Transfer to *in vitro* Conditions Influences Expression and Intracellular Distribution of Galectin-3 in Murine Peritoneal Macrophages

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Galectin-3 is a β-galactoside-binding lectin that has been implicated in numerous physiological processes, including mRNA splicing, cell differentiation, tumor metastasis and the stress response. We have studied effects of transfer of resident murine peritoneal macrophages to *in vitro* conditions on galectin-3 in different cell compartments. Galectin-3 was purified by immunoprecipitation with rat monoclonal antibody M3/38, and analyzed by immunoblotting using the same antibody. Transfer to *in vitro* conditions nearly doubled the total amount of galectin-3 in cells, and caused significant alterations in its intracellular distribution, indicating that galectin-3 is involved in the adaptation of peritoneal macrophages to *in vitro* conditions.

Introduction

Numerous lectins exist in various tissues where they function as specific receptors for carbohydrate moieties of glycoconjugates on other cells, or in the extracellular matrix. They are thought to be involved in regulation of numerous processes, from fertilization and development, to inflammation and the formation of memory. A family of soluble lectins that share affinity for β-galactosides has been named galectins, and the prominent member of this family is galectin-3, a ≈32 kDa lectin first described by Ho and Springer (1982).

Galectin-3 is expressed by many cell types including monocytes, macrophages, mast cells and all granulocyte lines. Various functions have been suggested for galectin-3, and it appears to be involved in numerous biological processes, including growth regulation, cell differentiation, adhesion, neoplastic transformation and tumor metastasis. It has also been suggested that galectin-3 is involved in the cellular stress response, but the results from various cell types are somewhat conflicting.

Galectin-3 can be found in cytoplasm, nucleus, as well as on the cell surface. Although galectin-3 is synthesized without a signal sequence and lacks a transmembrane sequence, it is secreted from cytoplasm and it can be found both in the extracellular space and on the outer side of the cell membrane, where it is bound to specific glycoprotein receptors. Intracellular galectin-3 in 3T3 fibroblasts has been suggested to play a role in transcription control and its synthesis was shown to be up-regulated in proliferating and transformed cells. Galectin-3 is involved in pre-mRNA splicing and is defined as a splicing factor. The expression of galectin-3 has been found to be markedly increased in peritoneal macrophages from thioglycolate-stimulated mice as compared with resting macrophages.

Many studies of various aspects of galectin-3 function involved primary cell cultures. We have noticed that simple transfer of cells from *in vivo* to *in vitro* conditions can influence galectin-3, and here we report that stress caused by transfer to *in vitro* conditions significantly changes expression and the intracellular distribution of galectin-3 in resting murine peritoneal macrophages.

Materials and Methods

Materials

All chemicals used were of analytical grade and obtained from Sigma (St. Louis, MO, USA). Protein G-Sepharose® was obtained from Pharmacia-

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Amersham (Uppsala, Sweden), polyvinylidene difluoride (PVDF) membranes (Immobilon-P) from Millipore Corp. (Bedford, MA, USA), and heat-inactivated fetal calf serum (FCS) from Life Technologies (Rockville, MD, USA). Rat monoclonal antibody M3/38 (anti-galectin-3) was kindly provided by Dr. I. Rosenberg (Harvard Medical School, Boston, MA). Goat anti-rat IgG conjugated to alkaline phosphatase was obtained from Medac (Hamburg, Germany).

Animals

All experiments were performed on male BALB/c mice, 14 weeks old. The animals were maintained under 12 h light/dark cycle at temperature 21±2° C and humidity of 50±10%. They were given water and food *ad libitum* (standard laboratory pellets, PLIVA d.d., Zagreb, Croatia) and regular animal care.

Peritoneal exudate cell preparation

Macrophages were collected by peritoneal lavage with 5 ml of RPMI 1640 medium (Gibco, Paisley, Scotland) containing 100 U/ml heparin (sodium benzylalcohol) per mouse and the cell suspensions were pooled. After centrifugation at 600×*g* for 5 min, pellet was resuspended in *culture medium* consisting of 15% phosphate buffered saline (PBS), 1% fetal calf serum (FCS), and 0.01% 2-mercaptoethanol in RPMI 1640 medium.

