ANTICANCER RESEARCH *25*: 3303-3308 (2005)

Binding of Recombinant Mistletoe Lectin (Aviscumine) to Resected Human Adenocarcinoma of the Lung

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Abstract. *Background: Lectins, carbohydrate proteins, bind to glycoconjugates of all mammalian cells, including cancer cells. Aberrant glycosylation, detected by lectin histochemistry, can predict outcome in some tumour entities. One such lectin is aviscumine (recombinant mistletoe lectin). Aviscumine has cytotoxic effects and can therefore be used as anti-tumour therapy. Materials and Methods: Lectin histochemistry with aviscumine was performed on primary tumour sections from resected adenocarcinoma of the lung. Staining results were then correlated with the clinical course of the patients. Results: Most of the adenocarcinomas (92.5%) bound aviscumine. Kaplan-Meier analysis revealed no correlation between aviscumine binding and progression-free survival or overall survival. Conclusion: These results suggest that for the selected group of patients with adenocarcinoma of the lung aviscumine binding activity can not serve as a prognostic factor. More strikingly, however, aviscumine binds to malignant cells in 92.5% of the patients. This is an indicator for the use of aviscumine as a possible target for tumour therapy.*

Abbreviations: MLs, mistletoe lectins; NSCLC, non-small cell lung cancers; RIP, ribosome-inactivating proteins; rML, recombinant mistletoe lectin; SC, Spearman coefficient.

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Key Words: Adenocarcinoma of the lung, aviscumine, recombinant mistletoe lectin, prognostic factors, non-small cell lung cancer.

Lectins are carbohydrate-binding proteins of non-immunological origin, which often multivalently bind to carbohydrate residues of glycoconjugates. This study focuses on the mistletoe lectins (MLs) derived from the European mistletoe (*Viscum album*). Three different MLs (ML-I, ML-II, ML-III) have been isolated and all three belong to the type-2 ribosome-inactivating proteins (RIPs), and are thus highly cytotoxic. ML-I is one of the best characterised lectins and has attracted considerable interest as it was shown to be the main biologically active component of aqueous mistletoe extracts (2-4). In addition to its cytotoxicity, ML-I displays a broad spectrum of immunomodulatory effects, which are additionally thought to be of benefit to cancer patients treated with aqueous mistletoe extracts (4). Because of these broad anti-tumour actions, ML-I has now been made available as a recombinant mistletoe lectin (rML) called rViscumin or aviscumine (INN). Aviscumine differs from the plant-derived ML-I, mainly in its lack of glycosylation, while the other features, such as carbohydrate-binding specificity and cytotoxicity, are identical for plant-derived ML-I and aviscumine (5, 6). Aviscumine has successfully passed phase I clinical trial and is presently being considered for phase II studies (7).

Type-2 RIPs consist of two protein chains, the B-chain which contains the carbohydrate-recognising domain (*i.e.* the lectin activity) and the A-chain, which exhibits rRNA-N-glycosidase activity (1). For the cytotoxic effect of avisumine, the B-chain initially has to bind to the carbohydrate coat of the cell, the glycocalyx. By a so-far unknown mechanism, the aviscumine molecule crosses the lipid bilayer of the cell membrane in the endosome/lysosome compartment and enters the cytoplasm, where the A-chain irreversibly inactivates the ribosomes. As surface

