Presence of serum carbonic anhydrase autoantibodies in patients relapsed after autologous stem cell transplantation indicates an improved prognosis

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Received April 10, 2008

Here we report patients with Hodgkin's disease and multiple myeloma, who relapsed/progressed after high dose therapy and autologous stem cell transplantation. In patients who developed aplastic anemia type syndrome, spontaneous tumor regression was observed and concomitantly high titers of serum autoantibodies were found. In order to identify the antibody specificity, two-dimensional electrophoresis, blotting and immunoreactions were used to analyze the peripheral blood stem cell extract with autoantibodies containing serum. The unique protein spot visualized exclusively by serum of patients with aplastic anemia type syndrome was identified as carbonic anhydrase I (CA I, accession No. P00915 and Q7M316) by means of mass spectrometry. The specificity of autoantibodies was confirmed by reaction with commercial CAs I, II, IX and XII. Immunoreaction in Western blots with these CA isoforms differed in sera obtained from patients with various types of the disease. Sera of Hodgkin's disease patients reacted with CA I, II and XII; sera of multiple myeloma patients reacted with the CA I, II, XII and IX. Patients developing and/or possessing CA autoantibodies had a significant survival benefit over those who did not develop CA anhydrase autoantibodies. Possible relevance of the presence of CA autoantibodies and clinical outcome is discussed.

Key words: stem cell transplantation, spontaneous remission, serum autoantibodies, carbonic anhydrases.

High dose therapy (HDT) with autologous stem cell transplantation (SCT) has become a routine method for the treatment of chemosensitive malignant diseases [1, 2]. The relapse/progression of the disease after SCT remains the major concern of this treatment. During 15 years of HDT and autologous SCT at the National Cancer Institute we have observed patients who relapsed/progressed shortly after SCT. Some of these patients developed an aplastic anemia like syndrome concomitant with the relapse/progression of the disease. In each instance spontaneous remission of the disease was observed in these patients [3]. The possible antitumor effect of aplastic anemia in myelodysplasia has been previously described by Nissen and Schubert [4]. It is well known that autoimmunity may be a potential benefit in treatment of some tumors. The induced autoimmunity by high dose interferon alpha treatment proved to be a good prognostic factor in melanoma patients [5]. Both inherited and acquired autoimmunity were beneficial with metastatic melanoma [6], during interferon therapy in hematological patients [7] and with metastatic renal cell cancer [8].

Blood serum analysis revealed that autoantibodies developed in several patients against proteins derived from cells collected and used for stem cell transplantation. Here we report the presence of high titers of serum autoantibodies directed against CAs in some patients treated with HDT and autologous SCT for progressive Hodgkin's disease, multiple myeloma and some other malignant diseases. The patients developing and/or possessing CA autoantibodies had a significant survival benefit over those who did not develop these autoantibodies.

Patients and methods

Patients. The patients who were eligible for HDT and autologous SCT were treated according to standard European Bone Marrow Transplantation group protocols employed at the National Cancer Institute. Written informed consent was

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obtained from all patients according to the institutional guidelines. After separation the collected peripheral blood stem cells (PBSC) were cryopreserved in 2.2% dimethyl sulphoxide [9] and stored in liquid nitrogen vapor.

One- and two-dimensional gel electrophoresis and Western blot analysis. PBSC were thawed and lysed in a solution containing 8% SDS, 20 mM TRIS-Cl pH 6.8, 40% glycerol and 10mM EDTA. The proteins were separated by SDS PAGE in 12.5% polyacrylamide slab gels. For two-dimensional electrophoresis, one volume of stem cells was lysed with four volumes of solution containing 9.6 M urea, 5% CHAPS, 25 mM spermine and 50 mM DTT for 60 min. at room temperature [10]. The solution was centrifuged for 250 000xg at 20° C for 60 min. After extraction, carrier ampholytes (pH 3-10) were added to bring the final concentration to 0.4%. The proteins were separated by two-dimensional electrophoresis as previously described [11] except 3 % CHAPS was used instead of NP- 40. After 1-D or 2-D gel electrophoresis, the proteins were transferred to nitrocellulose membranes (Schleicher Schuell). The quality of protein transfer was controlled by staining with 0.1 % Ponceau S (Sigma) in 5% acetic acid. The membranes were blocked with 1% Blot Quantified BSA (Promega) and then incubated overnight with diluted (500 times) human sera. After washing, the blots were incubated with Antihuman IgG (H&L) AP conjugate (Promega) at a 5000-fold dilution and the signal was developed with BCIP-NBT reagent (Promega).

