Galectin-7 (PIG1) Exhibits Pro-apoptotic Function through JNK Activation and Mitochondrial Cytochrome c Release*\(\text{S}\)

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Galectin-7 is normally expressed in all types of stratified epithelia, but is significantly down-regulated in squamous cell carcinomas. This protein was recently found to be highly inducible by p53 in a colon carcinoma cell line, DLD-1, and designated as PIG1 (for p53-induced gene 1). We studied transfectants of HeLa and DLD-1 cells ectopically expressing this protein and found that they were more susceptible to apoptosis than control transfectants. This was observed in apoptosis induced by mechanistically distinct stimuli, suggesting that galectin-7 acts on a common point in the apoptosis signaling pathways. Further analyses of actinomycin D-induced apoptosis demonstrated that galectin-7 expression causes enhanced caspase-3 activity and poly(ADP-ribose) polymerase cleavage, and the potentiation of apoptosis by galectin-7 was completely abrogated by a caspase inhibitor, benzylxoycarbonyl-Val-Ala-Asp-fluoromethyl ketone. In addition, galectin-7 transfectants displayed accelerated mitochondrial cytochrome c release and up-regulated JNK activity upon apoptosis induction. Several lines of evidence indicate that the effect on apoptosis is not due to the lectin functioning extracellularly through interactions with cell surface glycoconjugates. In fact, this lectin is found to localize in nuclei and cytoplasm of the transfectants and the transformed keratinocyte line HaCaT. Therefore, galectin-7 is a pro-apoptotic protein that functions intracellularly upstream of JNK activation and cytochrome c release. DNA microarray analysis revealed genes that are differentially expressed between galectin-7 and control transfectants. Some of them are potentially contributory to this lectin’s proapoptotic function and these include redox-related genes monoamine oxidase B, ryanodine receptor 2, and glutathione S-transferase Mu 3.

Galectins are a growing family of β-galactoside-binding animal lectins (1), which have been shown to have diverse biological functions, including regulation of cell adhesion, cell growth, and apoptosis (2–5). Family members have sequence similarity to each other and are evolutionarily highly conserved, because they are present in organisms from nematodes to vertebrates. These proteins all lack a classical signal peptide and are mainly localized in the cytoplasm, but they can be secreted, possibly through a nonclassical secretory pathway (6). To date 12 mammalian galectins have been cloned and characterized, but a large number of family candidates have been identified in nucleic acid data bases (4).

Galectin-1 and -3 have been extensively characterized, and the studies have suggested important roles for these proteins in cell-cell and cell-extracellular matrix interactions (7–10), which are mediated by their carbohydrate-binding properties. In addition, galectin-1 has been shown to induce apoptosis in activated T cells and thymocytes (11, 12), in a fashion that is dependent on the lectin function of the molecule (13). However, this protein has also been shown to suppress cell growth and block cell cycle progression of fibroblasts in a carbohydrate-independent manner (14). Induction of apoptosis has also been observed when galectin-1 is overexpressed in a prostate cancer cell line (15). On the other hand, galectin-3 has been shown to be anti-apoptotic when ectopically expressed in Jurkat T lymphoma cells (16) and breast carcinoma cells (17, 18).

Galectin-7 is a 15-kDa protein with a single carbohydrate recognition domain initially identified in human epidermis (19, 20). It is expressed in all types of stratified epithelial cells found in the skin, tongue, esophagus, and Hassal’s corpuscles in the thymus (21), but is strikingly down-regulated in SV40-transformed human keratinocytes (19) and squamous cell carcinomas (21). Thus, this protein may function in the maintenance of the normal phenotype of these epithelial cells. Recently, it has been shown that the galectin-7 mRNA is highly induced by p53, and it has been postulated that the protein might be related to the pro-apoptotic function of p53 (22). More recently, galectin-7 has been shown to be rapidly inducible in skin keratinocytes exposed to UV irradiation in association with p53 stabilization, and the increased levels of the protein was mainly found in sunburned apoptotic keratinocytes (23). Furthermore, overexpression of galectin-7 in a squamous cell line by transient transfection resulted in increased apoptosis, suggesting a pro-apoptotic function of this protein (23).

To investigate the mechanism of galectin-7’s pro-apoptotic function, we have generated and studied stable transfectants of HeLa and DLD-1 cells ectopically expressing this protein. We found that galectin-7 expression results in increased apoptosis induced by stimuli that operate through distinct mechanisms, suggesting that galectin-7 acts on a common point in
the apoptosis signaling pathways. Additional mechanistic analyses revealed that the accelerated apoptosis caused by galectin-7 is caspase-dependent and associated with mitochondrial cytochrome c release as well as heightened JNK activation. Finally, DNA microarray analysis of mRNA differentially expressed in galectin-7 and control transfectants showed up-regulation of several gene groups suggestive of galectin-7's involvement in tissue remodeling processes. The analysis also revealed genes that are potentially connected to the pro-apoptotic function of galectin-7, and these include a set of redox-related genes that are either up- or downregulated as a result of the ectopic expression of galectin-7.

