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Immunomodulatory effects of *Viscum album* agglutinin-I on natural immunity

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In 24 h cultured human peripheral blood mononuclear cells, treated with various (1 μg/ml to 1 ng/ml) concentrations of *Viscum album* agglutinin-I, quantitative assessment of DNA breaks labelled with terminal deoxynucleotidyl transferase revealed a dose-dependent *Viscum album* agglutinin-I-induced apoptosis above a lectin concentration of 10 ng/ml. After 24 h incubation of peripheral blood mononuclear cells with non-cytotoxic concentrations of *Viscum album* agglutinin-I (10 and 1 ng/ml), messenger (m)RNA expression and secretion of a panel of cytokines were evaluated by reverse polymerase chain reaction and by enzyme-linked immunosorbent assay (ELISA), respectively. The lectin induced expression of interleukin-1α, interleukin-1β, interleukin-6, tumour necrosis factor-α, interferon-γ, granulocyte–monocyte colony stimulating factor and interleukin-10 genes, but no expression of interleukin-2 or interferon-γ production could be detected. In addition, cellular components of the natural immune system (such as monocytes and granulocytes) bound *Viscum album* agglutinin-I molecules to a higher degree than lymphocytes. To establish the modulatory potency of *Viscum album* agglutinin-I on the natural immunity of human subjects, four randomized, double-blind crossover trials were performed on healthy volunteers. In contrast to the significant lectin-induced increases in number and activity of natural killer cells observed in animal models, in the first and second trial human healthy individuals showed no significant differences between their natural killer responses following an injection of lectin-enriched preparation or saline. Due to considerable intrinsic fluctuation of these parameters, a third and fourth double-blind trial with freshly isolated *Viscum album* agglutinin-I was performed using a more rapidly detectable parameter, the priming of granulocytes. Here, significant lectin-induced increases were found.

Keywords: Mistletoe lectin, pro-inflammatory cytokines, natural killer cells, priming of granulocytes.

Isolation, identification and characterization of the galactoside-specific *Viscum album* agglutinin-I investigated in our studies

Isolation of *Viscum album* agglutinin-I

Homogenates from the leaves of *Viscum album* plants were prepared by overnight extraction at 4°C in phosphate buffered salt solution (pH 7.2). The crude extract was ultrafiltrated by the Sartocon® mini-crossflow system (Sartorius AG, Göttingen, Germany). Two filtration modules were used: cellulose triacetate with a 20 kDa cut-off and polysulphon with a 100 kDa cut-off, respectively. The product was further purified on lactose-agarose columns (Sigma-Aldrich Handels GmbH, Vienna, Austria) as follows. The ultrafiltrate diluted with a buffered salt solution [1], consisting of 138 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1 mmol/l $MgCl_2$ and 0.6 mmol/l $CaCl_2$, pH 7.4 (PiCM) was recirculated overnight with a flow rate of 1.5 ml/h. Galactose or lactose containing buffer (0.1 mol/l) was used to elute high affinity bound *Viscum album* agglutinin-I [2]. The pure *Viscum album* agglutinin-I was dialysed against the PiCM buffer and concentrated to 0.8 mg/ml.

Identification and characterization of *Viscum album* agglutinin-I

Molecular weight was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate according to Laemmli [3]. After reductive cleavage of the disulphide bonds, the toxic A chain with an apparent molecule weight of 29 kD and the sugar binding B subunit with an apparent molecular weight of 34 kD were detected. Agglutination capacity of *Viscum album* agglutinin-I was examined by the haemagglutination test: 0.025 ml two-fold dilution series of 0.8 mg/ml lectin solution in PiCM buffer were mixed with 0.025 ml 2% washed human erythrocyte suspension. The degree of agglutination was estimated after 1 h incubation at room temperature. Agglutinating capacity was regularly effective above a concentration of 6.25 μg/ml. Protein concentrations were determined by the Lowry method with bovine serum albumin as the standard [4]. The sugar binding lectin content was measured by the enzyme-linked lectin assay as described previously [5]. Briefly, lectin was bound to an immobilized ligand (asialofetuin) and polyclonal rabbit antibody to *Viscum album* agglutinin-I was added. Enzyme conjugated goat anti-rabbit immunoglobulin G was added to the bound antigen–antibody complex and the active lectin content measured at a wavelength of 492 nm. The polyclonal antiserum to *Viscum album* agglutinin-I was prepared in rabbits as described elsewhere [5]. Endotoxin contamination was

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measured with a quantitative kinetic limulus amoebocyte (LAL) assay [6] and was regularly less than 5 pg/ml.