Cells collected from 16 animals were pooled and the viability was determined by Trypan-blue staining. 1×10^7 living cells were transferred into each of 3 plastic tissue-culture treated Petri-dishes (Greiner 60/15, C. A. Greiner, Nürtingen, Germany). Control cells (1×10^7) were washed twice with ice-cold *cell washing buffer* (CWB; PBS, pH 7.4 supplemented with 5% FCS and 0.05% 2-mercaptoethanol). Although erythrocytes were not macroscopically visible in the samples, the small amount of residual erythrocytes that could have entered peritoneal cavity due to micro-injuries were removed by lysis in 0.155 mol/l NH_4Cl, 0.01 mol/l $KHCO_3$, 0.13 mmol/l EDTA at 4° C for 5 min. Remaining macrophages were then washed twice with CWB and resuspended in the final volume of 1 ml ice-cold CWB.

The cells in Petri-dishes were pre-incubated for 30 min at 37° C in humidified atmosphere of 5% CO_2 and 95% air. The culture media was removed and the adhered cells were washed five times with warm, sterile CWB. The equal volumes of fresh culture medium were added in each Petri-dish and incubation was continued for (i) 1 h, (ii) 2.5 h and (iii) 23.5 h. At the end of incubation culture medium was collected and centrifuged at 600×*g* for 10 min. The supernatants were collected and PMSF (phenylmethylsulfonyl fluoride) was added to the final concentration of 1 mmol/l. An equal proportion (3 ml) of each supernatant was lyophilized and redissolved in 0.5 ml bidistilled water. The cell monolayers were washed twice with ice-cold CWB. The cells were scraped from the culture dishes using cell scraper and isolated by centrifugation at 600×*g* for 5 min. The pellet was resuspended in final volume of 1 ml ice-cold cell wash buffer. The viability of cell was determined by Trypan blue exclusion and measurement of LDH activity in cells and the culture medium.

Subcellular fractionation

For preparation of the cell homogenate an aliquot (0.1 ml) from the suspensions of uncultivated and cultivated cells was removed and centrifugated at 600×*g* for 5 min. The cell pellet was resuspended in 0.04 ml of *lysis buffer* (0.5% (v/v) Triton X-100 in RSB; reticulocyte saline buffer; 0.01 mol/l tris(hydroxymethyl)aminomethane/HCl (hereafter referred to as Tris/HCl), pH 7.4, 3 mmol/l $MgCl_2$, 0.01 mol/l NaCl) containing 1 mmol/l PMSF and additionally homogenized in ultrasound disintegrator (MSE, 100 W, London, England) for 2 min (amplitude 5 μm, ν=22 kHz).

The remaining part of cell samples was centrifuged at 600×*g* for 5 min. The pellet was diluted in 1 ml of 0.3×RSB containing 1 mmol/l PMSF and homogenized in ultrasonic desintegrator for 2 min (amplitude 5 μm, ν=22 kHz). Nuclear fraction obtained by centrifugation at 600×*g* for 10 min was additionally homogenized by sonication for 2 min (amplitude 50 μm, ν=22 kHz) in 0.3×RSB containing 1 mmol/l PMSF. The supernatant was centrifuged at 100 000×*g* for 1 hour (Beckman Instruments, Inc., Palo Alto, CA, USA; TLA-100 rotor). The supernatant was saved as the cytosolic fraction. The pellet was washed once with 0.3×RSB containing 1 mmol/l PMSF and the wash was added to the cytosolic fraction. The final pellet was

extracted in *lysis buffer* containing 1 mmol/l PMSF and sonicated for 2 min (amplitude 4 μm, ν=25 kHz). After spinning down insoluble material (at 13 200×*g* for 20 min, Eppendorf centrifuge 5414S, Eppendorf, Hamburg, Germany), the supernatant was saved as the membrane fraction.

Immunoprecipitation and immunoblot analysis of galectin-3

Membrane, nuclei and cytosolic fractions were pre-incubated with culture supernatant from hybridoma M3/38 (anti-galectin-3) at final dilution 1:25 for 1 h at 4° C. A 1:5 slurry of protein G-Sepharose (Pharmacia, Uppsala, Sweden) in PBS was added to the samples (1:3 (v/v)) and incubation was carried out by shaking overnight at 4° C. The protein G-Sepharose beads were washed four times with *wash buffer* (0.05 mol/l Tris/HCl, pH 8.2, 0.5 mol/l NaCl, 5 mmol/l EDTA, 0.02% NaN_3, 0.5% Triton X-100, 0.5% sodium desoxcholate, 0.1% SDS; sodium dodecyl sulfate) and than resuspended and boiled for 5 min in 15 μl of 3×SDS-polyacrylamide gel electrophoresis (PAGE) *sample buffer* (0.15 mol/l Tris/HCl, pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, 0.05% bromophenol blue). The last step was repeated tree times and supernatants were pooled.

