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TUMOR REGRESSIVE EFFECTS OF *VISCUM ALBUM* PREPARATIONS

EXPLORATION OF IMMUNOMODULATORY MECHANISMS

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Abstract *Viscum album* is a parasitic plant that grows on apple or pine trees. It is commonly known as European mistletoe. *Viscum album* (VA) preparations are the aqueous extracts used as a complementary medicine in cancer therapy. Various clinical studies have shown that VA preparations improve the quality of life in different cancer patients. Biologically active components of VA extracts include Mistletoe lectins (ML), viscotoxins, polyphenols and polysaccharides. The treatment with VA preparations or with purified ML has been shown to be associated with tumor regression in several *in vivo* experimental models of tumoral implantation. Various *in vitro* studies have shown the cytotoxic effects of VA preparations on various tumor cells. The mechanisms underlying the anti-tumoral activity of VA preparations or ML are complex and involve apoptosis, angiogenesis and immunomodulation. The immunomodulatory mechanisms mediated by the VA preparations can complement the anti-tumoral effects, resulting in effective tumor regression. This review provides an account of the current status of the understanding of the VA-associated immunomodulation in various cell types including lymphoblastoid, monocytic and endothelial cell lines.

Key words: *Viscum album*, cytotoxicity, apoptosis, endothelium, immune system

Resumen ***Efecto antitumoral de extractos de* Viscum album. *Búsqueda de efectos inmunomoduladores.*** *Viscum album* es una planta parasitaria que crece en los pinos y manzanos. Se conoce comúnmente como *European mistletoe*. Las preparaciones de *Viscum album* (VA) son extractos acuosos que se emplean como medicinas complementarias en la terapia del cáncer. Varios estudios clínicos han demostrado que las preparaciones de VA mejoran la calidad de vida de los pacientes oncológicos. Entre los componentes biológicamente activos presentes en los extractos acuosos de VA se pueden mencionar las lectinas de Mistletoe (ML), viscotoxinas, polifenoles y polisacáridos. En varios modelos experimentales de implantación tumoral *in vivo*, el tratamiento con preparaciones de VA o con la lectina ML purificada se asoció con regresión tumoral. Asimismo, diversos estudios *in vitro* demostraron que las preparaciones de Va tienen efectos citotóxicos sobre varias células tumorales. Los mecanismos implicados en la actividad antitumoral de las preparaciones de VA son complejos e involucran apoptosis, angiogénesis e inmunomodulación. Se propone que los mecanismos inmunomodulatorios podrían complementar los efectos antitumorales, resultando en una efectiva regresión del tumor. Este trabajo revisa el estado actual del conocimiento sobre la inmunomodulación asociada al VA en diferentes tipos celulares incluyendo líneas linfoblastoideas, monocíticas y endoteliales.

Palabras clave: *Viscum album*, citotoxicidad, apoptosis, endotelio, sistema inmune

Viscum Album (VA) preparations are aqueous extracts from *Viscum album* or European mistletoe consisting of different types of lectins[1-3]. Mistletoe lectin (ML) I, II, and III belong to the ribosome-inactivating protein (RIP) family of type II. RIP of type II are composed of an N-glycosidase (A chain) and a galactoside-recognizing lectin (B-chain) connected by a disulfide bridge[4]. The entire preparations used therapeutically consist in addition to mistletoe lectins and viscotoxins, several enzymes, peptides (for eg., viscumamide), amino acids, thiols, amines, polysaccharides, cyclitoles, lipids, phytosterols, triterpines, flavonoids, phenylpropanes and minerals. Several studies have reported the clinical benefits of preparations consisting of VA extracts in cancer patients[5-7]. Treatment with VA extracts or by purified ML has also been shown to be associated with tumor regression in several *in vivo* experimental models of tumoral implantation[8-10].

Experimental anti-tumoral effect of VA extracts may be supported by the direct cytotoxic properties of ML towards tumor cell lines[1, 11, 12]. It is well established that the cytotoxicity of VA extracts is dependent on the induction

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of apoptosis. However, the mechanisms underlying the VA extract-induced apoptosis have not been fully elucidated[10, 13-20]. More recently, several studies have demonstrated that VA extracts and purified ML have immunomodulatory properties[14, 17, 18, 21-27]. VA preparations or purified ML have been shown to induce activation of transcription and secretion of pro-inflammatory cytokines such as IL-1, IL-6 and, TNFα in human PBMC, and endothelial cells[17, 22, 24, 26]. Moreover, several studies have shown that VA extracts exert immunomodulatory properties i.e. enhancement of NK cell activity or modulation of TH polarization[28, 29]. However, the *in vitro* cytotoxic properties of VA extracts towards cancer cell lines, raise the question concerning the interaction of VA preparations with the survival of cells implicated in the immune and inflammatory systems i.e. lymphocytes, monocytes, endothelial cells (EC). To address this question, we have conducted several *in vitro* studies on the interaction of VA extracts with various established cell lines (monocytic and lymphoblastoid cell lines, EC lines) or primary cultures (normal murine splenocytes, HUVEC).

