The La Autoantigen Is a Malignancy-Associated Cell Death Target That Is Induced by DNA-Damaging Drugs
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Abstract

Purpose: To evaluate the La autoantigen as a target for specific monoclonal antibody (mAb) binding in dead cancer cells after use of DNA-damaging chemotherapy.

Experimental Design: In vitro studies of La-specific 3B9 mAb binding to malignant and normal primary cells with and without cytotoxic drug treatment were done using immunoblotting and flow cytometry. Chromatin-binding studies and immunofluorescence detection of γH2AX as a marker of DNA double-stranded breaks together with 3B9 binding assays were done to measure DNA damage responses. Incorporation of a transglutaminase 2 (TG2) substrate and TG2 inhibition were studied to measure protein cross-linking in dead cells.

Results: La was overexpressed in human cancer cell lines with respect to normal primary cells. Within 3 h of the DNA-damaging stimulus, La became chromatin bound when it colocalized with γH2AX. Later, after the stimulus produced cell death, La-specific 3B9 mAb bound specifically and preferentially in the cytoplasm of dead cancer cells. Moreover, 3B9 binding to dead cancer cells increased with increasing DNA damage. Both La and 3B9 became cross-linked in dead cancer cells via TG2 activity.

Conclusion: La autoantigen represents a promising cancer cell death target to determine chemotherapy response because its expression was selectively induced in dead cancer cells after DNA-damaging chemotherapy.

Among the nuclear structural changes that typify malignancy is the enlargement of nucleoli, which are the ribosome factories of the cell. Although it remains unclear whether it is cause or effect, increased ribosomal biogenesis and protein synthesis are invariant features of malignancy (1). Thus, it may not be surprising that various nuclear and nucleolar proteins, including the ribonucleoproteins, which are major constituents of the nucleolus and which regulate gene expression via assembly and organization of the RNA processing machinery, are often overexpressed in malignancy (reviewed in refs. 2, 3). Although overexpression of nuclear and nucleolar proteins could mark malignant cells, these antigens are not available for detection by antibodies under physiologic conditions in vivo (4–6). During apoptosis, the ribonucleoprotein-containing structures of perichromatin and interchromatin granules (7) together with nucleolar components reorganize and aggregate in the interchromatin space as heterogeneous ectopic ribonucleoprotein-derived structures. Subsequently, heterogeneous ectopic ribonucleoprotein-derived structures are transported toward the cell surface to be packaged as blebs and apoptotic bodies where they are potentially accessible to specific monoclonal antibody (mAb; refs. 8, 9). These dramatic morphologic changes are mediated by molecular events, such as DNA and RNA cleavage, posttranslational modification, and proteolysis of several nuclear proteins (reviewed in refs. 10–12). Consequently, the RNA recognition motif of ribonucleoprotein is contained in ~40% of proteins modified during apoptosis (13, 14). Another striking characteristic of apoptotic bodies is their resistance to solubilization in nonionic detergents (15), which is mediated by the calcium-dependent protein-protein cross-linking activity of type 2 tissue transglutaminase (TG2; ref. 16) that may protect against autoimmunity and inflammation (17). TG2 expression is induced early during apoptosis by DNA-damaging stimuli, such as radiation (18) and cytotoxic drugs (19), and only certain substrate proteins, such as actin, β-tubulin, vinculin, fibronectin, troponin, histone 2B, and retinoblastoma protein, are extensively polymerized throughout the cell (16) to create detergent-insoluble apoptotic bodies (20). Overexpression of TG2 is associated with the malignant phenotype of drug resistance, evasion of apoptosis, and increased metastatic potential (21).

Originally, it was observed that radiolabeled antibodies could enter necrotic cells in vivo and bind cognate antigens, such as the myosin heavy chain, in cardiac skeletal muscle after experimental infarction (22, 23). Epstein et al. (24, 25) confirmed and extended this finding when they showed that cell ghosts in necrotic and degenerating areas of tumors also harbored insoluble antigens that were accessible to mAbs as a
method to target cancer. We hypothesized that mRNA-binding proteins contained within apoptotic cancer cells may present useful targets for cancer diagnosis and therapy. This hypothesis is based on the abundance and overexpression of this class of protein in malignant cells together with the retention and accessibility of these targets during apoptosis. We chose to investigate the potential of targeting a ubiquitous and exceedingly abundant ribonucleoprotein, Lα/SSB (26), which is essential for early mammalian development (27). Lα acts as a molecular chaperone for RNA polymerase III, which catalyzes synthesis of transfer and rRNA species and which is overactive in malignancy (28). Lα plays a dynamic role in RNA biogenesis by acting as a transient interaction partner for a variety of RNA, ribonucleoprotein, and other proteins (29–31), which facilitate assembly of small RNA into functional ribonucleoprotein (32) and help to construct the protein translation apparatus (33, 34). Corresponding with its diverse interactions, Lα shuttles between nucleus and cytoplasm and is believed to play an important role in the coupling of transcription and translation (34), which are biological processes otherwise separated by the double-layer nuclear membrane but necessarily integrated for normal and malignant growth. Here, we report that the 3B9 mAb directed toward Lα/SSB is a malignancy-associated cell death ligand. We found that Lα is overexpressed in malignant cells compared with primary cells and report that 3B9 binding to Lα may provide a useful “readout” of chemoresponsiveness.