**EXPERIMENTAL PROCEDURES**

*Cell Lines and Reagents—* HeLa and DLD-1 cells were obtained from ATCC (Manassas, VA). HaCaT cells were kindly provided by Dr. N. Fusseneg (Deutsche Krebsforschungszentrum, Heidelberg, Germany) (24). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Actinomycin D, etoposide, camptothecin, cycloheximide, digitonin, and human recombinant TNF-α were from Sigma. zVAD-fmk (benzoyloxycarbonyl-Ala-Val-Asp-fluoromethyl ketone) was from Keynote (Seattle, WA). Anti-caspase-3 (C-32P32) mouse monoclonal antibody (clone 19) was from Transduction Laboratories (Lexington, KY). Antibodies against PARP (clone C2–20), caspase-8 (clone B9–2), and cytochrome c (clone THS.2CL2) were from BD PharMingen (San Diego, CA). Antibodies against JNK1 (polyclonal goat and mouse sera) and p38 (polyclonal rabbit serum) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). CRP-conjugated mouse anti-goat IgG and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). HRP-conjugated goat anti-rabbit IgG was from Bio-Rad. HRP-conjugated goat anti-mouse IgG was from Chemicon (Temecula, CA).

Galectin-7 and -9 cDNA were obtained as expressed sequence tag clones from ATCC for antibody production. Galectin-7 and -9 cDNA were cloned into pET25b (Novagen, Madison, WI) and pTrcHis2C (Invitrogen), respectively. These recombinant proteins were used to produce goat and rabbit antibodies against galectin-7 and -9, respectively. For transfection, cDNA was cloned into pEF1-neo (25).

**Generation of Stable Transfectants—** Cells were cultured at 37 °C in 25-cm well-dish for 6-week dishes for 1 day and then transfected with 2.5 µg of pEF1-neo-galectin-9 or pEF1-neo-galectin-7 using Superfect (Qiagen, Inc., Valencia, CA), according to the manufacturer's instructions. Stable transfectants were selected in medium containing 1 mg/ml G418 (Calbiochem, San Diego, CA). The medium was changed every 3–4 days. When numerous G418-resistant colonies appeared (usually after 2 weeks), the cells in the colonies were mixed. The mixed population of G418-resistant cells was stored in liquid nitrogen until used for studies. After the aliquots were thawed, the cells were cultured in the presence of 0.3 mg/ml G418 and used in experiments within 2 months to avoid possible phenotypic changes after long term culture.

**Immunoblotting—** Cells were solubilized in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline, pH 7.4) containing 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin at 4 °C for 20 min. After centrifugation at 10,000 × g for 15 min, the supernatant was removed and the protein concentration was determined by BCA assay (Pierce). Samples containing 30–60 µg of proteins were boiled for 5 min in SDS sample buffer and resolved by SDS-PAGE under reducing conditions. For the analysis of galectin-9 expression, the lysates were mixed with lactate- Sepharose 4B and the absorbed proteins were resolved by SDS-PAGE. For immunoblotting of both galectins, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for more than 30 min at room temperature and probed overnight at 4 °C with appropriate dilutions of the primary antibodies in TBS-T, followed by incubation with HRP-conjugated secondary antibodies. After extensive washes with TBS-T, proteins were visualized by the ECL protein detection system (Amersham Biosciences, Inc.).

**Assessment of Apoptosis—** Apoptosis was induced by incubating cells with DMEM containing 10% FBS in the presence of actinomycin D, TNF-α plus cycloheximide, etoposide, or camptothecin at the indicated concentrations. For the induction of apoptosis with ultraviolet (UV) irradiation, the culture medium was aspirated and the cells were exposed to UVB using a UV cross-linker apparatus (Fisher Scientific, Pittsburgh, PA) at 190 J/m2. After UV irradiation, fresh DMEM containing 10% FBS was added to the cells, and the cells were cultured for the indicated periods. After induction of apoptosis, both detached and attached cells were harvested and combined. Mitochondrial transmembrane potential was measured using the DiOC6 (3) and JC-1 (Molecular Probes, Eugene, OR) at 190 J/m2. After UVB irradiation, fresh DMEM containing 10% FBS was added to the cells, and the cells were cultured for the indicated periods. After induction of apoptosis, both detached and attached cells were harvested and combined. Mitochondrial transmembrane potential was measured using the DiOC6 (3) and JC-1 (Molecular Probes, Eugene, OR) at 190 J/m2.
RESULTS