In-gel digestion. The selected spot was excised from Coomasie-stained gels and destained with 50 mM Tris/HCl (pH 8.5) in 50% (v/v) acetonitrile. The gel piece was shrunk by dehydration in acetonitrile. The solvent was then discarded and a volume of 10 mM dithiotreitol (DTT) in 100 mM ammonium hydrogen carbonate sufficient to cover the gel pieces was added to reduce the protein for 1 hour at 56° C. The DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM ammonium hydrogen carbonate at room temperature and incubated for 45 min. at ambient temperature in the dark. The gel piece was washed with 50-100 μl of equilibration buffer (50 mM ammonium bicarbonate, pH 7.8, in 5% acetonitrile) for 10 min., dehydrated with acetonitrile, rehydrated in equilibration buffer and dehydrated again in the same volume of acetonitrile. The liquid phase was removed and the gel piece was subsequently dried in a lyophilizer. The gel piece was swollen at 4° C in digestion buffer containing 0.1 μg of sequencing grade trypsin (Promega, Madison, WI) and 4.5 μl of equilibration buffer. After 20 min., 15 μl of equilibration buffer was added. The tube with the gel piece was mildly shaken overnight at 37° C. Enzymatic cleavage was terminated by adding 20 μl of 5% formic acid, vortexed and incubated at room temperature for 10 min. Supernatant was recovered after a brief spin. Peptides were extracted from the gel matrix by adding 30-40 μl of 100% acetonitrile, vortexed and incubated for 20 min. at room temperature. The peptide mixture was then spun down and the supernatant recovered. The peptide extraction was repeated twice. Pooled supernatants were concentrated in lyophilizator and subjected to mass spectrometric analysis.

Mass spectrometric analysis. The extracted peptide mixture was separated on nanoAcquity UPLC system (Waters, USA) using precolumn concentration (Atlantis C18, 180 μm x 20 mm, 5 μm nanoAcquity trap column, Waters, USA) and gradient elution on analytical nanoAcquity UPLC column BEH 130 C18 (100 μm x 100 mm, 1.7 μm particle size) at flow rate 1 μl/min. The gradient of buffer B (100% Acetonitrile, 0.1% Formic Acid) consisted of a linear increase from 3% to 50% in 15 min. The column was connected to the PicoTip emitters (New Objective, USA) mounted into the nanospray of the Qtof Premier (Waters, UK). The data acquisition was performed in a data dependent manner for the time of the separation, collecting up to 3 MSMS events at the same time and a maximum of two seconds for each precursor. Data were processed by ProteinLynx Global Server v. 2.2 (Waters) that provided background subtraction (polynomial order 5 and threshold 35%), smoothing (Savitzky Golay, twice, over three channels) and centroiding (top, 80%, minimal peak width at half height 4). Resulting data were searched against human (Uniprot_Sprot_human, Swiss Institute of Bioinformatics) and all species (Uniprot_Sprot, Swiss Institute of Bioinformatics) databases with the following criteria: fixed carbamidomethylation of Cys, variable Met oxidation, tryptic fragments with 1 misscleavage, peptide mass tolerance 100 ppm, fragment mass tolerance 0.1 Da. The results were validated by identification of three or more consecutive fragment ions from the same series.