Materials and Methods

Materials. Suppliers of materials are identified for each material. Malignant cell lines were obtained from the American Type Culture Collection. Cell culture medium, RPMI 1640, DMEM and Ham’s F12, and FCS were from JRH Biosciences, Inc. Trypsin-EDTA solution, trypsin blue, propidium iodide, bovine serum albumin, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium premixed substrate solution for alkaline phosphatase, hydrocortisone, monodansylcadaverine (MDC), and staurosporine were from Sigma-Aldrich Co. Hybrid-B membrane (polyvinylidene difluoride) and protein G purification columns were from Amersham Biosciences. Bicinchoninic acid protein reagent assay was from Pierce Biotechnology, Inc. Anti-poly(ADP-ribose) polymerase mAb clone C-2-10 and anti–primary proliferating cell nuclear antigen mAb clone PC10 were from Oncogene Research Products. Trichostatin A (TSA), anti-human histone 2A (H2A) polyclonal antibody, and biotin-conjugated anti-phosphorylated histone H2AX (Ser139) mAb (clone JBW301) were from Millipore, Inc. Anti-α-fodrin mAb was from Chemicon International. Anti-β-actin affinity-purified rabbit polyclonal antibody, FITC-conjugated goat anti-rabbit IgG, alkaline phosphatase–conjugated goat anti-rabbit IgG, and alkaline phosphatase–conjugated rabbit anti-mouse IgG antibodies were from Rockland. The anti-Lα/SSB 3B9 mAb hybridoma (35) is a murine IgG2a autoantibody cross-reactive with human Lα, which was prepared by Dr. M. Bachmann (Oklahoma Medical Research Foundation, Oklahoma City, OK) and was a kind gift from Dr. Tom P. Gordon (Department of Immunology, Allergy and Arthritis, Flinders Medical Centre, Adelaide, South Australia, Australia). The isotype control Sal5 (1D4.3) mAb hybridoma, prepared by Dr. I.K. Ashman (Medical Science Building, University of Newcastle, Newcastle, New South Wales, Australia), was kindly supplied by Dr. S. McColl (School of Molecular Biosciences, University of Adelaide, Adelaide, South Australia, Australia). The mAbs were affinity purified using protein G columns and FITC conjugates were prepared according to the manufacturer’s instructions (Sigma-Aldrich). Anti-human nucleolin mAb, anti-human nucleophosmin mAb, anti-mouse IgG antibody conjugated to Alexa Fluor 488, streptavidin-Alexa Fluor 488, streptavidin-phycerythrin, 7-aminomycin D (7-AAD), SytoxGreen DNA-dye, and biotinylated cadaverine were from Invitrogen. Lymphoprep (Axis-Shield PoC AS), etoposide (Pfizer, Inc.), cyclophosphamide and cisplatin (Bristol-Myers Squibb Co.), and gemcitabine (Eli Lilly) were obtained from the Royal Adelaide Hospital Cytotoxics Pharmacy (Adelaide, South Australia, Australia).

Cell culture. Suspension cultures of Jurkat and U937 leukemia cell lines were maintained in RPMI 1640 containing 5% FCS and passaged by splitting at 1:10 every 72 h. Cultures of adherent MDA-MB-231, MCF-7, PC-3, LNCaP, and A549 cancer cell lines were maintained in RPMI 1640 containing 5% FCS, and adherent cultures of Panc-1 cells were maintained in RPMI 1640 containing 10% FCS. Cell culture conditions were maintained in culture medium, RPMI 1640, DMEM and Ham’s F12 medium supplemented with 400 ng/mL hydrocortisone and 5% FCS. Adherent cells were passaged every 48 to 72 h at a 1:4 dilution after detachment with trypsin-EDTA solution. Peripheral blood mononuclear cells were isolated from fresh heparinized blood obtained from normal healthy volunteer donors using Lymphoprep separation and cultured overnight in RPMI 1640 containing 5% FCS to separate adherent cells from those remaining in suspension. Cytologic analysis indicated that ≥70% of both suspension and adherent cells were CD3+ and CD14+, respectively. Hence, CD3- and CD14-enriched peripheral blood mononuclear cell preparations will be described as peripheral blood lymphocytes and monocytes, respectively. Clonetics conditioned cultures of primary human cells were maintained according to the manufacturer’s instructions (Cambrex Corp.) and included cultures of human mammary epithelial cells, prostate epithelial cells, and normal human bronchial epithelium. Finally, buccal cells were isolated from the gum lining of normal healthy volunteer donors as described (36).

