shown to be cleaved from its 55-kD membrane-bound counterpart. Loenen et al49 showed that the sCD27 and the mCD27 were derived from the same mRNA. Furthermore, both soluble and membrane forms of the TNF receptor type I were described. They were probably derived from the same transcript and are indistinguishable by molecular weight and hydrophobicity.29,52

A soluble form of APO-1 was recently described that is generated by differential splicing, yielding a secreted molecule lacking the transmembrane region.53 This deletion of 19 amino acids would not be resolved on SDS-PAGE (Fig 3). We found a similar molecular weight of sAPO-1 and mAPO-1 in Western blot analysis. Culture of SKW6.4 in the presence of protease inhibitors did not result in a change of the amount of sAPO-1 detected (data not shown). Therefore, our data are consistent with the finding that sAPO-1 is a splice variant of mAPO-1 rather than a product of proteolytic cleavage.
The amount of sAPO-1 detected in culture supernatants of various cell lines was about 3 U/mL. However, in sera of leukemic patients, levels of up to 210 U/mL sAPO-1 were observed. The variations seen in serum samples from individual patients within the same leukemia group correspond to variations seen with other soluble receptors such as sTNF receptors in HCL. All cell lines examined showed strong mAPO-1 expression. In contrast, mAPO-1 expression of leukemic cells from patients with B-lineage leukemias/lymphomas (CALL, CLL, and HCL) was low or negative. Only cells from patients with T-ALL were strongly APO-1 positive. Thus, sAPO-1 in serum of leukemic patients may not always be derived from the leukemic cells and serum levels may not always be correlated with mAPO-1 expression on the leukemic cells. Another putative source of sAPO-1 may be T and B cells, reflecting the activation status of these cells. However, because sAPO-1 was constantly high in patients relatively resistant to antileukemic therapy, sAPO-1 detection in sera could serve as a putative marker for an active persisting leukemia. This hypothesis is supported by the finding that, after successful therapy, sAPO-1 levels returned to normal values. In addition, sAPO-1 levels are not elevated in sera from a cohort of HIV-infected children (n = 367), in which mAPO-1 expression is greatly increased on the patients' T cells. Because lymphocyte depletion by apoptosis is observed in acquired immunodeficiency syndrome (AIDS) and may involve the APO-1 system, this observation also supports the concept that sAPO-1 may prevent APO-1-mediated lymphocyte death.

Tumor cells may have a greater(1,9),(992,988)
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