Cytotoxicity of VA extracts towards human cell lines derived from T and B lymphocytes, and from monocytes in vitro

Several authors have established that the cytotoxicity of VA extracts could greatly differ depending on the type of preparation and to the investigated cell. The induction of cell necrosis by VA extracts has been reported in human peripheral blood lymphocytes (PBL), human peripheral blood monocytes (PBM), murine thymocytes, human monocytic THP1 cells and mononuclear leukemia cells MOLT4[14, 16-18].

We conducted a study to compare the cytotoxic properties of different VA preparations: VA Qu FrF, Qu Spez, M Spez and VA P in a large representative panel of lymphoblastoid cell of T and B origins and in monocytic cell lines[19, 30]. The induction of cell toxicity by various VA extracts was assessed *in vitro* on the human T cell lines CEM and Jurkat, B lymphoblastoid lines Raji, BC36, BC28, BC41, WW2-LCL, and in HL-60 and MM-6 monocytic cell lines[19, 30]. Cell death was measured by the uptake of propidium iodide followed by flow cytometry analysis. VA Qu FrF, Qu Spez and M Spez induced a dose-dependent cell death in both T cell lines, and in monocytic cell lines. Cell toxicity was associated with a dose-dependent inhibition of cell proliferation. As expected, VA P was devoid of cytotoxicity in all tested cell lines. This may be in part explained by the low concentrations of cytotoxic ML in VA P preparations.

The involvement of apoptosis in the cell toxicity induced by VA extracts have been well demonstrated in various tumoral cell lines[13-17, 31]. The molecular mechanisms are not fully understood and conflicting results have been published. Concerning lymphoblastoid cells, using Fas-resistant HuT78.B1 T cell line, we have demonstrated that Fas pathway is not involved in VA Qu FrF apoptosis as previously reported by others[32]. We have also shown that VA QuFrF induces a dramatic decrease in the amount of anti-apoptotic proteins Bcl-2 and Bcl-X proteins in T lymphocytes[19], a finding consistent with the release of cytochrome from mitochondria induced by ML I[13].

At the concentrations studied, nearly all B lymphoblastoid cell lines were resistant to all the VA preparations (only BC41 cell line was fully sensitive to the three preparations) studied. Our findings clearly confirm that cell lines of B lymphocyte origin are refractory to the cell death induced by VA extracts. Similar results were obtained by Bantel et al[13]. The B lymphocyte cell line, BJAB, required approximately 1000-fold higher amounts of ML-I than that required by Jurkat cells to attain an optimal level of apoptosis[13]. The molecular mechanisms of such resistance of cell lines of B-cell origin are currently unknown.

Results from preliminary experiments conducted in our laboratory may provide new insight into the interaction of VA extracts with the immune system. These preliminary

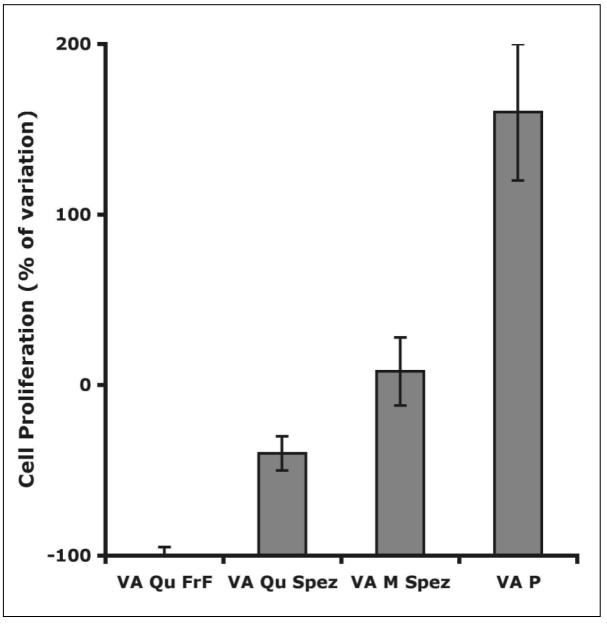

Fig. 1.– **Differential modulation of murine splenocyte proliferation by VA extracts.** Murine splenocytes were obtained from normal C57BL6 mice (6 mice). Cells were cultured for 72h in the presence of 50 µg/ml of VA Qu FrF, Qu Spez, M Spez and P. After 48h, [^{3}H]thymidine (0.5 µCi) was added to the medium. The cells were then cultivated for further 24h. The splenocytes were then collected and the incorporated [^{3}H]thymidine was measured. The results are represented as a percentage of variation as compared to untreated controls. After 72h of culture, VA Qu FrF and Q Spez strongly inhibited murine splenocyte proliferation, whereas VA P dramatically induced cell proliferation.

ex vivo experiments suggest that VA preparations may induce cell proliferation in normal C57BL6 murine splenocytes as measured by cellular incorporation of tritiated thymidine. However, these data clearly show a balance between inhibitions or induction of proliferation and induction of apoptosis that varied depending on the preparation used, the concentration and the incubation time (Fig. 1). The results emphasize the necessity to design the adequate schedule to achieve immunostimulation in both *in vivo* and *in vitro* experiments.

