In Vitro Response of Stimulated B-CLL Lymphocytes of Patients Treated with *Viscum album* L. Extracts

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Abstract. *Background: Extracts from Viscum album (VA-E) have been shown to induce apoptosis and immunoactivation. To exclude possible B-CLL propagating effects, the in vitro reactions of cultured peripheral blood B-CLL cells were analysed. Patients and Methods: Intracellular expression of apoptosis-associated mitochondrial Apo2.7 and proliferation-associated Ki-67 molecules in B-CLL cells from patients treated with VA-E for 12 months were measured after incubation with various stimuli. Results: Within the observation period, the susceptibility of the B-CLL cells towards the apoptosis-inducing potential of the VA-E significantly decreased. This effect could be due to the presence of physiologically-induced anti-mistletoe lectin antibodies which may block the effects of cytotoxic mistletoe lectins. No significant induction of Ki-67 was observed, but an increase of non-specific binding, even in untreated medium controls, did occur within the last months. Conclusion: In this in vitro setting of the observational study, no stimulation of leukemic cells from the patients treated with VA-E was profound.*

Malignant B lymphocytes in chronic lymphatic leukaemia (CLL) are small resting, long living cells which accumulate in the peripheral blood and bone marrow (1). These cells are not 'frozen' at an early stage of differentiation, because it has been accumulated that they are able to differentiate (1). A variable pattern in the response to different cytokines such as interleukin (IL)-2, IL-4, interferon-alpha and interferon-gamma has been reported in B-CLL (1); this may be indicative of discrete stages of maturation and activation in B-CLL patients. Among these cytokines, IL-2 appears to be the most consistent activator, whereas IL-4 down-regulates CLL B lymphocytes, in contrast to its effect on normal B cells. Tumour necrosis factor (TNF)-alpha acted as a growth stimulatory factor on leukaemic B lymphocytes from many patients (2-5). Because TNF induces production of IL-6, which has been shown to be a growth factor for myeloma and other transformed B cells (5), Aderka *et al.* (2) examined the possibility that IL-6 mediates the growth-stimulatory effect of TNF on B-CLL cells, and found that IL-6 is an inhibitor of B-CLL growth (2). Reittie *et al.* (5) reported that IL-6 inhibits apoptosis and TNF induced proliferation of B-CLL. In fact, recent findings indicate that endothelial cells produce IL-6 dimers which inhibit apoptosis of B-chronic lymphocytic leukemia cells (6), and may thus promote their survival in B-CLL patients.

Apart from conventional treatments, patients with B cell lymphoma and B-CLL are treated in some cases additionally with extracts from *Viscum album* L. (VA-E) (7-10). Unfortunately, there are only a few investigations concerning the efficacy or safety of this treatment in lymphoma/leukaemia patients. Criticism arose from early findings that components from *Viscum album*, *i.e.* the cytotoxic mistletoe lectins (ML) (11, 12) and polysaccharides (13), may induce pro-inflammatory TNF-alpha, IL-1 and IL-6 which could promote the growth of B-CLL/lymphoma. This speculation was substantiated by Hagenah *et al.* (14), who observed subcutaneous nodes of centrocytic non-Hodgkin's lymphoma at the exact same sites of previous regular injections of a mistletoe preparation. However, the patient had multiple courses of ineffective chemotherapy due to a progression of the lymphoma (even prior to the onset of VA-E application), and thus a depletion of T lymphocytes and granulocytes. The residual population of immune cells were the centrocytic non-Hodgkin's lymphoma cells, which were found at the injection sites. The authors concluded that high concentrations of mistletoe preparations subcutaneously injected can have a growth-promoting action on cells of a centrocytic lymphoma, and suggested that this proliferative stimulus may have been mediated by a high local concentration

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of IL-6 liberated from the skin by mistletoe lectins (14). In contradiction, Kuehn concluded that "due to the final leukaemic flushing out NHL-infiltrations would have been detected on additional body areas except the mistletoe injection sites if a biopsy had been done" (8). In a retrospective study by Stumpf and co-workers enrolling more than 200 patients with malignant haematological and lymphatic diseases, no indication of risk of a mistletoe therapy on progress of the disease and the survival time was found (10).