SDS-polyacrylamide gel electrophoresis was carried out in 12% polyacrylamide gel according to Laemmli (1970). The separated components were transferred onto PVDF – membrane by semi-dry transfer technique. After protein transfer, the membranes were incubated with 3% BSA (bovine serum albumin) in TBS (Tris buffered saline; 0.05 mol/l Tris/HCl, pH 7.5, 0.15 mol/l NaCl) overnight at room temperature. Detection of galectin-3 on the membranes was achieved by incubation with primary antibody (culture supernatant from hybridoma M3/38 diluted 1:25 in 3% BSA in TBS) at room temperature for 3 h followed by five washes in TBST (0.1% Tween-20 in TBS) for 10 min each. The membranes were incubated with alkaline phosphatase-labeled goat anti-rat IgG diluted 1:2000 in TBS for 1 h at room temperature and washed with TBST five times for 10 min each. Galectin-3 was visualized with 0.02 g/l 5-bromo-4-chloro-3-indolyl phosphate and 0.04 g/l nitro-blue tetrazolium in 0.05 mol/l Tris/HCl, pH 9.5, 0.1 mol/l NaCl, 5 mmol/l $MgCl_2$. The membranes were incubated in dark until the color developed.

A Mr≈32 000 protein reactive with M3/38 monoclonal antibody was considered to be positively identified galectin-3. Quantification of the bands corresponding to galectin-3 from immunoblots was performed by densitometric analysis of stained membranes in LKB Ultroscan XL® laser densitometer (Pharmacia, LKB, Bromma, Sweden). For maximal precision, two-dimensional scanning (0.4 mm resolution on the X axis, and 0.2 mm resolution on the Y axis) was performed. Scans were analyzed in Gel Scan™ software (Pharmacia, LKB, Uppsala, Sweden).

Other procedures

Protein concentrations in cell homogenates were determined by the method of Lowry. Lactate-dehydrogenase (LDH) activity was determined using a colorimetric assay as described by the manufacturer (Sigma, St. Louis, MO, USA).

Results

The influence of stress caused by transfer from *in vivo* to *in vitro* conditions on galectin-3 was analyzed by comparing levels of galectin-3 in freshly isolated murine resident peritoneal macrophages with those cultured for 1½, 3 and 24 h in plastic dishes. Over 98% of adhered cells were viable, and as judged by both Trypan blue staining, and the activity of lactate-dehydrogenase in the culture medium, there was no decrease in viability during 24 h of cultivation (data not shown). In the same time there was progressive increase in overall galectin-3 levels with 18%, 63% and 94% increase after 1½, 3, and 24 h in culture (Figs. 1,2). The increase of galectin-3 in the cells was accompanied

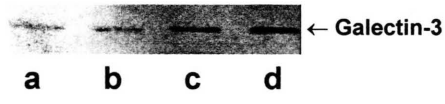

Fig. 1. An example of galectin-3 quantitation in macrophage samples. Murine resident peritoneal macrophages have been isolated by peritoneal lavage and cultivated for 0 (a), 1.5 (b), 3 (c), and 24 h (d). The cells were homogenized and proteins (10 μg per lane) separated by SDS polyacrylamide electrophoresis. Following transfer to PVDF membranes, galectin-3 was identified with M3/38 monoclonal antibody.

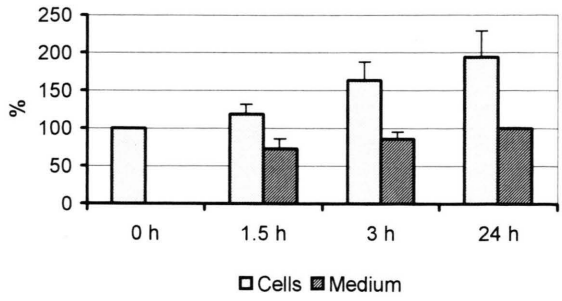

Fig. 2. Effects of stress due to transfer to *in vitro* conditions on galectin-3. Murine resident peritoneal macrophages have been transferred to *in vitro* conditions and the amount of galectin-3 in cell homogenates, and in the culture medium was measured using immunobloting with M3/38 monoclonal antibody. The results are expressed as percentages of initial (pre-cultivation) values for cells, and final values for culture medium to enable comparison of results from three independent experiments.