Galectin-7 Expression Confers Susceptibility to UV-induced Apoptosis in Tumor Cells—We generated galectin-7 and -9 transfectants from HeLa cells, which are commonly used as a model for studying apoptosis (e.g. Ref. 26). Immunoblot analysis showed that the level of galectin-7 in galectin-7 transfectants was lower than that in HaCaT, which is a spontaneously transformed keratinocyte line constitutively expressing this protein (Fig. 1A). Galectin-9 transfectants express galectin-9 but not galectin-7, and control transfectants express neither protein (Fig. 1B). Galectin-7 transfectants showed a slightly slower growth rate than control transfectants or parental HeLa cells, particularly when the cells were cultured in suboptimal conditions (e.g. low concentration of FBS) (data not shown). The cell cycle distribution of the transfectants was studied, but no difference was found between galectin-7 and control transfectants (data not shown). Therefore, we suspected that the lower growth rate might be related to an altered apoptosis susceptibility of galectin-7 transfectants. To test this possibility, we treated these cells with UV irradiation, and the degree of apoptosis was assessed by flow cytometry following FITC-annexin V/PI double staining. As shown in Fig. 2A, after UV treatment, galectin-7 transfectants demonstrated enhanced apoptosis, as indicated by the increase in the total percentage of cells stained by annexin V, compared with galectin-9 or control transfectants. The difference is mainly displayed in the annexin V-positive fraction, which corresponds to cells in the early phase of apoptosis (29) (Fig. 2B, upper panel), and not by the PI-positive fraction, which corresponds to cells in the late phase of apoptosis or necrosis (Fig. 2B, lower panel). Morphological changes characteristic of apoptosis, such as membrane blebbing and nuclear condensation, were more pronounced in galectin-7 transfectants than control cells. Trypan blue staining 24 h after UV irradiation showed that the total number of nonviable cells was ~2-fold higher in galectin-7 transfectants compared with control transfectants (data not shown).

Galectin-7 Transfectants Are More Susceptible to Apoptosis Induced by Various Agents—We then examined the apoptosis susceptibility of HeLa transfectants upon treatment with various agents. When actinomycin D (1 μM) was used as the apoptosis inducer, significantly more cells became annexin V-positive at 6 and 12 h in galectin-7 transfectants compared with control transfectants (Fig. 3A). The difference in apoptosis susceptibility between galectin-7 and control transfectants was also observed when these cells were exposed to etoposide (Fig. 3B), camptothecin (Fig. 3C), and a combination of TNF-α and cycloheximide (Fig. 3D).

Polyak et al. reported that overexpression of p53 in DLD-1, a colon carcinoma cell line, results in induction of many genes and galectin-7 (p53-induced gene 1, PIG1) is among the most highly induced (22). To test the effect of galectin-7 expression in this cell line, we obtained stable transfectants expressing galectin-7. Immunoblotting showed relatively high expression of galectin-7 in the transfectants compared with HaCaT cells (Fig. 1A). As shown in Fig. 4, actinomycin D-induced apoptosis was potentiated in galectin-7 transfectants (Fig. 4A). We noted that, even in cells not treated with actinomycin D, there was a significantly higher percentage of cells that were annexin V-positive among galectin-7 transfectants compared with control transfectants. This portion was dominated by cells stained positively by both annexin V and PI (Fig. 4B), and thus in late apoptosis or necrosis. We also obtained similar results in apoptosis induced by camptothecin and etoposide (data not shown).

Galectin-7 Transfectants Exhibit Accelerated Caspase-3 Activation and PARP Cleavage upon Apoptosis Induction—To identify a common apoptosis signaling point at which galectin-7 might act, additional analyses were conducted using actinomycin D as the apoptosis inducer. First, we examined the cleavage of procaspase-3 and its substrate PARP. Caspase-3 is a cell death executor that is crucially activated in apoptosis by various stimuli, leading to the cleavage of many substrates, including PARP (30, 31). As shown in Fig. 5, when the cells were exposed to actinomycin D for 6 and 12 h, the cleavage of procaspase-3 was enhanced in galectin-7 transfectants over control transfectants, as demonstrated by the decrease in the intensity of the procaspase-3 signal. The enhanced procaspase-3 cleavage was accompanied by increased PARP cleavage. The results indicate that galectin-7 expression results in accelerated kinetics of apoptosis.