Carbonic anhydrases. Human carbonic anhydrase I and II were purchased from Sigma. Human carbonic anhydrase IX was a personal gift from Dr. S. Pastorekova (Institute of Virology, Slovak Academy of Sciences, Bratislava) and human carbonic anhydrase XII was purchased from R&D Systems.

Results

Previously we reported on patients who relapsed and/or progressed after autologous stem cell transplantation (SCT) and then spontaneously achieved complete remission [3]. Their blood counts resemble an aplastic anemia type syndrome. In order to identify any marker(s) in the serum of these patients after autologous SCT, we prepared cell extracts from their autologous peripheral blood stem cells (the back up bag). The extracted proteins were resolved by SDS PAGE, transferred to nitrocellulose membrane and allowed to react with the particular serum obtained from each patient. A protein band of about 30 kDa was detected in these Western blots (Fig. 1A, lanes 1, 5).

A relationship between presence of the antibodies against the 30 kDa protein band and the status of the disease has been observed. In patients who progressed/relapsed very quickly after the SCT (progression group), the presence of antibodies was not observed (Fig. 1A, lanes 2, 3, 4, 6). More-

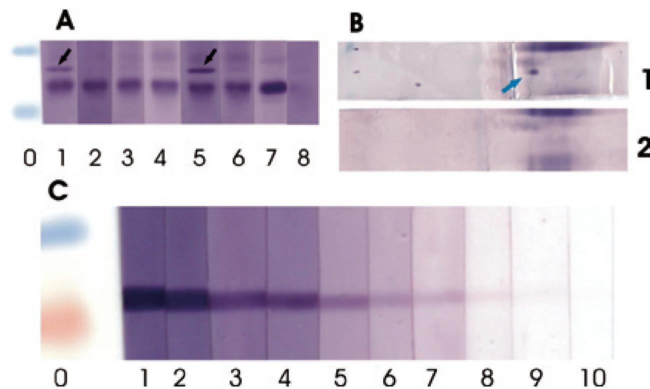

Figure 1. A – Western blots of PBSC extracts with various patients' sera. Lane 0 – molecular weight standards (33 and 24 kDa); lanes 1, 2, 3, 4 – Hodgkin's disease patients; lanes 5, 6 – multiple myeloma patients; lane 7 – healthy person; lane 8 – anti-human IgG only. Patients' sera were diluted 500 times. Arrows indicate 30 kDa band in patients' sera with positive autoantibodies. B – Western blots of two-dimensional electrophoresis of PBSC extract with positive serum autoantibodies (#1) and negative serum autoantibodies (#2). Arrow indicates the protein spot used for protein identification by mass spectroscopy. C – Titration of positive serum with commercial human CA I on Western blots. Lane 0 – molecular weight standards (36 and 28 kDa); lane 1 – 100 fold dilution; 2– 200 fold dilution; 3 – 400 fold dilution; 4 – 800 fold dilution; 5 – 1600 fold dilution; 6 – 3200 fold dilution; 7 – 6400 fold dilution; 8 – 12800 fold dilution; 9 – 25600 fold dilution; 10 – 51200 fold dilution.

over, the blood counts of the antibody-positive patients (regression group) corresponded to the previously described aplastic anemia type syndrome. Sera of healthy persons did not react with peripheral blood stem cell extract (Fig. 1A, lane 7).