Induction of apoptosis by various *Viscum album* extracts on both immortalized human venous endothelial cell lines and HUVEC

In addition to its gate-keeping role between blood and tissue, the endothelium plays a pivotal role in several biological processes. EC actively participate in the regulation of blood flow and coagulation, in initiation and enhancement of inflammation, and in angiogenesis that is fundamental to reproduction, development and repair[33, 34]. The contribution of an EC-mediated effect in the anti-tumoral properties of VA extracts thus needs to be investigated. In this respect, we assessed the interactions between VA extracts and EC.

Our *in vitro* study focused on the ability of VA extracts to induce EC death. Using primary human venous endothelial cells (HUVEC) and the immortalized human venous endothelial cell lines IVEC and EA-hy926, we have shown that VA extracts induce EC death in a dose- and time-dependent manner[20]. Fig. 2 depicts the cytotoxic properties of VA extracts towards EA-hy926 as assessed by the uptake of PI. Three VA extracts induced a potent and dose mortality of EA-hy926 as assessed by the uptake of PI. VA Qu FrF and VA Qu Spez were the most effective preparations. Iscador M Spez had less cytotoxic properties. EA-hy926 cells were insensitive to Iscador P at any tested concentration. Similar results were obtained with the IVEC cell lines. Other authors have also reported the sensitivity of various EC lines to VA extracts[35].

We then demonstrated that VA-mediated EC death involves apoptosis, using various methods i.e DNA ladder formation, annexin V labeling, and western blot analysis for poly(ADP)-ribose polymerase (PARP) cleavage. Fig. 3A illustrates the cytological aspect of HUVEC incubated with high concentrations of VA Qu FrF[20]. Numerous typical blebs were observed around the cell. As illustrated in Fig. 3B, HUVEC cultured in the presence of VA extracts exhibited the typical apoptotic bodies. Annexin V labeling with flow cytometry analysis and DNA laddering experiments also confirmed the involvement of apoptosis in the EC cell death induced by VA extracts. The percentage of EC undergoing apoptosis (An V+/PI-) increased in a time- and dose-dependent manner for VA extracts. Western blot analysis also demonstrated that the cleavage of PARP is involved in VA-induced HUVEC apoptosis.

Can *Viscum album* extracts be used for anti-angiogenic therapy of cancer?

Angiogenesis is the process by which new blood vessels are formed by sprouting pre-existing vessels[33, 34, 36, 37]. Tumor angiogenesis plays an essential role in tumor progression and metastasis. Clinical applications of research on angiogenesis have emerged towards the diagnosis, prognosis or therapy of neoplasia[38, 39]. The mechanisms involved in tumor angiogenesis consist of a wide range of

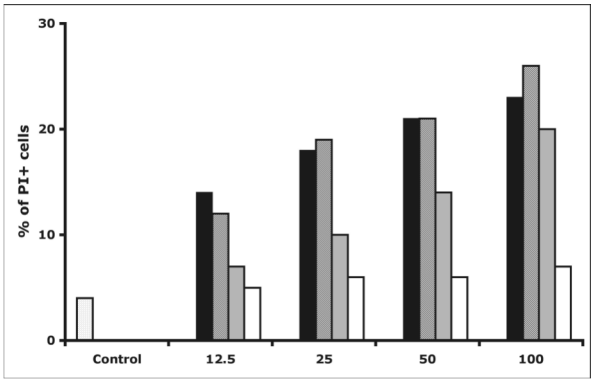

Fig. 2.– **Induction of cell death in endothelial cell line EA-hy926 by VA extracts.** Sub-confluent EA-hy926 cells were cultured for 48h in the presence of increasing concentration of VA extracts: VA Qu FrF (black bars), Qu Spez (shaded bars), M Spez (grey bars) and P (open bars), or were left untreated (Control). Cell death was then quantitated by measuring the uptake of PI and expressed as a percentage of dye-positive cells. VA Qu FrF, Qu Spez and M Spez induced dose-dependent cell toxicity in EA-hy926 cells. EC were fully resistant to VA P.