In contrast, the protein level of procaspase-8 was unaltered in either galectin-7 or vector-transfected cells, upon exposure to actinomycin D for 12 h. This is consistent with the fact that caspase-8 is not a primary mediator of apoptosis induced by...
this drug (26). However, at 24 h, procaspase-8 was no longer detectable in both galectin-7 and control transfectants, suggesting that cleavage had occurred by this time, probably by the mechanism of post-mitochondrial activation of caspases (32). The results suggest that galectin-7 does not function through caspase-8 activation.

**Galectin-7-mediated Apoptotic Susceptibility Is zVAD-fmk-dependent**—Next, we tested whether the potentiated apoptotic susceptibility induced by galectin-7 is caspase-dependent. Cells were exposed to actinomycin D in the presence of the caspase inhibitor zVAD-fmk, and annexin V-stained cells were analyzed by flow cytometry. As shown in Fig. 6, the addition of zVAD-fmk inhibited apoptosis in both control and galectin-7 transfectants, suggesting that actinomycin D-induced apoptosis is caspase-dependent, which is consistent with a previous report (26). More importantly, because apoptosis in galectin-7-transfected cells was completely abrogated in the presence of zVAD-fmk, the results suggest that caspase-independent pathways are not activated in galectin-7 transfectants in response to actinomycin D.

**Mitochondrial Cytochrome c Release Is Up-regulated in Galectin-7 Transfectants**—We then analyzed galectin-7’s effect upstream of caspase-3 activation. Because cytochrome c release is known to play a central role in the apoptosis-signaling pathways leading to caspase-3 activation, we tested whether cytochrome c release is affected in cells expressing galectin-7. As
shown in Fig. 7, as early as 6 h after exposure to actinomycin D, cytochrome c release to the cytosol. Significantly more cytochrome c was released in galectin-7 transfectants compared with control cells at the 6-, 9-, and 12-h time points. Because the same result was obtained in apoptosis induced by the combination of TNF-α/H9251 and cycloheximide (data not shown), which is a mechanistically different stimulus, this up-regulated cytochrome c release is possibly a common mechanism for the apoptosis susceptibility induced by galectin-7. Galectin-7 thus appears to function upstream of cytochrome c release. The pan-caspase inhibitor zVAD-fmk had no inhibitory effect on cytochrome c release observed in control transfected cells nor on the enhanced release in galectin-7 transfectants (Fig. 7). The results suggest that caspases are not involved in the apoptosis signaling pathway upstream of cytochrome c release through which galectin-7 functions.

Up-regulation of the JNK Pathway in Galectin-7 Transfectants—MAPks are kinases that respond to various extracellular stimuli and transmit cellular responses by cascades of phosphorylation events (33–35). They are composed of three major subfamilies, namely extracellular signal-regulated kinase, p38, and JNK. A number of different stimuli can activate these...
MAPKs, leading to cell proliferation, differentiation, and apoptosis. Recently, interrelationships between cytochrome c release and MAPK pathways have been reported (36–38), and MAPKs appear to work upstream of cytochrome c release. Thus, we wished to determine whether the MAPK signaling pathways are involved in the galectin-7’s pro-apoptotic manifestation. Following apoptosis induction by actinomycin D, JNK and p38 activities were assayed using GST-c-Jun and ATF-2, respectively, as substrate. The same membranes were re-probed with anti-JNK and anti-p38 antibodies and visualized by HRP-conjugated secondary antibodies and the ECL system. The results are representative of three independent experiments.

Galectin-7 Localizes in the Nucleus and the Cytoplasm—The primary translation products of all galectins do not contain a classical signal peptide and, therefore, these proteins reside normally intracellularly (39). However, galectin-1 and -3 are known to be secreted from the cell (40–43) and exist extracellularly (44, 45). Therefore, a possibility exists that galectins mediate extracellular functions. Indeed, it has been shown that recombinant galectin-1 and -9 induce apoptosis when added exogenously to thymocytes (12, 46), presumably by binding to and causing aggregation of cell surface glycoproteins through lectin-carbohydrate interactions (47). Thus, it is possible that galectin-7 is secreted from cells or released by damaged cells undergoing apoptosis and enhances apoptosis by interacting with cell surface apoptosis receptors. However, treatment of cells with 20 mM lactose, a concentration known to inhibit galectin-carbohydrate interactions (10), had no effect on the enhanced apoptosis susceptibility of galectin-7 transfectants, compared with treatment with 20 mM sucrose, which does not inhibit the galectin activities (data not shown).