In order to better characterize a protein that reacted with the antibodies present in sera of regression group patients, the selected peripheral blood stem cell extract was separated in two-dimensional electrophoresis, blotted and allowed to react with the patient's serum. Two equivalent blots developed when sera from the patients in the progression group were compared with sera from the regression group. A unique protein spot with approximate molecular weight of 30 kDa and pI value of 6.8 -7.0 was noticed by the sera of patients from the regression group only (Fig. 1B). The protein spot was cut out from the preparative gel, decolorized and digested by trypsin. Generated peptides were analyzed by tandem mass spectrometry in a data dependent manner. Carbonic anhydrase I (CA I, accession No. P00915 or Q7M316) was unambiguously identified based on fragment ions (MS/MS) spectra. As many as 12 CA I peptides were recognized. Their characteristics are shown in Table I. Sequence coverage (Fig. 2) was 61% and the probability score was 99.9%. A database search for possible posttranslational modifications of identified peptide sequences revealed prediction of possibly three O-glycosylation (NetOGlyc 3.1 Server, Technical University of Denmark), one N-glycosylation (NetNGlyc 1.0 Server, Technical University of Denmark) and 19 phosphorylation (NetPhos 2.0 Server, Technical University of Denmark) sites. The peptides identified by MS/MS in the current study did not show any of these posttranslational modifications.

The findings were confirmed by Western blots using the commercial human CA I (Sigma). The titer of antibodies presented in sera assayed with 40 ng of pure human CA I was high. The band was visualized up to 25 600-fold dilution of the serum (Fig. 1C).

To see whether the antibody would react with various isoenzymes of CA we separated CAs: I, II, IX and XII by electrophoresis. The sera obtained from regression group patients with various types of the diseases reacted diversely in Western blots with these CAs. Sera from patients with Hodgkin's disease reacted with CA I, II and XII. Sera from patients with multiple myeloma reacted with CA I, II, XII and CA IX as well. The sera of the patients from the progression group did not react with any tested CA isoenzymes.

Out of 16 patients with Hodgkin's disease treated in the last two years at the National Cancer Institute who relapsed/

Table 1. Some characteristics of the identified peptides produced by tryptic digestion of the gel spot containing carbonic anhydrase Type I

Submitted Mass	Submitted	Experimental Charge	Theoretical Mass	Delta Mass	Sequence (ppm)	Start	End	Modifications
473.2249	2	944.4342	944.4352	1.034	(K)NGPEQWSK(L)	11	18	None
871.9562	2	1741.8967	1741.8999	1.8221	(K)LYPIANGNNQSPVDIK(T)	19	34	None
619.569	4	2474.2446	2474.2651	8.2885	(K)TSETKHDTSLKPISVSYNPATAK(E)	35	57	None
643.6735	3	1927.9971	1928.0004	1.7095	(K)HDTSLKPISVSYNPATAK(E)	40	57	None
752.6931	3	2255.0559	2255.0354	-9.0941	(K)EIINVGHSFHVNFEDNDNR(S)	58	76	None
493.2251	2	984.4346	984.4301	-4.526	(K)GGPFSDSYR(L)	81	89	None
538.265	3	1611.7716	1611.7794	4.8472	(K)YSAELHVAHWNSAK(Y)	114	127	None
513.7574	2	1025.4991	1025.5029	3.6901	(K)YSSLAEAASK(A)	128	137	None
593.8484	2	1185.6812	1185.6792	-1.6473	(K)ADGLAVIGVLMK(V)	138	149	None
485.7975	2	969.5793	969.5859	6.7357	(K)VLDALQAIK(T)	160	168	None
790.9104	2	1579.8052	1579.7842	-13.2903	(K)ESISVSSEQLAQFR(S)	214	227	None
690.5986	4	2758.363	2758.3821	6.9037	(R)SLLSNVEGDNAVPMQHNNRPTQPLK(G)	228	252	None