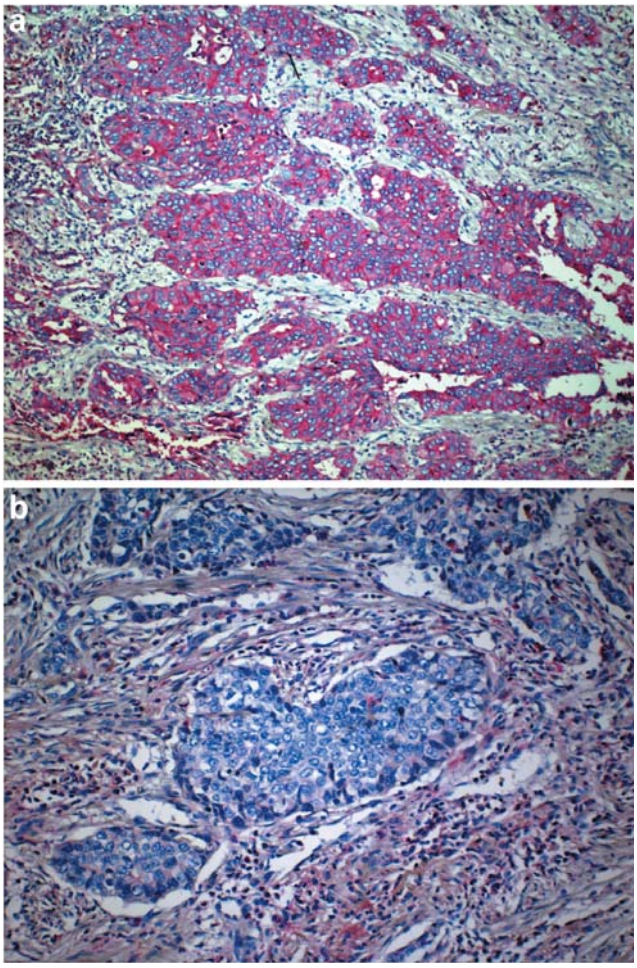

Figure 1. *Two cases of adenocarcinoma of the lung stained with aviscumine. (a) The majority of the tumour cells show intense binding, (b) no binding of the tumour cells for aviscumine.*

Table I. *Patient characteristics.*

Characteristics	Number of patients
Total number of patients	93
Gender	
Male	63 (68%)
Female	30 (32%)
Stage*	
IA	20 (22%)
IB	32 (34%)
IIA	4 (4%)
IIB	16 (17%)
IIIA	19 (21%)
IIIB	1 (1%)
IV	1 (1%)
Grade of differentiation	
Well-differentiated	10 (11%)
Moderately-differentiated	37 (40%)
Poorly-differentiated	33 (35%)
Undifferentiated	5 (5%)
Unknown	8 (9%)
Blood group type	
0	33 (35%)
A	42 (45%)
B	9 (10%)
A/B	2 (2%)
Unknown	7 (8%)

*= 5th edition of the TNM classification of lung cancer, 1997.

binding is a prerequisite for its internalisation and subsequent cytotoxic action, it would be an advantage to know whether a particular tumour entity has binding sites for aviscumine.

The first aim of the present investigation was to evaluate the presence of aviscumine binding sites in adenocarcinoma of the lung, which is currently the main histological subtype of non-small cell lung cancers (NSCLC). In NSCLC, surgical resection followed by adjuvant chemotherapy is the therapy of choice in early stages of the disease (8). However, even in this selected group of patients, about half relapse after complete resection (9, 10), indicating that the tumour has already spread beyond its anatomical site at the time of surgery, and resulting in a poor prognosis particularly warranting the testing of new systemic approaches for adjuvant treatment.

The second aim of the study was to analyse whether aviscumine binding has prognostic implications. This analysis was performed as lectin binding to the primary tumour has been of prognostic significance in resected adenocarcinomas of the lung (11).

Materials and Methods

The binding of aviscumine was studied in resected adenocarcinomas of the lung. The complete series of paraffin wax blocks retrieved from the files of the Department of Pathology (General Hospital Harburg, Hamburg, Germany) consisted of samples from 103 resected specimens of adenocarcinomas of the lung. All patients who underwent surgery between 1990 and 1995 in the General Hospital Harburg, were consecutively included in the series. Of these, a complete follow-up could be retrieved from 93 patients, whose tissue blocks were further investigated. No other selection criteria were applied.

Lectin histochemistry. Sections were cut at 5 μm and then deparaffinized in xylene and rehydrated in a series of graded ethanols to distilled water. Afterwards, the slides were incubated

in 0.1% trypsin (Biochrom KG, Berlin, Germany) dissolved in Tris-buffered saline (TBS; pH 7.6) with calcium chloride (1 mM) and magnesium chloride added (1 mM) (Merck, Darmstadt, Germany) ("lectin buffer", pH 7.6), warmed to 37˚C in a waterbath. The slides were washed in running tap water for 15 min to stop the trypsin reaction, followed by washing three times in lectin buffer for 5 min each. Biotin-conjugated aviscumine was diluted 1:100 (10 μg/ml) in lectin buffer in which the slides were incubated for 1 h in a humid chamber (for all incubations a humid chamber was used). The slides were washed three times for 5 min in TBS. The biotin-conjugated aviscumine binding sites were visualised using an alkaline phosphatase-labelled streptavidin by incubating the slides with the Vectastain® ABC KIT (Vectastain®, Vector, Burlingame, CA, USA) solution for 30 min.

Naphtol-AS-biphosphate together with hexatozised New fuchsin was used as a substrate solution. The slides were covered by this substrate solution and incubated in the dark for 20 min. To stop the reaction, the slides were washed in running tap water for 10 min and were then transferred into distilled water.

The slides were counterstained in Mayer's hemalum solution (Merck). After aqueous mounting media precipitation (crystal/mount™, Biømeda, Foster City, CA, USA), the slides were slipped using a resinous permanent mounting media (Clariøn, Biømeda).

To identify all cancer cells within each slide, the slides were additionally stained with hematoxylin and eosin. The sugar specificity of the lectins was tested with galactose (200 mM) (Serva, Heidelberg, Germany). Each staining procedure included a negative control in which incubation with the lectin was omitted.