Next, we examined the ability of recombinant galectin-7 to induce apoptosis in HeLa cells when added to the culture medium of the cells. The recombinant galectin-7 up to 1 μM had no effect, but, at higher doses, it induced appreciable levels of apoptosis (data not shown). This may be attributable to the binding of the lectin to cell surface receptors involved in apoptosis. However, it is doubtful that such a large amount of galectin-7 exists in the culture media of the transfected cells even during apoptosis, where the intracellular proteins could be released. This is because galectin-7 is undetectable in the culture medium in either UV-treated or nontreated galectin-7-transfected HeLa cells by immunoblot analysis, which has a sensitivity as low as ~1 ng/ml. In addition, immunostaining of galectin-7 transfectants undergoing apoptosis showed no evidence for localization of galectin-7 in the cell membrane (data not shown). We also tested the effect of the medium obtained from a culture of galectin-7-transfected HeLa cells following UV irradiation. This medium, as well as that from similarly treated control transfectants, did not induce apoptosis when added to HeLa cells (data not shown). Therefore, it is unlikely that the released lectin is responsible for the enhanced apoptosis observed in galectin-7-transfected cells.

We thus analyzed the intracellular localization of galectin-7 by confocal microscopy. As shown in Fig. 9, both endogenous galectin-7 in HaCaT cells and transfected galectin-7 in HeLa cells localize predominantly in the nuclei. There was also positive staining in the cytosol, especially around the densely stained nuclei in some cells, but there was no distinct staining on the plasma membrane. No galectin-7 staining was observed in control transfectants (data not shown). We also recognized a similar pattern of staining in galectin-7-transfected DLD-1 cells (data not shown).

Gene Array Experiments Show Modulation of Several Gene Groups in Galectin-7-transfected HeLa Cells—Information about genes that are up- or down-regulated upon galectin-7 expression is of great value, as it is possible that such genes may contribute to the functional phenotypes of cells or may cooperate with galectin-7 in manifestation of its function. Recent developments of DNA microarray technologies have facilitated very large scale analysis of gene expression. Therefore, we carried out DNA microarray analysis to identify genes that are altered because of galectin-7 expression. cRNA from galectin-7-and vector-transfected HeLa cells were hybridized to Afinymetrix U95Av2 GeneChips, which contain ~12,000 human gene transcripts. Changes in gene expression were analyzed by
a pairwise comparison from the computer-processed data between vector- and galectin-7-transfected HeLa cells. We identified a subset of genes whose expression were up- or down-regulated at least 3-fold upon expression of galectin-7 in duplicate experiments, and they are listed in Table I. In this list, the number of up-regulated genes was far greater than down-regulated ones. Among them galectin-7 itself is the one that is the most up-regulated, attesting to the validity of the analysis. As a reference, other galectin family genes found on the GeneChips were listed in Table I. The expression levels of these galectins were not significantly different between vector- and galectin-7-transfected HeLa cells. A functional categorization of the genes in the list identifies several distinct groups of genes. The first group of up-regulated genes encodes proteins that are components of the extracellular matrix, including procollagen α2(V), fibrillin, osteonidogen, vitronectin, and α1 type XV collagen. The second contains metabolic enzymes. Many are mitochondrial enzymes and are particularly interesting with respect to apoptosis susceptibility. They include monoamine oxidase B, which is a mitochondrial outer membrane enzyme catalyzing oxidative deamination of biogenic amines. The resultant product, $\text{H}_2\text{O}_2$, contributes to an increase of steady state level of reactive oxygen species (48, 49). The third group consists of secreted proteins including thyroid-stimulating hormone α subunit, neutrosensin, fibroblast growth factor homologue factor 2, semaphorin E, dickkopf-1, connective tissue growth factor-related protein (WISP-2), and bone morphogenetic protein 5. Most of this group of genes are involved in cell growth/development and differentiation. The fourth group encodes proteins involved in signal transduction, including G protein γ-11 subunit, tyrosine phosphatase, and MAP kinase phosphatase-4. The fifth group contains tumor antigens including prostate stem cell antigen and three MAGE genes (MAGE-3, -5a, and -6). The latter are frequently overexpressed in prostate cancer, and secreted proteins involved in cell growth/development and differentiation. The combination of TNF-α and cycloheximide, which then directly activate effector caspases (52), also enhances susceptibility of the cells to apoptosis induced by various agents, including UV, actinomycin D, etoposide, camptothecin, and a combination of TNF-α and cycloheximide. The potentiation of apoptosis as a result of galectin-7 expression is caspase-dependent, because it is abrogated by the pan-caspase inhibitor zVAD-fmk. When treated with apoptotic stimuli, galectin-7 transfectants exhibit enhanced caspase-3 activity, but not enhanced caspase-8 activation, and up-regulated cytochrome c release that is not inhibited by zVAD-fmk. Finally, galectin-7 expression is associated with up-regulated JNK activity in cells upon apoptosis induction.