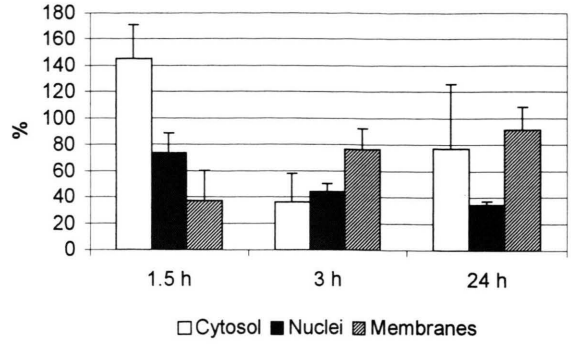

Fig. 3. Redistribution of galectin-3 within peritoneal macrophages during transfer to *in vitro* condition. Murine resident peritoneal macrophages (1×10^7) were transferred to *in vitro* conditions, and following different cultivation times, nuclear, cytoplasmatic and membrane fractions were separated by ultracentrifugation. Galectin-3 was purified by immunoprecipitation, and its amount measured by immunobloting with M3/38 monoclonal antibody. The amount of galectin-3 in resting macrophages was set to 100% and the changes that occurred during cultivation were expressed as percentages of this initial (pre-cultivation) value. All results are averages from three independent experiments involving total of 48 mice.

by a concurrent increase of galectin-3 secreted to the culture medium. The rate of secretion varied considerably. Most of galectin-3 was released within the first 1½ h, with only slight further increase in the concentration of galectin-3 in the culture medium till the end of the 24th h incubation.

Galectin-3 exists both in the nucleus and the cytoplasm, as well as on the cell membrane. To see whether stress caused by transfer to *in vitro* conditions influenced subcellular distribution of galectin-3, we have separated nuclear, cytoplasmatic and membrane fractions by differential centrifugation and analyzed levels of galectin-3 in these fractions, as well as in the culture medium. After 1½ h, which included the process of adhesion, completed within the first ½ h, and 1 h of cultivation in adhesive conditions, the level of galectin-3 in whole cell homogenate increased only slightly. However, there was a significant redistribution of galectin-3 within the cell. The most prominent change was in the membrane fraction where the amount of galectin-3 decreased to only 37% of the initial value. Another compartment where galectin-3 was decreased was the cell nucleus, but this decrease was not as strong as on the membranes (Fig. 3). The decrease of galectin-3 in the nuclei and on the membranes was accompanied with the concurrent increase of galectin-3 in the cytoplasm (45% increase), as well as with the appearance of a significant amount of galectin-3 in the culture medium.

After the initial decrease of galectin-3 in the membrane fraction, further cultivation was associated with a continuous increase in membrane galectin-3, which reached 91% of the initial amount after 24 h (Fig. 3). Changes of galectin-3 in cytosolic fraction were also very interesting. Initial 45% increase in the first 1½ h of culturing was followed by a rapid, nearly five-fold decrease to 36% of the initial amount after 3 h. During the next 21 h there was an increase to approximately 77% of the pre-cultivation level.

Discussion

By measuring overall levels of galectin-3 in peritoneal macrophages during first 24 h of cultivation we have found that stress caused by removal from physiological conditions, and the adaptation to *in vitro* culture are associated with the increase of galectin-3 concentration. Similar increase was reported to occur during *in vitro* cultivation of peripheral blood monocytes, and it was suggested that changes in galectin-3 level might be associated with differentiation of monocytes into macro-

phages. However, we have observed the same effect in fully differentiated peritoneal macrophages, suggesting that in addition to macrophage differentiation, the increase in galectin-3 might be also associated with the cellular response to changed environmental conditions.

Peritoneal macrophages from thioglycollate-stimulated mice and some murine macrophages cell lines specifically secrete galectin-3 to the extracellular medium. However, galectin-3 mRNA lacks the consensus signal sequence for transport out of the cell, and the exact secretion mechanism is not known. Our results suggest that the rate of secretion of galectin-3 varies significantly during the first 24 h of cultivation. There is a burst of galectin-3 release during the first 1½ h, which is followed by much slower, but continuous release in the following hours.

Galectin-3 is attached to the extracellular side of the macrophage membrane through specific interactions with membrane glycoproteins Mac-1, CD98 and Mac-3. In the first 1½ h after transfer onto the plastic surface the amount of galectin-3 on the cell membrane decreases rapidly, probably due to the release of galectin-3 into the culture medium. The exact mechanism of this release is not known, but it probably includes some form of molecular alteration of the carbohydrate – lectin interaction, either by changing the carbohydrate structures, or by modification of the galectin-3 to alter its binding properties.

Marked changes in concentration of galectin-3 were also observed in cell nuclei and the cytoplasm, suggesting that the stress response induced by transfer from *in vivo* to *in vitro* conditions involves specific changes in the regulation of expression and intracellular distribution of galectin-3. Although the role of galectin-3 in the cellular stress response processes is not known, changes observed during adaptation to culturing condition are far from negligible, and should not be ignored when studying effects of different parameters on the physiology of galectin-3 and other molecules in primary cell cultures.

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