

# Concentrations of Galectin-3 in the Sera of Normal Controls and Cancer Patients<sup>1</sup>

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## ABSTRACT

**Galectin-3, a member of the  $\beta$ -galactoside-binding animal lectins, has been implicated in tumor invasion and metastasis. Using an immunoligand assay, we assessed the circulating levels of galectin-3 in sera from cancer patients as well as from healthy controls. Low serum levels of galectin-3 were detected in healthy individuals (median, 62 ng/ml; range, 20–313 ng/ml; 95th percentile, 184.3 ng/ml). Compared with healthy individuals, galectin-3 serum levels in patients with breast, gastrointestinal, lung, or ovarian cancer, melanoma, and non-Hodgkin's lymphoma were significantly elevated ( $P = 0.014$ ). Moreover, galectin-3 concentrations in sera from patients with metastatic disease were higher than in sera from patients with localized tumors. Maximum serum concentrations of galectin-3 (median, 320 ng/ml; range, 20–950 ng/ml) were found in patients with metastatic gastrointestinal carcinoma. These results suggest that circulating galectin-3 may play a role in tumor progression. The possibility of using this assay in early-stage cancer to predict metastasis should be studied.**

## INTRODUCTION

Galectins constitute a gene family of widely distributed carbohydrate-binding proteins characterized by their affinity for  $\beta$ -galactoside-containing glycans. Galectin-3, formerly known as Mac-2, CBP-35, L-29, L-34, L-31,  $\epsilon$ BP, and other names, is isolated as a monomer of  $M_r \sim 30,000$  (1, 2). Galectin-3 is found in the cytoplasm, but depending on cell types and proliferative states, it can also be detected on the cell surface (3), within the nucleus (4), and in the extracellular compartment (5, 6). Galectin-3 acts as a receptor for ligands containing poly-*N*-acetylglucosamine sequences. To date, several ligands for galectin-3,

including lysosomal-associated membrane proteins 1 and 2, IgE, laminin, and Mac-2 BP<sup>3</sup> (also known as 90K), have been described (7, 8).

The biological functions of galectin-3 remain elusive. Studies from several groups suggest that galectin-3 may have a role in a variety of physiological and pathological processes. Of most relevance to the present study are experimental observations suggesting a relationship between galectin-3 and tumor progression and metastasis. For example, tumor cell variants demonstrating higher potential for lung colonization were found to express higher levels of galectin-3 on the cell surface (9). Similarly, increased galectin-3 expression has been correlated with the metastatic potential of several tumorigenic cells, possibly by affecting cell motility and invasion of extracellular matrices (10, 11). However, the generality of these findings in relation to human tumors of epithelial origin is not fully clear. For example, in human colorectal carcinoma, galectin-3 has been reported to increase (12) or decrease (13) with progression toward a metastatic state. In addition, decreased expression of this lectin compared with the normal tissue has been associated with the metastatic propensity of cancer cells in breast (14), endometrial (15), and ovary (16) carcinomas. Thus, the ability of galectin-3 to promote or inhibit invasion and metastasis may depend upon tumor-specific factors. To further address the role of galectin-3 in tumor progression and metastasis, we developed an immunoligand assay for the determination of soluble galectin-3 in the circulation. Using this assay, we measured the levels of galectin-3 in the serum of patients with various types of cancer. Serum specimens from healthy blood donors were used as controls. In addition, we looked for a correlation between galectin-3 levels, tumor type, and disease state.

## MATERIALS AND METHODS

**Sera and Patients.** Blood samples were obtained from patients being seen in the oncology day-hospital of the University of Chieti Medical School. A total of 99 patients with histologically proven diagnosis of cancer were included in this study: 35 with breast carcinoma, 25 with gastrointestinal carcinoma (4 patients with gastric carcinoma, 12 patients with colorectal carcinoma, 4 patients with pancreatic carcinoma, 3 patients with cholangiocellular carcinoma, and 2 patients with hepatocellular carcinoma), 26 patients with non-small cell lung carcinoma, 4 patients with melanoma, 4 patients with ovarian carcinoma, and 5 patients with non-Hodgkin's lymphoma. Approximately 70% of patients with epithelial cancer had clinical evidence of metastases. Samples were allowed to clot, and the serum was stored at  $-20^\circ\text{C}$  until assayed. In five patients with colorectal carcinoma, galectin-3 serum levels were measured

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<sup>3</sup> The abbreviation used is: Mac-2 BP, Mac-2-binding protein.