Toxicity of *Viscum album* agglutinin-I

To elucidate the cytotoxic/cytostatic effect of *Viscum album* agglutinin-I, the viability of peripheral blood mononuclear cells was determined *in vitro* in the presence of different lectin concentrations. Measurements with flow cytometry using propidium iodide (at a final concentration of 5 μg/ml) in a culture of human peripheral blood mononuclear cells (10×10^6/ml) demonstrated that the cell viability is significantly affected above a lectin concentration of 10 ng/ml (which corresponds to our reference standard *Viscum album* agglutinin-I). Using ^{3}H-thymidine incorporation, the cytotoxic effect of *Viscum album* agglutinin-I on peripheral blood mononuclear cells and on a human myeloid leukaemia cell line (K562) was also compared. In cultures of K562 cells, *Viscum album* agglutinin-I showed a cytotoxic/cytostatic effect three to four times higher than those found in cultures of peripheral blood mononuclear cells. *Viscum album* agglutinin-I in low concentrations which are non-toxic and slightly mitogenic for the lymphocytes but toxic for the natural killer-sensitive target cells, can enhance the cytotoxic effect on K562 cells as measured by ^{3}H-thymidine incorporation (unpublished data, 1996).

Viscum album agglutinin-I induces gene expression and secretion of pro-inflammatory cytokines

The effects of *Viscum album* agglutinin-I on cellular mechanisms of the natural immune system are not fully understood [5]. It has been suggested that lectin-induced pro-inflammatory cytokines such as interleukin-1, interleukin-6 and tumour necrosis factor-α are, at least in part, involved in its immunomodulatory potency [7]. Therefore, after 24 h incubation of peripheral blood mononuclear cells with non-cytotoxic concentrations of *Viscum album* agglutinin-I (10 and 1 ng/ml), mRNA expression and secretion of a panel of cytokines were evaluated by reverse polymerase chain reaction and by ELISA, respectively [8]. The lectin induced expression of interleukin-1α, interleukin-1β, interleukin-6, tumour necrosis factor-α, interferon-γ, granulocyte–monocyte colony stimulating factor and interleukin-10 genes, but no expression of interleukin-2 and interleukin-5 genes could be detected. Regarding cytokine secretion, interleukin-1β, interleukin-6 and tumour necrosis factor-α production was induced by 1 and 10 ng/ml *Viscum album* agglutinin-I. Interleukin-10 secretion was only stimulated by 1 ng/ml lectin. No interleukin-2 or interferon-γ production could be detected. This study suggests that the cell source of lectin-induced cytokine production is likely to reside in monocytes. This hypothesis is supported by the prevailing detection of monocyte-derived cytokines such as interleukin-1α, interleukin-1β, tumour necrosis factor-α, interleukin-10 and by the analysis of binding of fluorescein isothyocynate (FITC)-conjugated mistletoe lectin-1 to leukocytes [8]. These data are also in agreement with previous observations [7], in that the amount of tumour necrosis factor-α released from human peripheral blood mononuclear cells correlates with the percentage of monocytes in cell cultures. Under these conditions, D-galactose (a competitive inhibitor of binding of the lectin to galactoside) residues on the cell membrane caused a marked inhibition [7]. Thus, lectin–sugar interactions on the cell surface of monocytes may be primarily responsible for the cytokine secretion induced by *Viscum album* agglutinin-I. On the other hand, *Viscum album* agglutinin-I increased the percentage of HLA-DR+ T lymphocytes and natural killer cells *in vitro* suggesting that the effects of the lectin might extend beyond monocytes [8]. The higher affinity by binding assay of *Viscum album* agglutinin-I to cellular components of natural immunity was also supported, since monocytes and granulocytes bound the fluorescence conjugated mistletoe lectin-1 molecules to a considerably higher degree than lymphocytes [8]. These results suggest that lectin–sugar interactions on the cell surface of monocytes and granulocytes can play an important role in the lectin-induced modulation of the natural immune system mediated at least in part by pro-inflammatory cytokines.