Although VA-E were recognized to induce both apoptosis (15-22) and indirect immunoactivation (23, 24), it is of outstanding importance to exclude possible B-CLL propagating effects. In a previous study, we investigated leukaemic B cells from patients with B-CLL after *in vitro* incubation with several immunomodulators. In response to VA-E, the cells released IL-6 but underwent apoptosis, while a proliferation response was recognised only in response to mitogenic stimulation and not to the VA-E or IL-6 (17). The clinical situation is much more complex, and thus we investigated the *in vitro* responses of B-CLL cells from patients treated subcutaneously with VA-E for 12 months towards the applied VA-E, IL-6 and mitogens such as pokeweed mitogen and phytohaemagglutinin, *i.e.* expression of apoptosis-associated mitochondrial Apo2.7 molecules and intracellular expression of proliferation-associated Ki-67 molecules.

Patients and Methods

Patients. Patients with chronic B-cell leukaemia were recruited and treated in various medical wards (Table I). All patients were informed about the purpose of the study and gave informed consent to participate. The study was registered according the German law as an observational study. The primary aim of the study was the susceptibility of leukaemic B lymphocytes to VA-E induced apoptosis and proliferation response tests towards the *in vivo* applied VA-E. Within the 12 months' observation period, the study was not designed as a confirmatory study to prove efficacy, but as an explorative study to judge safety. Therefore, there was no control group without mistletoe extract application and the B-CLL cells responses were measured *in vitro*. The respective values of visit 1 were used as a reference for all further visits (visits 2-6).

Ten patients (Table I) were enrolled (mean age of 66.6±10.7 years), and contacted the medical wards within the observation period at least 4 times. According to the cytological staging systems, 7 patients had a low staging (Binet A), 2 had Binet B and one Binet C; according to the Rai classification, 4 patients were in the low-risk category (Rai 0), 5 in the intermediate-risk category (Rai I-II) and one in the high-risk category (Rai IV). All further details are given in Table I.

All patients were treated subcutaneously with VA-E at least three times per week up to daily in the morning: 7 patients with the commercially available whole plant extract HELIXOR® P (HP), produced from mistletoe grown on pine trees with a relatively high ML content (17), and 3 patients with HELIXOR® A (HA), an extract from mistletoe grown on fir trees with a low ML content (17). Among these 3 patients, two changed later to HELIXOR® P. For the medical doctors, no restrictions were imposed as to the applied VA-E concentrations, and thus the maximally applied concentrations differed (Table I).

Cells and reagents. Ficoll-isolated lymphocytes ($1.5x10^6$ cells/ml) from patients with leukaemic B-cells were incubated for 96 h in RPMI FG 1640 medium (Biochrom, Berlin, Germany) with 10% autologous plasma in a humidified 5% CO_2 atmosphere at 37˚C as described elsewhere (17). The aqueous mistletoe extracts HELIXOR® P and HELIXOR® A were kindly provided by HELIXOR Heilmittel GmbH & Co. KG, Rosenfeld (Germany) and added at final concentrations of 10, 100 and 1000 μg/ml for 96 h. Both, pokeweed mitogen (PWM; Sigma, Deisenhofen, Germany), which stimulates T-cell dependent B-cell proliferation (25, 26), and phytohaemagglutinin (PHA; Sigma), which stimulates mainly T-cells, were added at final concentrations of 2.5 μg/ml as independent positive controls for 96 h. IL-6 at concentrations of more than 100 U/ml was described to increase spontaneous DNA synthesis of B-CLL cells (5); IL-6 (Boehringer Mannheim, Germany) was added for 96 h as an additional control at a final concentration of 1000 U/ml as reported previously (17). Sixty-nine hours incubated RPMI FG 1640 medium served as a negative control for all cell cultures.

Flow cytometric analysis of intracellular marker. Flow cytometric analysis was performed on an EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany) according to the routine procedures. To detect intracytoplasmic proteins, cultured cells were permeabilised at 4˚C with digitonin as described elsewhere (17, 18). The mitochondrial membrane protein Apo2.7 (7A6 antigen), a 38 kDa protein localised on cells undergoing apoptosis (18, 20, 21, 26, 27), was measured by flow cytometry using the PE-labelled monoclonal antibody Apo2.7 (Coulter-Immunotech, Krefeld, Germany) as indicated. Ki-67 is a proliferation-associated marker expressed in the late G_1-, S-, M- and G_2-phase of the cell cycle, but not in resting (G_0) cells (28, 29), and was also measured intracellularly.