The staining of the cancer cells was analysed usind a semi-quantitative scale. If less than 5% of the tumour cells were stained, a negative symbol (–) was assigned , a plus (+) was assigned if up to 50% of the tumour cells were stained and, in the case that more than 50% of the tumour cell were stained, a double plus (++) was assigned (presented in Figure 1). The slides were examined under a Zeiss Axioplan photomicroscope (Carl Zeiss Jena GmbH, Jena, Germany) by two observers independently. Cases in which opinions differed were discussed and a consensus was achieved. Photographs were taken by the Axiocam MRc5 (Zeiss, Munich, Germany).

Statistical analysis. The clinical course of all patients with resected adenocarcinoma was followed-up for a minimum of 5 years.

The patients' overall survival time was defined as the interval from the date of diagnosis to death (of any cause) or to the last date of information for living patients (censored observation). Time to progression was defined, respectively, from diagnosis to the date of the first progression according to the WHO criteria. For both, survival curves were calculated and graphically presented using the Kaplan-Meier method for censored failure time data. Overall survival and progression curves, when stratified by prognostic factors, were compared using the log rank test. To evaluate the prognostic significance of aviscumine expression, univariate Cox-regression analysis was performed.

Spearman correlation coefficients were calculated to analyse the relationship between aviscumine binding and age, gender, blood group, tumour stage and grading. Statistical analyses were performed using the statistical packages SAS for Windows Version 8 (SAS Institute Inc., North Carolina, USA) and R Version 1.6.2 (http://www.r-project.org). *P*-values of less than 0.05 were considered statistically significant.

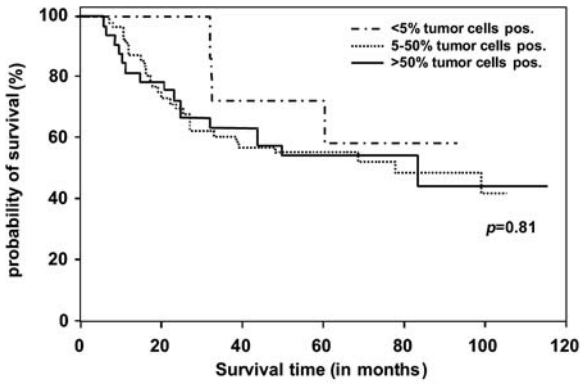

Figure 2. *Kaplan-Meier overall survival for aviscumine binding. Negative binding (<5%) versus moderate binding (5-50%) versus intense binding (>50%). Aviscumine binding showed no significant influence for patient survival.*

Results

Patient characteristics. The tumour tissues of 93 patients with adenocarcinomas of the lung were investigated. The majority of patients (68%) were male (Table I) with a median age of 59 years (range 27 to 81). Seventy-two patients (77%) were pathologically staged as early disease (stage I and II disease) with negative mediastinal lymph nodes, and 19 (21%) were staged as locally advanced disease with positive lymph nodes of the ipsilateral mediastinum (stage IIIA disease). No patient had tumour positive lymph nodes of the contralateral mediastinum or distant metastases. Nevertheless, one patient with a pulmonary metastasis of the same lobe as the primary tumour (stage IIIB disease) was included, as was one patient with a pulmonary metastasis of another lobe of the same side (stage IV disease). Patients with positive mediastinal lymph nodes received radiotherapy after surgery. The predominant grade of tumour differentiation was moderately-differentiated (40%), followed by poorly-differentiated tumours (35%). The most common blood group type was A (45%).

Lectin-binding characteristics. The tumours of only 7 patients (7.5%) showed no aviscumine binding (–), and the tumours of 86 patients (92.5%) demonstrated moderate (+) (n=54; 58%) to intense (++) (n=32; 34.5%) aviscumine binding to the tumour cells. No correlation was observed between aviscumine binding and age (Spearman coefficient (SC): –0.042), gender (SC: −0.045), blood group (SC: 0.008), tumour stage (SC: 0.016) and grading (SC: 0.054).

Survival analysis and prognostic impact. The overall 5-year survival rate of all 93 patients was 49.5%. Distant metastases or a local relapse was diagnosed in 49 patients

(53%). Twenty-six patients had distant metastases only, one patient presented with a local relapse only, while 22 patients suffered from distant metastases as well as a local recurrence. Tumor stage ($p<0.000005$), grade of tumour differentiation ($p=0.025$) and gender ($p=0.04$) had a significant influence on overall survival. The blood group showed no statistical association with survival ($p=0.36$).