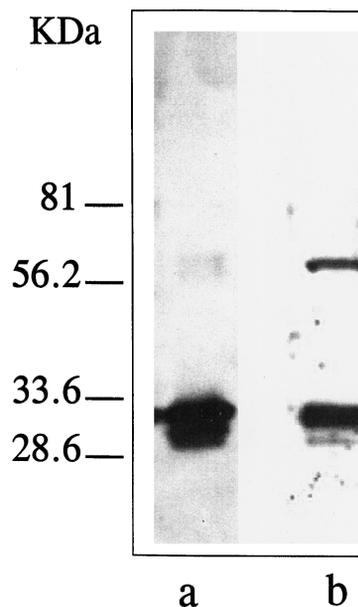
before and after resection of the primary tumor. Samples were also obtained from 50 blood donors (30 females and 20 males) with a median age of 42 years (range, 21–65 years).

**Human Recombinant Galectin-3 Expression and Purification.** Recombinant galectin-3 was expressed in *Escherichia coli* and purified by affinity chromatography as described in detail (17).

**Production and Purification of Recombinant Mac-2 BP.** Human embryonic kidney EBNA 293 cells transfected with the full-length cDNA encoding human Mac-2 BP/90K (18) were kindly supplied by Dr. Rupert Timpl (Martinsried, Germany). Mac-2 BP was isolated from cell culture supernatant as described previously (19). In brief, cells were seeded into cell factories in DMEM containing 10% FCS. When the cells reached confluence, the serum concentration was gradually reduced to 1%. Conditioned medium was collected every day for 3 consecutive days, centrifuged at  $10,000 \times g$  for 15 min, and stored at  $-80^{\circ}\text{C}$  after adding EDTA and Pefabloc (Boehringer Mannheim) to 1 and 0.4 mM, respectively. Mac-2 BP was precipitated from this medium by adding solid ammonium sulfate to 43% saturation (19). The precipitate was dissolved in PBS containing protease inhibitors and passed over a  $3 \times 12$ -cm column loaded with Sepharose CL-4B conjugated to anti-Mac-2 BP monoclonal antibody as described (20). After extensive washing with 0.5 M NaCl in PBS, Mac-2 BP was eluted with 20 ml of Actisep elution medium (Sterogene Bioseparation, Inc., Arcadia, CA). Purified material was aliquoted and stored at  $-80^{\circ}\text{C}$ .

**Western Blotting.** Serum (20 ml) from a healthy blood donor was adsorbed with lactosyl-Sepharose 4B (17), and the bound proteins eluted with 0.2 ml of 0.1 M lactose in PBS. Aliquots of the eluate were resolved by 12% SDS-PAGE and transferred to nitrocellulose. The membranes were then blocked with 1% BSA overnight at  $4^{\circ}\text{C}$  and probed at room temperature for 2 h with rat anti-galectin-3 monoclonal antibody M3/38 (1  $\mu\text{g}/\text{ml}$ ). Biotinylated goat antirat IgG was added as secondary antibody, followed by peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark). After extensive washing, the membranes were processed for chemiluminescent detection using an enhanced chemiluminescence kit (ECL; Amersham Life Sciences, Arlington Heights, IL) according to the manufacturer's instructions. Membranes were also probed with purified recombinant Mac-2 BP (5  $\mu\text{g}/\text{ml}$ ; 2 h), followed by sequential incubation with mouse anti-Mac-2 BP monoclonal antibody 1.A422 (Ref. 21; 1  $\mu\text{g}/\text{ml}$ ; 45 min), biotinylated goat antimouse IgG, peroxidase-conjugated streptavidin, and enhanced chemiluminescent reagents as above.