Statistics. Data were presented as mean±SD. Comparison of the *in vitro* results was performed by a nonparametric two-sample Wilcoxon signed rank test using WinStat Version 3.0 (Kalmia Co. Inc., CA, USA). A value of $p<0.05$ was considered to indicate significant differences between groups.

Results

The *in vitro* properties of cultured peripheral blood B-CLL cells from the patients treated *in vivo* with VA-E were analysed. Incubation of these cells with the VA-E resulted in a dose-dependent increase of apoptosis, with HP (which has a higher content of cytotoxic ML) being the most potent (Table II). Within the observation period, the susceptibility of the cells towards the apoptosis-inducing potential of the VA-E significantly decreased (Table II), *i.e.* visits 4-6. IL-6 did not induce apoptosis, but reduced the level of spontaneous apoptosis of cultured cells (Table II); the proportion of apoptotic cells did not significantly change during the time course. A similar effect was observed with the mitogens PHA and PWM which served as a positive control for proliferation (Table II).

Table I. *Patient data.*

Patient	Age (years)	Gender	Recruiting medical ward (city)	B-CLL diagnosed in	Stage	Previous treatment	Subcutaneously applied mistletoe extract	Maximal applied VA-E concentration within the observation period	Clinical course of disease within the observational period
01/ KS	44	F	Denkingen	06/2001	Binet: A Rai: I		HELIXOR® P (until 04/2004), Additionally Cefalektin®	10 mg	No objective clinical aggravation
03/ RW	69	M	Kreuzwertheim	12/1998	Binet: A Rai: I	None	HELIXOR® P	40 mg	No objective clinical aggravation
08/ HW	65	M	Gevelsberg	07/2002	Binet: A Rai: 0	None	HELIXOR® A -> HELIXOR® P	5 mg	No objective clinical aggravation
10/ HG	72	M	Denkingen	10/2002	Binet: A Rai: 0		HELIXOR® P	5 mg	No objective clinical aggravation
11/ KH	68	M	Bad Neuenahr	12/2002	Binet: B Rai: I	None	HELIXOR® P	10 mg	Increase of lymphocyte counts in 2004, no objective clinical aggravation
12/ KA	72	F	Aurich	01/2003	Binet: A Rai: 0	None	HELIXOR® A	10 mg	No objective clinical aggravation
15/ BW	59	M	Gevelsberg	03/1997	Binet: C Rai: IV	None	HELIXOR® P	5 mg	No objective clinical aggravation
16/ EK	75	M	Gevelsberg	05/2003	Binet: A Rai: 0	None	HELIXOR® P	5 mg	No objective clinical aggravation
17/ DJ	59	F	Schönebeck	05/2003	Binet: B Rai: II	None	HELIXOR® A (for 3 months) -> HELIXOR® P	150 mg	Increase of lymphocyte counts in 2004, no objective clinical aggravation
19/ LK	83	F	Gevelsberg	11/2003	Binet: A Rai: Ia		HELIXOR® P	5 mg	No objective clinical aggravation

The number of proliferating cells as measured by the intracellular expression of the proliferation-associated marker Ki-67 was analysed. First of all, the cells did not proliferate *per se* in the cell culture (Table III); a proliferation response was induced only by PHA and PWM, but not by IL-6. Within the observation period, some strong variances were found, reflecting individually higher Ki-67 expressions; in most cases these variances were due to an increase of non-specific binding of the added monoclonal Ki-67 and Apo2.7 antibodies, respectively (Table IV). At visits 4 and 6, in all cell cultures incubated with the VA-E, an increase of Ki-67 expression was observed (Table III), which was significantly higher only at the extremely high concentration of HP 1000 μg/ml. However, this increase of Ki-67 (and even Ki-67/Apo 2.7 double positive cells) binding was also observed in untreated medium controls, and thus is not an induced *in vitro* proliferation, but a consequence of other effects. It is apparent that this increase of non-specific binding coincidences with the decrease of mitochondrial Apo2.7 expression (Table II), and thus could be explained by the induction of anti-ML antibodies which block the ML cytotoxicity *in vivo* (30-32).

Due to the progressive nature of the disease, the mean values of peripheral CD5+ CD19+ B lymphocytes from the patients increased within the observation period from 18,205±12,836 cells/μl at visit 1, to 20,214±14,202 cells/μl at visit 2, 21,197±16,936 cells/μl at visit 3, 22,993±20,021 cells/μl at visit 4, 25,257±23,017 cells/μl at visit 5 and 29,042±28,490 cells/μl at visit 6. This increase was not statistically significant ($p=0.09$; Wilcoxon).