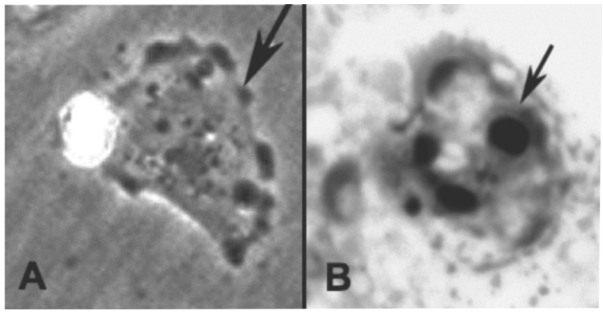

Fig. 3.– HUVEC undergoes apoptosis when treated with higher doses of VA Qu FrF. Sub-confluent HUVEC were cultivated with VA Qu FrF at 50 µg/ml for 48h. Panel A illustrates the features of EC under cytologic examination, with the presence of blebbing around the cells. Panel B confirmed the involvement of apoptosis with a typical pattern of apoptotic body.

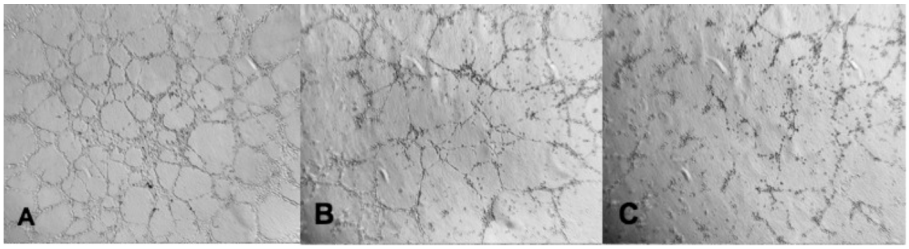

Fig. 4.– **VA Qu Spez inhibits angiogenesis under *in vitro* conditions.** EA-hy926 cells were seeded on Matrigel© which promotes *in vitro* angiogenesis. The matrigels were either untreated (panel A) or treated with different concentrations of VA P or VA QuSpez. After 24h, there is normal growth of vasculature in Control (Panel A), there is moderate decrease in the vascular growth in VA P treatment (Panel B- 50 µg/ml). But, there is a remarkable reduction in the vascular growth in VA QuSpez treatments (Panel D-50 µg/ml). Thus, the incubation with VA Qu Spez (50 µg/ml) partially abrogated capillary tube formation (C).

phenomena including enhanced division of endothelial cells (EC) within the tumor, up-regulation of cell adhesion molecules and production of angiogenic factors[33, 34].

The role of VA extracts in the process of angiogenesis is thus of interest to understand some of the molecular mechanisms involved in the anti-tumoral properties of VA extracts. Fig. 3 illustrates our preliminary results obtained in capillary tube formation experiments using EA-hy926 cell line. EC were seeded on matrigel and incubated with various concentrations of VA extracts. A well-organized capillary tube network was observed in control well (Fig. 4A). In contrast, the capillary tube formation was intermediate with VA P (50 µg/ml) (Fig. 4B), while it was abrogated in the presence of VA Qu Spez (50 µg/ml) (Fig. 4C). Thus, these preliminary results suggest that extracts inhibit *in vivo* angiogenesis in a differential manner. *In vivo* anti-angiogenesis effect of VA extracts have also been examined by choriallantoic membrane assays in C57BL6 mice inoculated with B16-BL6 melanoma cells and treated with Viscum album L. coloratum agglutinin[10]. In addition, VA *coloratum* extracts suppressed tumor growth *in vivo* and inhibited the number of blood vessels oriented towards the tumor mass[40].

The molecular mechanisms underlying the VA-induced inhibition of angiogenesis have not been fully elucidated. At the level of EC, our results may suggest the implication of VA-associated EC apoptosis, at least in part in its anti-angiogenic properties. Several studies have well demonstrated that EC apoptosis is implicated in the physiological inhibition of angiogenesis. Consistently, Yoon et al. have observed in *in vitro* experiments that VA coloratum extracts inhibit the proliferation of rat EC.

Conclusions

The molecular mechanisms underlying the anti-tumoral activity of VA or ML are complex and involve several inter-related biological phenomena including apoptosis, angiogenesis and immunomodulation. As in the case of other members of RIP II family, VA extracts exert cytotoxic activity towards cell lines derived from both human and rodent origins although to a lesser extent as compared to ricin. VA extracts and purified ML also induce activation of transcription and secretion of pro-inflammatory cytokines in human PBMC and endothelial cells. To address the interactions between immunomodulatory and anti-tumor properties of VA preparations, we have recently investigated the mechanisms underlying the effects of VA extracts on tumoral growth of melanoma implanted in mice. These studies are suggestive of the implication of certain anti-tumoral cytokines. Together, several lines of evidence that have been accumulated in the recent years, in favor of anti-tumoral and immunomodulatory properties of VA extracts, strongly encourage further intensive investigation.

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