The fact that galectin-7 transfectants exhibit enhanced sensitivity to apoptosis induced by mechanistically distinct stimuli suggests that this lectin acts on a common point in the apoptosis signaling pathways. Actinomycin D has been reported to induce apoptosis through an intrinsic pathway that involves mitochondrial cytochrome c release, followed by effector caspase activation (26). Therefore, galectin-7 appears to up-regulate the intrinsic pathway and sensitize the cells to apoptosis. The combination of TNF-α and cycloheximide induces apoptosis by an extrinsic mechanism that involves the receptor-mediated pathway and activation of caspase-8, which then directly activate effector caspases (52–54). Therefore, it appears that galectin-7 also affects receptor-mediated apoptosis.

The JNK signaling pathway is activated by a wide range of cellular stresses, including UV (55, 56) and γ irradiation (57), DNA-damaging drugs (58), osmotic stress (59), and cytokines, like TNF-α (60) and interleukin-1 (61). The transduced signals are believed to lead to apoptosis (62), although they may also lead to cell proliferation, depending on the character of stimuli and cell types (55, 63, 64). A recent report using primary murine embryonic fibroblasts from JNK1/2 double knockout-mice has provided convincing evidence demonstrating that activation of the JNK pathway results in apoptosis through cytochrome c release (36). In our experiments, the up-regulation of JNK activation in cells expressing galectin-7 appears to kinetically precede accelerated cytochrome c release. The up-regulation of JNK activation was observed at 4 h and peaked at 6 h, whereas accelerated cytochrome c release was observed at 6 h and peaked at 9 h (Figs. 7 and 8). These data suggest that the potentiated activation of the JNK pathway leads to up-regulation of cytochrome c release in galectin-7 transfectants. In addition, the kinetics of JNK activation argues against the possibility that JNK activation could be a second stress response to apoptosis (35), because up-regulation of JNK activation peaks at 6 h when the manifestation of apoptosis is still low.

Galectin-7 is one of 13 genes highly inducible by p53, as demonstrated by the analysis of the p53-transfected colon carcinoma cell line DLD-1, using the serial analysis of gene expression technique (22). In the context of interaction between tumor suppressor proteins and JNK activation, it is interesting that another p53-induced gene product, GADD45, and another tumor suppressor, BRCA1, have also been reported to induce apoptosis via JNK (65, 66). Apoptosis inducibility of these proteins may be related to the persistent activation of JNK, a status suggested in apoptosis induced by UV and DNA-damaging agents (57). Likewise, galectin-7’s pro-apoptotic function appears to be exerted by influencing the amplitude and the duration of JNK activation.

Immunostaining of HaCaT cells and galectin-7-transfected HeLa cells showed that galectin-7 localizes in the nucleus and cytoplasm. This subcellular distribution is consistent with an intracellular function of the protein and may argue against the possibility that secreted or released galectin-7 during apoptosis exerts its effect extracellularly. Several lines of evidence indicate that the observed effect of galectin-7 on apoptosis is not exerted through the extracellular mechanism. First, addition of lactose to the culture medium does not affect the apoptosis susceptibility. Second, the culture medium from the UV-treated transfectants has no detectable effect on apoptosis. Third, the culture media do not contain adequate levels of galectin-7 that are required to induce apoptosis. The total amount of galectin-7 is $\approx 2 \text{ ng (0.013% of the total cell protein)}$ in $10^6$ galectin-7 transfected HeLa cells, as estimated by immunoblot analysis standardized with recombinant galectin-7. If all the galectin-7 is distributed into the culture medium, the concentration of this protein would be 0.13 nM, which is far below the levels that can cause apoptosis (i.e. 5 μM). Therefore, the pro-apoptotic effect of galectin-7 appears to be exerted intracellularly.

Analysis of the gene expression profile using DNA array between vector- and galectin-7-transfected HeLa cells revealed interesting changes resulting from galectin-7 expression. Many up-regulated genes belong to distinct categories such as extracellular matrix proteins, tumor antigens, metabolic enzymes, and secreted proteins involved in cell growth/development and differentiation. Up-regulation of secreted proteins may be re-
Genes affected by galectin-7 expression in HeLa cells

Genes showing at least a 3-fold increase or decrease in duplicate experiments in galectin-7-transfected HeLa cells compared to vector-transfected HeLa cells are grouped by general functions and sorted by the levels of expression. The presence (P), marginal presence (M), or absence (A) of each gene in vector- and galectin-7 transfected cells was determined by using the Absolute Call (AC) matrix in Affymetrix software. The increase (I), decrease (D), or no change (NC) in each gene in galectin-7-transfected cells compared to vector-transfected cells was determined by using Difference Call (DC) matrix in the same software.