**Immunoligand Assay for Galectin-3.** Serum galectin-3 concentrations were assayed with a newly developed immunoligand assay. The assay uses Mac-2 BP immobilized to plastics as galectin-3 capture protein, followed by incubation with rat anti-galectin-3 antibody and peroxidase-labeled goat antirat IgG as detecting antibody. Ninety-six-well microtiter plates (Nunc-Maxisorp; Life Technologies, Inc., Rockville, MD) were coated with 5  $\mu\text{g}/\text{ml}$  purified Mac-2 BP in PBS by incubation overnight at  $4^{\circ}\text{C}$  and then blocked with 1% BSA (Sigma Chemical Co., St. Louis, MO) in PBS for 2 h at room temperature. For the assay, 100  $\mu\text{l}$  of samples and serially diluted human recombinant galectin-3 (standards) in dilution buffer (PBS containing 0.1%



*Fig. 1* Binding specificity of Mac-2 BP to galectin-3 in human serum. Serum was adsorbed with lactosyl-Sepharose CL-4B, and the bound proteins eluted with 0.1 M lactose. Proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Blots were developed with anti-galectin-3 monoclonal antibody M3/38 (a) or purified recombinant Mac-2 BP (b). Bands were visualized as specified in "Materials and Methods."

BSA and 5% glycerol) were added to the wells and incubated for 2 h at room temperature. After washing with PBS containing 0.02% Tween 20 (three 5-min washes each, with constant shaking), 100  $\mu\text{l}$  of monoclonal antibody M3/38 (1  $\mu\text{g}/\text{ml}$ ) were added and incubated for 1 h at room temperature. After washing the wells three times as above, 100  $\mu\text{l}$  of peroxidase-labeled antirat IgG were added to each well and incubated for 45 min at room temperature. Wells were then washed three times, and then the enzyme reaction was carried out at room temperature for 30 min with diaminobenzidine as a substrate. The reaction was then stopped with 1 N  $\text{H}_2\text{SO}_4$ , and absorbance at 450 nm was read on a microplate reader. The amount of galectin-3 from the samples was estimated by extrapolation from a log:log linear regression curve determined from the serially diluted human recombinant galectin-3 ranging from 625 to 0 ng/ml. Samples containing galectin-3  $>625$  ng/ml were diluted with dilution buffer and reassayed.

**Statistical Analyses.** Because galectin-3 values were not normally distributed, the 5th and 95th percentile values were chosen for data description. Differences between patient groups were tested with the nonparametric Mann-Whitney (nonparametric) *U* test. All *P*s given are used in a descriptive manner, because no adjustment for multiple testing was performed.

## RESULTS

**Binding Specificity of Mac-2 BP.** Western blotting was carried out to determine binding specificity of Mac-2 BP used in this study. As shown in Fig. 1, immunostaining of blotted serum proteins with anti-galectin-3 monoclonal antibody M3/38 re-

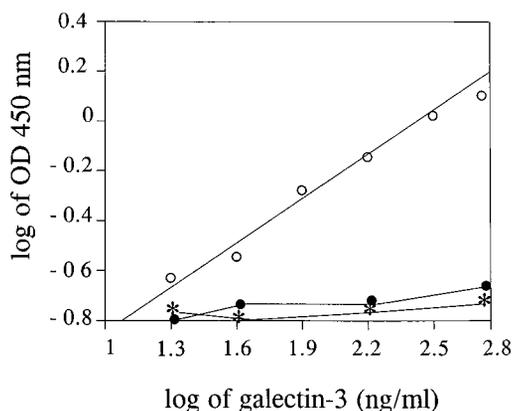


Fig. 2 Standard curve for immunoligand assay of galectin-3. The plot of the absorbance at 450 nm versus galectin-3 concentration is represented in a log:log scale. As a control, the assay was carried out when the Mac-2 BP-catching protein was substituted with human serum albumin (\*) and when rat antigalectin-3 antibody M3/38 was substituted with rat antitumor necrosis factor- $\alpha$  antibody (●).

vealed one major band with  $M_r \sim 31,000$  and a minor component of  $M_r \sim 60,000$  representing galectin-3 homodimers (17). A superimposable staining pattern was obtained after reacting the blotting membrane with Mac-2 BP. These results indicate that Mac-2 BP does not react with serum components other than galectin-3.

**Galectin-3 Immunoligand Assay.** The galectin-3 used to prepare the standard curve of galectin-3 ELISA was >95% pure as judged by SDS-PAGE. The dose-response curve obtained with this material was linear on a log:log scale from 20 to 625 ng/ml (Fig. 2). When plates were coated with human serum albumin instead of Mac-2 BP or when rat antigalectin-3 antibody M3/38 was substituted with a rat antitumor necrosis factor- $\alpha$  antibody, no signal above background level was observed. The intra-assay and interassay coefficients of variation were less than 12 and 18% throughout the range, respectively. Long-term storage (>6 months) of frozen serum or 1 week of storage at 4°C failed to significantly alter galectin-3 content. No difference in galectin-3 measurements was observed after two freeze-thaw cycles.