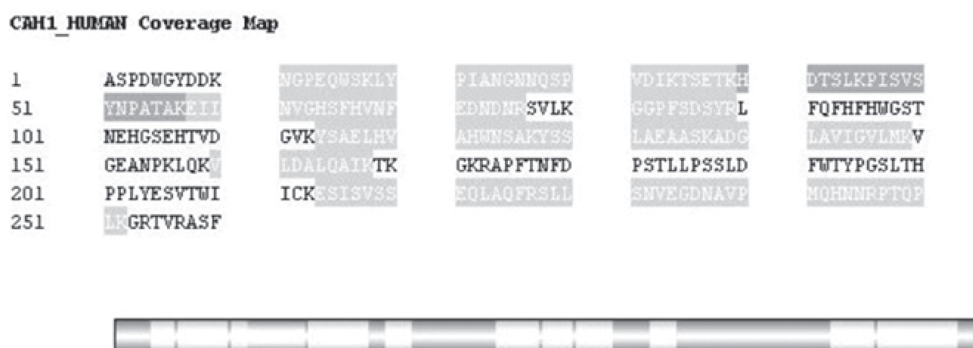

Figure 2. Protein coverage map of human carbonic anhydrase type I with peptide matched (highlighted) demonstrating sequence coverage of 61%.

progressed after HDT and autologous SCT, five patients with autoantibodies present have been in complete remission without additional treatment. The other 11 patients had no autoimmune response. Seven have died and four are rapidly deteriorating despite salvage therapy. We observed similar results in patients with multiple myeloma who relapsed/progressed after autologous HDT and SCT. Two patients with antibodies against CAs have been in complete remission without treatment. The other four patients without the antibodies died after disease progression despite salvage therapy.

We were interested to know whether regression group patients treated more than five years ago [3] possess the anti CA autoantibodies. In all surviving patients living in complete remission; Hodgkin's disease (CA I, II, XII), non-Hodgkin's lymphoma (CA I, II, IX, XII), Ewing's sarcoma (CA I, II, IX, XII), the anti CA autoantibodies were detected.

Discussion

The pathogenic effect of serum antibodies against CAs has been reported in patients with several autoimmune pathogenic diseases like autoimmune pancreatitis [12], chronic viral hepatitis [13] or Sjđrgren's syndrome [14]. On the other hand, spontaneous autoimmune disorders have been associated with a better prognosis in patients with melanoma [6, 15]. Recently, autoimmunity was proven to be a prognostic factor in melanoma patients treated with adjuvant low-dose interferon alpha [5]. CA II was found to be highly expressed in glial tumors where it represents a major target antigen stimulating the autoantibodies [16].

Our study showed that the presence of serum autoantibodies directed against CAs in patients treated with high dose therapy and autologous stem cell transplantation is connected with better outcome. This finding agrees well with better prognosis of those melanoma patients who developed autoimmunity during treatment with interferon alpha [5]. We cannot exclude that we deal with a naturally occurring antibody to CA, which was highly upregulated in those patients entering remission. Naturally occurring carbonic anhydrase antibodies have been found to be upregulated in certain tumor patiens [17] and the occurrence of IgM antibodies was also observed in mice [18].

It is interesting to mention that the disease progressed in Hodgkin's disease patient who did not develop autoantibodies shortly after autologous stem cell transplantation. However, when the autoantibody was induced, the disease regressed. When the disease started to progress again, the antibody level decreased to an undetectable level and the blood counts normalized. High expression of various isoenzymes of CAs seems to be quite frequent in various types of malignant tumors [19, 20, 21, 22, 23].

It may be possible that a high level of CA antibodies keep the tumor cells in a dormant state. If we take into account that after HDT only cancer stem cells can survive [24], it could be speculated that the CA antibodies are either cytostatic to cancer stem cells or are creating a microenviroment that does not allow the asymetric cell division and further proliferation of the truncated progenitor cells. The obtained data also suggest a potential use of CA antibodies for tumor treatment [25, 26, 27]. The presence of upregulated carbonic anhydrase is a prognostic marker for renal clear cells carcinoma [28]. Indeed, the overexpression of membrane-bound CA IX in several types of cancer seems to be an attractive target for therapy [29].

Authors thank Dr. S. Pastorekova for gift of CA IX and to Susan Hellenbrand and Dan Bruneau for language revision. The work was partially supported by the Foundation for Transplantation (to JL) and SPP Foundation (to CA).

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