“/H11011” in -fold change (FC) indicates an approximate -fold change calculated using the noise threshold instead of the gene signal when the signal intensity is below the noise threshold. As a reference, human protein genes used as controls in Affymetrix U95A GeneChips as well as galectin family genes found on the GeneChips are included. The levels of these genes were not significantly different between vector- and galectin-7-transfected cells.

| Accession no. | Description | Function | Exp. 1 | Exp. 2 |
|---------------|-------------|----------|--------|--------|
| X98330        | Ryanodine receptor 2 | Calcium channel | 23.3 P | 89.4 P |
| X68742        | Integrin α1 subunit | Cell adhesion | 13.8 A | 82.8 P |
| M31452        | C4b-binding protein | Complement regulation | 3.6 A | 92 P |
| J00073        | α-Cardiac actin | Cytoskeleton | 136.8 P | 780 P |
| U27185        | TIG1 | Differentiation, skin | 12.8 A | 70.1 P |
| Y14690        | Procollagen α2(V) | ECM | 33.2 P | 881.9 P |
| X63556        | Fibrillin | ECM | 1.5 A | 185.3 P |
| D86425        | Osteonidogen | ECM | 18.2 A | 164.9 P |
| X03168        | Vitronectin | ECM | 0.3 A | 98.7 P |
| L25286        | Fibrillin | ECM | 11.7 A | 164.3 P |
| AA010777      | Galectin-7 | Galectin | 162.2 A | 90.7 P |
| U10492        | Mox1 | Homeobox, development | 7.7 A | 458.8 P |
| X62822        | β-Galactoside α-2,6-sialyltransferase | Metabolism | 24.2 A | 433 P |
| D86324        | CMP-N-acetylneuraminic acid hydroxylase | Metabolism | 2.8 A | 119.7 P |
| D31628        | 4-Hydroxynonenoylpyruvic acid dioxygenase (HDPI) | Metabolism | 14.9 A | 187.6 P |
| X66435        | HMG-CoA-synthase | Metabolism | 7 A | 117.6 P |
| U07919        | Aldehyde dehydrogenase 6 | Metabolism | 39.4 A | 211.9 P |
| D10040        | Long-chain acyl-CoA synthetase | Metabolism | 35.6 P | 160.8 P |
| U54617        | Pyruvate dehydrogenase kinase isoenzyme 4 | Metabolism | 36 P | 134.5 P |
| U84573        | Lysyl hydroxylase isoenzyme 2 (PLOD2) | Metabolism | 108.8 P | 336.3 P |
| M69177        | Monoamine oxidase B (MAO-B) | Metabolism | 120.7 P | 382.5 P |
| X81892        | HE6 Tm7 receptor | Receptor | 203.4 P | 667.3 P |
| S70585        | Thyroid-stimulating hormone α subunit | Secreted, hormone | 3 A | 117.3 P |
| U91618        | Proneurotensin | Secreted, hormone | 264.8 P | 3330.3 P |
| U66198        | Fibroblast growth factor homologous factor 2 (FGF-2) | Secreted | 24.9 A | 57.9 M |
| AB000220      | Semaphorin E | Secreted | 26.8 A | 65.6 P |
| AB020315      | Dickkopf-1 (dkkk-1) | Secreted | 15.8 A | 124.7 P |
| AF100780      | Connective tissue growth factor-related protein WISP-2 | Secreted | 306.5 P | 2212.9 P |
sponsible for the induction of other listed genes because of autocrine or paracrine mechanisms. It has been shown that UV irradiation causes an increase in galectin-7 expression in epidermal keratinocytes (23) and HaCaT cells. Therefore, synthesis of extracellular matrix components and secretion of factors that facilitate cell growthdevelopment and differentiation might suggest that galectin-7 expression results in accumulation of proteins that contribute to the tissue remodeling processes following tissue damage that involves apoptotic cell death.