**Galectin-3 from Normal Controls and Patients with Cancer.** The levels of galectin-3 in sera of 50 healthy controls varied between 20 and 313 ng/ml (median, 62 ng/ml; 95th percentile, 184.3 ng/ml; Table 1). The 95th percentile of galectin-3 levels (184.3 ng/ml) was arbitrarily taken as the upper limit of normal. There was no significant difference in galectin-3 serum concentration between males (median, 61.5 ng/ml; range, 20–313 ng/ml) and females (median, 64 ng/ml; range, 20–230 ng/ml), nor was there a correlation between serum galectin-3 levels and age or blood group. Serum galectin-3 concentrations of healthy individuals and patients with cancer are summarized in Table 1.

In all types of cancer patients, several cases showed an increase in galectin-3 levels in sera. Overall, galectin-3 serum levels in patients with cancer were significantly higher than those in healthy individuals ( $P = 0.014$ ). In 35 patients with localized or metastatic breast carcinoma, the median galectin-3

concentration in serum was 100 ng/ml (range, 20–620 ng/ml). One (12%) of the 8 patients with local disease and 4 (14%) of the 27 patients with metastatic disease showed serum galectin-3 levels above the upper limit of normal. As a whole, there was no a significant difference between healthy individuals and patients with breast carcinoma. However, a significant difference in serum galectin-3 was seen between breast carcinoma patients with nonmetastatic and those with metastatic disease ( $P < 0.032$ ).

Three (30%) of 10 patients with nonmetastatic gastrointestinal carcinomas but 10 (66%) of 15 with metastatic disease had serum galectin-3 concentrations above the upper limit of normal. Like the breast carcinoma patients, patients with metastatic colorectal carcinomas showed significantly higher galectin-3 serum levels than did those with nonmetastatic disease ( $P < 0.026$ ). Notably, maximum serum concentrations of galectin-3 occurred in patients with metastatic gastrointestinal carcinoma (median, 320 ng/ml; range, 20–950 ng/ml; Table 1). Galectin-3 serum levels did not differ among the various types of gastrointestinal neoplasia.

Thirteen (50%) of 26 patients with metastatic non-small cell lung cancer, 1 (25%) of 4 patients with metastatic melanoma, 1 (25%) of 4 patients with metastatic ovarian carcinoma, and 1 (20%) of 5 patients with stage IV non-Hodgkin's lymphoma showed increased galectin-3 concentrations over the upper normal limit.

In five colorectal carcinoma patients, serum galectin-3 levels were measured before and 2 days after tumor resection. In four of these cases, there was a decrease in the galectin-3 serum concentrations after surgery (Fig. 3). One patient with regional lymph node involvement had a preoperative galectin-3 serum level of 570 ng/ml that dropped to 185 ng/ml within 3 days after surgery. In contrast, in one patient with localized tumor and normal galectin-3 serum level before surgery, no significant change was observed in galectin-3 serum concentration.

## DISCUSSION

In this study, we have determined serum galectin-3 concentrations in patients with various types of cancer. Compared with the levels in healthy individuals, serum galectin-3 levels were significantly higher in subpopulations of patients having each type of tumor. In breast cancer, only about 10% of examined cases showed galectin-3 concentrations of more than the cutoff level; however, the incidence of supranormal levels of galectin-3 was elevated in relation to tumor progression. Serum galectin-3 levels were significantly higher in patients with metastatic disease compared with patients with localized tumors. This tendency, that the increase in serum galectin-3 levels was associated with the occurrence of metastasis, was also observed in gastrointestinal cancer patients.

The biological role of galectin-3 remains elusive. Galectin-3 is found at elevated levels in a variety of neoplastic cells, and several experimental observations suggest that it is involved in tumor metastasis *in vivo* (22, 23). Different modalities have been proposed to explain how this lectin might be involved in the metastatic process: (a) the potential of galectin-3 to interact with extracellular matrix proteins, such as laminin, fibronectin, and vitronectin (24, 25), on one hand, and with cell surface