Discussion

To more clearly define the effects of VA-E on B-CLL cells, we incubated peripheral lymphocytes from patients with B-CLL treated *in vivo* with VA-E and incubated these cells in the presence of these VA-E, IL-6 and two mitogens which served as a positive control. In general,

Table II. *Intracellular expression of mitochondrial Apo2.7 molecules (% of medium control) of cultured B-CLL cells.*

	Visit 1 Screening	Visit 2 1 month later	Visit 3 3 months later	Visit 4 6 months later	Visit 5 9 months later	Visit 6 12 months later
Number of patients per visit	10	9	9	10	7	7 #
HA 10 μg/ml	96.8±14.9	93.4±16.5	99.3±11.1	103.2±9.7	106.2±7.2	108.7±17.9
HP 10 μg/ml	140.6±59.9	101.3±14.8	123.2±38.1	101.0±14.6*	108.2±9.1	102.3±27.5*
HA 100 μg/ml	167.0±102.0	106.3±23.3*	133.0±47.0	105.3±11.7*	107.2±9.5*	91.2±26.3*
HP 100 μg/ml	249.2±119.6	156.5±64.1	160.7±117.0	106.5±14.0**	122.8±24.3*	95.2±31.4*
HA 1000 μg/ml	329.4±409.4	195.5±93.6	207.3±116.4	160.3±98.5*	125.9±50.0	143.1±116.4
HP 1000 μg/ml	325.7±201.0	275.3±183.3	280.8±136.6	200.0±70.6	187.4±105.6	138.8±100.5
IL-6 (1000 U/ml)	86.5±19.0	69.7±29.2	81.2±22.5	79.2±11.7	91.4±15.4	74.4±14.4
PHA (2.5 μg/ml)	68.4±27.8	66.1±25.4	106.9±48.9	97.8±78.4	66.2±23.9	65.2±32.7
PWM (2.5 μg/ml)	67.3±36.5	64.9±24.5	73.5±31.5	62.4±31.2	51.2±25.8	44.9±28.4

Results are means±standard deviation and are significantly different from the screening value (visit 1) at *$p<0.05$, **$p<0.01$ (Wilcoxon). # Two patients with missing data at visit 6 contacted the tumor out-patient clinic later at visit 7 but were not included in the statisticall analysis.

Table III. *Intracellular Ki-67 expression (%) of cultured B-CLL cells.*

	Visit 1 Screening	Visit 2 1 month later	Visit 3 3 months later	Visit 4 6 months later	Visit 5 9 months later	Visit 6 12 months later
Number of patients per visit	10	9	9	10	7	7 #
Medium	0.47±0.41	0.77±0.71	0.41±0.28	1.48±1.49	0.73±0.45	1.10±0.62
HA 10 μg/ml	0.44±0.35	0.59±0.45	0.48±0.45	1.24±1.20	0.73±0.43	1.03±0.64
HP 10 μg/ml	0.49±0.44	0.67±0.68	0.44±0.40	1.11±1.08	0.93±0.71	1.16±0.91
HA 100 μg/ml	0.51±0.48	0.52±0.42	0.43±0.34	1.46±1.79	0.84±0.63	1.09±0.63
HP 100 μg/ml	0.51±0.58	0.86±1.09	0.39±0.25	1.18±1.30	0.69±0.47	1.36±0.97
HA 1000 μg/ml	1.05±1.80	0.63±0.56	0.52±0.41	2.30±3.87	2.47±2.19	1.54±1.21
HP 1000 μg/ml	0.42±0.53	1.03±1.23	0.41±0.29	2.32±3.85	0.81±0.62	1.16±0.86*
IL-6 (1000 U/ml)	0.47±0.46	1.03±1.08	0.39±0.39	1.10±1.04	0.60±0.49	0.70±0.47
PHA (2.5 μg/ml)	12.2±16.1	12.9±14.6	11.5±13.7	13.3±17.5	11.7±20.2	4.84±4.96
PWM (2.5 g/ml)	4.45±4.94	4.50±4.88	3.51±3.09	4.87±5.04	4.69±8.68	1.53±0.92*

Results are means±standard deviation and are significantly different from the screening value (visit 1) at *$p<0.05$, **$p<0.01$ (Wilcoxon). # Two patients with missing data at visit 6 contacted the tumor out-patient clinic later at visit 7 but were not included in the statisticall analysis.