With regard to the apoptosis susceptibility of galectin-7 transfectants, up-regulation of monoamine oxidase B, ryano-

| Accession no. | Description | Function | Exp. 1 | Exp. 2 |
|---------------|-------------|----------|--------|--------|
| M60314        | Bone morphogenetic protein 5 (BMP-5) | Secreted |        |        |
| U31384        | G protein γ-11 subunit | Signal transduction |        |        |
| D64053        | Tyrosine phosphatase | Signal transduction |        |        |
| Y08302        | MAP kinase phosphatase | Signal transduction |        |        |
|               |              |          |        |        |
| M65292        | Factor H | Complement |        |        |
| M94856        | Fatty acid-binding protein homologue | Keratinocyte protein |        |        |
| Y07604        | Nucleotide-diphosphate kinase, mm23-H4 | Metabolism |        |        |
| AF037335      | Carbonic anhydrase precursor (CA 12) | Metabolism |        |        |
| X05409        | Mitochondrial aldehyde dehydrogenase I (ALDH 1) | Metabolism |        |        |
| AF043105      | Glutathione S-transferase Mu 3 (GSTM3) | Metabolism |        |        |
| U81992        | C2H2 zinc finger protein PLAGL1 (PLAGL1) | Transcriptional regulation |        |        |
| D50495        | Transcription elongation factor S-II, hS-II-T1 | Translational regulation |        |        |
| M69023        | Globin regulator | Unassigned |        |        |
|               |              |          |        |        |
| X00351        | β-Actin | Cytoskeleton |        |        |
| M33197        | Glyceraldehyde-3-phosphate dehydrogenase | Metabolism |        |        |
| M97935        | Transcription factor ISGF-3 | Transcription |        |        |
| M11507        | Transferrin receptor | Receptor |        |        |
| M14087        | Galectin-1 | Galectin |        |        |
| AL023315      | Galectin-2 | Galectin |        |        |
| AB006780      | Galectin-3 | Galectin |        |        |
| AB006781      | Galectin-4 | Galectin |        |        |
| Z49107        | Galectin-9 | Galectin |        |        |

2 I. Kuwabara, Y. Kuwabara, and F.-T. Liu, unpublished data.
dine receptor 2, and down-regulation of glutathione S-transferase Mu 3 are of considerable interest. Monoamine oxidase B (MAO-B) has been known to cause oxidative stress leading to apoptotic cell death in neurodegenerative disorders such as Parkinson’s disease (67–69). A recent report demonstrates the relationship between MAO and MAP kinase pathways, in that MAO was identified as a potential target of pro-apoptotic signaling of p38 MAP kinase pathway in nerve growth factor withdrawal-induced apoptosis in PC12 cells (70).

Repressed expression of glutathione transferase Mu 3 gene is interesting in considering resistance versus susceptibility to apoptosis. An analysis of apoptosis resistance of murine B lymphoma cell line using DNA array showed elevated levels of a mouse glutathione S-transferase homolog mRNA together with genes regulating cellular redox potential (71). Down-regulation of the glutathione S-transferase gene may be related to the opposite situation, i.e. apoptosis susceptibility. The involvement of glutathione S-transferase in MAP kinase signaling pathways has been demonstrated, in that murine glutathione S-transferase Mu 1–1 regulates apoptosis by binding to ASK1 kinase, which activates JNK and p38 apoptosis signaling pathways, and suppress its activity (72). Thus, if glutathione S-transferase Mu 3 functions in a similar fashion, an interesting possibility exists that the down-regulation of this enzyme by galectin-7 in turn results in up-regulation of JNK, which we indeed observed. Ryanodine receptors (RyR) regulate intracellular Ca2+ levels, and Ca2+ has been suggested to be important in the apoptosis signaling pathways (73–75). Cell redox changes have been known to influence Ca2+ release by affecting reactive thiol groups of RyR, and RyR is demonstrated to be redox sensor to control cytoplasmic Ca2+ concentration (76–78).

Taken together, an intriguing aspect of these genes modulated in galectin-7 transfectants is that they are redox-related and may affect cellular redox status. Interestingly, recent DNA array analyses of apoptosis-sensitive and -resistant cell lines have identified redox-related genes that are differentially expressed between the two types (71, 79), although the exact mechanisms by which these redox-related genes can affect susceptibility or resistance to apoptosis have not been elucidated. In our study, the expression of a number of mitochondrial enzymes are also modulated in apoptosis-susceptible galectin-7-transfectants. Some are possibly involved in cellular redox regulation.

In summary, galectin-7 is a pro-apoptotic protein inducible by p53, whose expression results in enhanced cytochrome c release during apoptosis. The protein appears to function intracellularly and upstream of JNK activation. DNA microarray studies demonstrate a pattern suggestive of tissue remodeling following apoptotic cell damage and a set of redox-related genes potentially contributory to galectin-7’s pro-apoptotic function. Other galectins have been shown to regulate apoptosis. Galectin-1 and -9 induce thymocyte cell death (12, 46), and galectin-8 inhibits cell adhesion and induces apoptosis in a human carcinoma cell line (80), when added exogenously to the cells; galectin-3 protects cells from death, apparently by functioning inside the cell and interacting with intracellular proteins. Thus, a picture that emerges is that galectins are a family of apoptosis regulators.

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