Kaplan-Meier survival curves (Figure 2) of time to death in months for aviscumine-negative-(−)-patients *versus* aviscumine-(+)-positive patients *versus* aviscumine-intensively-positive-(++)-patients showed no significant difference in overall survival between the three different groups ($p=0.81$). No significant differences were found for relapse-free survival ($p=0.96$). These results were confirmed using univariate Cox regression for aviscumine binding categorized into negative, positive and intensively positive. No difference in prognostic significance was observed: aviscumine-negative *versus* aviscumine-intensively-positive patients $p=0.56$, Hazard ratio 0.69, aviscumine-positive *versus* aviscumine-intensively-positive patients $p=0.94$, Hazard ratio 1.03.

Discussion

The present study was undertaken to analyse the extent of aviscumine binding to adenocarcinoma cells of the bronchus. This tumour entity was chosen since it is the main histological sub-type of non-small cell lung cancer. Thus, even if the primary tumour is so small that surgery with the intent to cure is performed as in our series, only half of the patients are alive after 5 years, thus stressing the need for new treatment options. That aviscumine might be such an option is indicated by the fact that tumour cells in the vast majority of cases of adenocarcinoma of the lung bound aviscumine (92.5%). Since binding is necessary for the cytotoxic action, the results presented here indicate that adenocarcinomas of the lung could be a suitable choice for clinical studies of the cytotoxic action of aviscumine.

Aviscumine has been shown to bind to both glycoproteins and glycolipids (12). Therefore, one would assume that the extent of aviscumine binding to tumour cells *in vivo* might be even larger as indicated in this study because only paraffin wax material has been used for aviscumine-binding-site analysis. Paraffin wax-embedded tissue is devoid of lipids, as these are dissolved during the wax embedding process and are thus absent in the slides analysed. Comparing ML-I binding to living cells incubated at 37˚C in tissue culture with formalin-fixed cells, Valentiner *et al.* (13) found consistently more ML-I binding sites in the living cells than in the formalin-fixed cells, supporting the hypothesis that lectin binding sites are more abundant in living cells than in fixed ones. However, as cells were not only fixed but also wax-embedded in the present study, the situation becomes more complex as wax embedding and sectioning of the cells may influence the number of lectin binding sites in a complex way. Lectin binding sites present on glycolipids are, on the one hand, diminished by the embedding procedure, however, lectin binding sites on the internal membranes are made more accessable by the sectioning process. As a result of these conflicting procedures, no such clear correlation between ML-I binding and fixation could be found if living cells were compared with paraffin wax-embedded cells, where in general even a more intensive staining was observed compared to the native cells (14). However, the interpretation of these data becomes even more complex as lectin binding sites may differ between *in vivo* and *in vitro* (4).

The prognostic ability of aviscumine was also evaluated here, since a previous study using the same patient cohort had demonstrated that lectin binding can be a prognostic factor in resected adenocarcinoma of the lung (11). In that study, the lectin HPA with sugar specificity for N-acetylgalactosamine showed a prognostic value. However, no association between aviscumine binding and prognosis could be detected in our study. Our results are in contrast to the findings of Thies *et al.*, who found ML-I to be of prognostic significance in malignant melanoma (15). One explanation for the observed difference in the prognostic ability of ML-I binding to tumour cells could rest in the differences in the glycocalyx composition of the tumour cells. Melanoma cells are derived from the neural crest, while adenocarcinoma cells of the lung are derived from the endoderm, and considerable cell- and tissue-specific expressions of glycosyltransferases, which determine the carbohydrate residues on the glycocalyx have been reported (16). The latter point is of particular interest as the sugar-binding specificity of aviscumine has very recently been redefined. Müthing *et al.* (12) showed that aviscumine bound to terminal Neu5Aca2-6Galb1-4GlcNac residues and not primarily to galactose residues, as previously described (4, 17). The difference in the carbohydrate specificity of aviscumine and the galactose-specific type-2 RIP ricin was supported by Moisenovich *et al.* (18), who found differences in the binding and cellular uptake of the two type-2 RIPs. Aviscumine binding was compared to SNA-I binding from a previous study by Laack *et al.* (11) using the same patient cohort. A moderate correlation ($p=0.0003$) was found between aviscumine and SNA-I binding, the latter being a lectin, which has a very similar sugar specificity, namely neuraminic acid, bound by a α2-6 bond to a galactosyl residue. In contrast, no association was found between aviscumine and MAA, a lectin which is specific for neuraminic acid, bound by an α2-3 bond to a galactosyl residue. This finding again emphasises the high specificity of the neuraminic acid-binding lectins.

Aviscumine is thus a cytotoxic lectin which probably binds with high specificity to terminal Neu5Acα2-6Galβ1-4GlcNac

residues, which are relatively widely distributed amongst adenocarcinomas of the lung. The high prevalence of aviscumine binding to wax-embedded sections of adenocarcinoma of the lung indicates a possible clinical relevance for the aviscumine treatment of patients of this tumour entity. For this, complementary studies are needed with larger numbers of patients, followed by *in vivo* models and clinical trials.

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Received March 16, 2005
Accepted May 30, 2005