Table IV. *Intracellular expression of Ki-67 / Apo2.7 (%) of cultured B-CLL cells (double-positive).*

	Visit 1 Screening	Visit 2 1 month later	Visit 3 3 months later	Visit 4 6 months later	Visit 5 9 months later	Visit 6 12 months later
Number of patients per visit	10	9	9	10	7	7 #
Medium	1.16±1.08	1.41±1.85	0.81±0.93	3.02±3.17 *	4.51±6.54	1.61±1.13
HA 10 μg/ml	1.09±1.00	0.90±0.92	1.07±1.07	2.44±3.09 *	4.33±7.22	1.24±0.79
HP 10 μg/ml	0.80±0.71	0.87±0.84	0.69±0.79	1.26±1.18	2.24±3.16	1.00±0.74
HA 100 μg/ml	1.55±1.29	1.09±1.23	0.98±0.81	1.64±1.65	2.94±3.71	0.96±0.69
HP 100 μg/ml	1.55±2.04	1.09±2.22	0.98±0.59	1.64±5.55	2.94±4.63	0.96±0.58
HA 1000 μg/ml	4.62±5.35	4.74±5.94	2.58±3.10	6.25±8.48	15.66±22.18	2.50±3.50
HP 1000 μg/ml	3.48±2.94	3.09±4.66	2.06±2.32	6.38±6.73	6.44±7.38	2.24±1.69
IL-6 (1000 U/ml)	0.52±0.47	0.42±0.33	0.44±0.49	0.96±0.69 *	1.73±3.08	0.53±0.36
PHA (2.5 μg/ml)	0.78±0.62	1.03±0.53	0.83±0.50	0.95±0.72	1.02±0.93	0.47±0.50
PWM (2.5 g/ml)	0.53±0.62	0.62±0.53	0.58±0.50	0.75±0.72	0.87±0.93	0.40±0.50

Results are means±standard deviation and are significantly different from the screening value (visit 1) at *$p<0.05$ (Wilcoxon). # Two patients with missing data at visit 6 contacted the tumor out-patient clinic later at visit 7 but were not included in the statisticall analysis.

VA-E induces apoptotic cell death (15-22), and thus the observed decrease of VA-E-induced apoptosis in the cultured B-CLL cells within the 12 months observation period might be due to the development of physiologically-induced anti-ML antibodies (30-32) in the added plasma, which could have decrease cytotoxicity of ML present in the added VA-E. However, in line with this decline of cellular susceptibility towards VA-E-induced apoptosis, we observed a marginal increase of Ki-67 expression which was significantly lower than the Ki-67 expression induced by the mitogens. The increase of this proliferation-associated marker was observed also in medium controls, particularly in the cultures with the extremely high VA-E concentrations, and thus represents an increase of non-specific binding of the marker antibodies (including Apo2.7$^+$ Ki-67$^+$ cells). However, IL-6, even at 1000 μg/ml, did not induce proliferation of the B-CLL cells from the patients but reduced spontaneous apoptosis. An inhibition of apoptosis by IL-6 is in accordance with the findings of others (5).

Hugo *et al.* (33) investigated whether VA-E may influence the expression of IL-6 and its receptor components in follicular B-NHL cell lines and found no altered expression level of IL-6 or its receptor components at any time nor with any of the applied VA extract concentrations. Thus, clinically relevant doses of VA-E do not trigger an autocrine or paracrine IL-6 loop nor do they initiate IL-6 trans-signalling in follicular B-NHL cell lines. However, we reported previously (17) that IL-6 might be induced in cultured B-CLL cells during the process of apoptosis, but that this secretion did not lead to a proliferation response of the leukaemic B cells. In this study again we were unable to observe significant proliferation responses towards the applied VA-E. The clinical course of the investigated patients will be addressed in another paper (Gutsch *et al.*, in preparation).

Nevertheless, although potential risks in this *in vitro* setting were not confirmed, the clinical situation of B-CLL patients intended to be treated with VA-E has been under careful consideration. In a retrospective study with more than 200 patients with malignant haematological and lymphatic diseases, Stumpf *et al.* (10) found no evidence for risk of subcutaneously VA-E application on progress of the disease and the survival time; however, the survival time of VA-E treated patients was higher.

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Conflict of interest

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