Table 1 Levels of galectin-3 in the sera of patients and healthy controls

Diagnosis	No. of patients	Galectin-3 (ng/ml)				<i>P</i> <sup>a</sup>
		Median	5th percentile	95th percentile	Range	
Healthy individuals	50	62	20	184.3	20–313	0.014 <sup>b</sup>
Breast cancer	35	100	23.5	478	20–620	
Nonmetastatic	8	40	24.2	177.3	20–170	
Metastatic	27	170	25	502	20–620	0.032 <sup>c</sup>
Gastrointestinal cancer	25	185	20	532.4	20–950	
Nonmetastatic	5	75	20	280	20–328	
Metastatic	20	320	27	684	20–950	0.026 <sup>c</sup>
Lung cancer	26	171.5	21.7	749	20–807	
Melanoma	4	48.5	35.1	214	33–243	
Ovarian cancer	4	94.5	36	333.1	35–366	
Non-Hodgkin's lymphoma	5	50	21.4	187	20–203	

<sup>a-c</sup> *P*s refer to the difference between the cohorts of healthy individuals and all patients with cancer<sup>b</sup> or to the difference between the groups of patients with nonmetastatic and metastatic disease<sup>c</sup>.

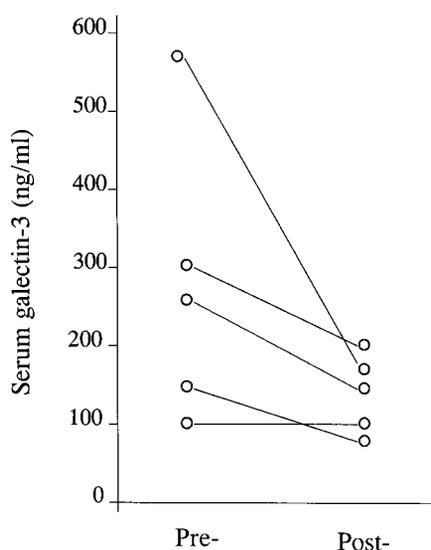


Fig. 3 Serum galectin-3 concentrations in five patients with colorectal carcinoma before (*Pre-*) and 2 days after (*Post-*) removal of the primary tumor.

proteins such as lysosomal-associated membrane proteins (1), on the other hand, suggests that galectin-3 acts as a bridge, linking cells to the extracellular matrix or other cells; (b) the data demonstrating that galectin-3 is able to mediate homotypic cell-cell adhesion through interaction with complementary glycoproteins (26, 27) lend credence to the theory that this lectin is involved in the formation of tumor emboli and dissemination of tumor cells in the circulation; and (c) the recent observations that galectin-3 is able to protect against apoptosis induced by the loss of cell anchorage (anoikis; Refs. 25 and 28) suggest that the expression of galectin-3 in tumor cells may provide a critical determinant for cell survival of disseminating cancer cells in the circulation during metastasis.

The results of the present study could imply that the metastatic spread of malignant tumors involves, among other factors, a higher level of expression of galectin-3 in the circulation. We are proposing that changes in the level of expression of

galectin-3 may favor metastasis by either one or all of the above-mentioned modalities, *i.e.*, by: (a) enhancing the adhesive interactions between tumor cells and the extracellular matrix; (b) promoting tumor cell embolization through increased cell-cell adhesion; and (c) conferring a selective survival advantage to metastatic cells. Alternatively, galectin-3 serum levels may reflect an immune reaction to the tumor load from inflammatory cells that are known to express galectin-3. However, this seems unlikely because we found no correlation between the extent of the inflammatory response in operable breast cancer and further disease progression.<sup>4</sup>

The source of increased serum galectin-3 in cancer patients remains unclear. According to our results, that removal of the tumor decreased serum galectin-3 concentrations, tumor tissues are likely to produce and secrete galectin-3 in sera. However, immunostaining of cancerous tissue with antigalectin-3 antibody showed that galectin-3 was expressed not only on malignant cells but also in macrophages and stromal cells (mainly fibroblasts) near cancer nests, and the stromal cells immediately adjacent to cancer nests have a higher galectin-3 expression in comparison to those cells farther away from the nests.<sup>5</sup> These results suggest that circulating galectin-3 is generated not only by tumor cells but also from peritumoral inflammatory cells and stromal cells.

In summary, the data presented above indicate that the detection of increased galectin-3 levels in the serum of certain patients with cancer may reflect biological aspects of tumor behavior associated with a metastasizing phenotype. Additional studies are warranted to determine the clinical value of circulating galectin-3 in patients with early-stage cancer as a predictor of tumor invasion and metastasis.

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<sup>4</sup> Manuscript in preparation.

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