Effect of *Viscum album* agglutinin-I on cellular parameters of natural immunity

In cancer patients, mechanisms of natural immunity, believed to serve as essential functions for their survival, are often significantly decreased [9,10]. Investigations on animal models and clinical observations with extracts standardized in terms of galactoside-binding lectin activity suggest that the immunomodulatory efficacy of *Viscum album* agglutinin-I is necessary for its beneficial effects [11–13]. Therefore, the proof of an immunomodulatory effect of *Viscum album* agglutinin-I on human subjects requires reliable and reproducible cellular analysis. In animal models, the natural killer system was found to be a suitable tool for immunological monitoring of the effect of lectin [5,11].

To establish the modulatory potency of *Viscum album* agglutinin-I on the natural immunity of human subjects, four randomized, double-blind crossover pilot trials were performed on healthy volunteers. In contrast to the significant lectin-induced increases in number and activity of natural killer cells observed in animal models, in the first and second trial, human healthy individuals

showed no significant differences between their natural killer responses following an injection of lectin enriched preparation or saline. However, high intrinsic fluctuations of immune parameters were observed after saline injection in the blood of healthy persons. It should not be overlooked that the biological vulnerability of natural killer cells has been well established in various human studies [14–16], demonstrating an association between various effects of stress and a significantly reduced natural killer activity. These data may also help to determine why it has often been so difficult to prove the effect of a biomodulator on host defences.

Due to the considerable intrinsic fluctuation of these parameters, a third and fourth double-blind trial with freshly isolated *Viscum album* agglutinin-I was performed using a more rapidly detectable parameter, the priming of granulocytes. Significant lectin-induced increases were found. The results of the third and the confirmatory fourth trial suggest that priming of polymorphonuclear cells (PMNs) may be more suitable for immunological monitoring of *Viscum album* agglutinin-I rather than the natural killer system [17]. In addition, mistletoe extracts were also able to activate the priming of granulocytes in blood of cancer patients [18].

Clinical perspectives of the immunological research of *Viscum album* agglutinin-I

Preclinical observations

Previous preclinical data demonstrate that immunologically active application of *Viscum album* agglutinin-I to balb/c and nude mice can induce an antimetastatic/antitumour effect on a lymphosarcoma (RAW 117), a fibrosarcoma (L1) and a xenotransplanted leiomyosarcoma model system [11,12].

Clinical observations

Several years ago, a pilot study was carried out with mistletoe extracts (Iscador M). The galactoside-binding, active lectin content was determined with an optimized enzyme-linked lectin assay [13]. After finding a most effective immunological dose schedule, the therapeutic efficacy of these mistletoe preparations was studied in 12 patients with measurable lesions of advanced malignancy. One complete, three partial (at least 50%) and three minor remissions (less than 50%) were established [13].

Conclusions

(1) The prevailing detection of monocyte-derived cytokines suggests that the cell source of lectin-(*Viscum album* agglutinin-I)-induced cytokine production is likely to reside in monocytes; (2) cellular components of the natural immune system (such as monocytes and granulocytes) bound *Viscum album* agglutinin-I molecules to a higher degree than lymphocytes; (3) natural killer cells in animal models and patients and priming of granulocytes in healthy human subjects appear to be suitable cellular parameters of natural immunity for the immunological dose finding of *Viscum album* agglutinin-I; and (4) further clinical research requires biological active and reproducible standardized preparations.

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