Induction of apoptosis or necrosis and cell permeabilization. Apoptosis or primary necrosis of cells, which were maintained in culture medium, was induced by cytoxic treatments using the specified conditions. In some experiments, cells were starved by serum deprivation. In other experiments, TSA used at the specified concentrations was prepared from 1 mg/mL stock solution in absolute ethanol. Control (untreated) cells had ≥90% viability determined by propidium iodide or trypan blue staining (data not shown). Viable cells were fixed and permeabilized by incubating cells at 5 × 10^5/mL in 2% (w/v) paraformaldehyde solution in PBS [150 mmol/L sodium phosphate and 150 mmol/L sodium chloride (pH 7.2)] for 10 min followed by 1:10 dilution in absolute methanol (at -20°C) for 1 to 3 min before a final wash with PBS.

Flow cytometry. Indirect immunofluorescence staining was done using purified mouse antibodies at 5 μg/mL for 30 min at room temperature in PBS followed by Alexa Fluor 488–conjugated anti-mouse IgG at 2 μg/mL for 30 min at room temperature in PBS. Cell viability was assessed by exclusion of propidium iodide (0.5 μg/mL) or by exclusion of 7-AAD (2 μg/mL for 15 min at room temperature). Annexin V-biotin staining was done according to the manufacturer’s instructions (BD Biosciences), and after washing, staining was revealed with 0.2 μg/mL streptavidin-Alexa Fluor 488. Staining for H2AX was done using 0.2 μg/mL of anti-H2AX-biotin for 30 min at room temperature followed by 2 μg/mL of streptavidin-phycerythrin or streptavidin-Alexa Fluor 488. Staining of polyclonal antibody (anti-actin or anti-H2A) was done using 5 μg/mL of antibody solution for 30 min at room temperature. Control incubations were done using protein G–purified rabbit IgG from normal rabbit serum (Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia). Cells were washed and then incubated (30 min at room temperature) with 2 μg/mL of FITC-conjugated anti-rabbit IgG antibody. Samples were acquired immediately by a Becton Dickinson FACSscan flow cytometry system (BD Biosciences). Acquisition was standardized to 10,000 events or in some cases standardized to a set time for acquisition (in seconds) at a flow rate of 60 μL/s to allow comparison of cell counts for different incubation conditions. Flow cytometry data were analyzed using WinMDI v2.8 (Scripps Research Institute). Unless
otherwise specified, no gating was done in any of the reported analyses. Quadrants were set where ≤3% of cells were positive using control antibody. Specific binding of antibodies was calculated as the difference in mean fluorescence intensity (MFI) between the test antibody and the isotype control antibody and expressed as the net MFI ± SE, which was calculated from replicate incubations (n > 2).

**SDS-PAGE and immunoblotting.** Cell lysates were prepared in SDS lysis solution [2% (w/v) SDS, 10% (v/v) glycerol, and 62.5 mmol/L Tris-HCl], and protein concentrations were determined using bichinchonic acid protein assay kit according to the manufacturer's instructions. Bromphenol blue (0.05%, w/v) and β-mercaptoethanol (5%, v/v) were added to lysates after bichinchonic acid assay measurement. Reducing 12% resolving SDS-PAGE as per Laemmli's method (37) and transfer onto polyvinylidene difluoride membranes were done according to the manufacturer’s instructions with the Hoefer Mighty Small II transfer unit (Amersham Biosciences), respectively. Immunoblotting was done using standard methods and blots were developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium premixed solution (Amersham Biosciences), respectively. Immunoblotting was done using standard methods and blots were developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium premixed solution as specified by the manufacturer and analyzed using GelPro Analyzer v3.1 (Media Cybernetics, Inc.).

**Chromatin-binding assay.** Jurkat cells were incubated in the presence or absence of 20 µg/mL cisplatin for 3 h, and soluble nuclear and chromatin fractions were prepared as described (38). Samples were fractionated using 12% SDS-PAGE for immunoblotting as described above. Membranes were probed with 1 µg/mL 3B9, 2 µg/mL anti-H2A antibodies, or 0.2 µg/mL anti-γH2AX-biotin followed by the appropriate alkaline phosphatase–conjugated secondary antibodies or streptavidin-alkaline phosphatase. The presence of H2A in chromatin and not in soluble fractions was used to assess the quality of preparation of the chromatin fractions (38).

**Assays of transglutaminase-mediated cross-linking.** TG2-mediated protein-protein cross-linking in apoptotic cells was investigated using a modification of a previously described method (39). Briefly, cells were resuspended at 1 × 10^6/mL in PBS containing 1% Triton X-100, 0.2 µg/mL sulforhodamine-101, and 0.1 µmol/L SytoxGreen. Samples were vortexed and incubated for 10 min before analysis by flow cytometry. The degree of cross-linking in apoptotic cells was calculated as the ratio of the MFI of sulforhodamine staining in apoptotic cells to the MFI in control cells (protein cross-linking ratio). Incorporation of the TG2 substrate, biotinylated cadaverine, in cellular proteins was used as an index of TG2 activity. Cells were incubated with increasing concentrations of cisplatin with or without 100 µmol/L cadaverine-biotin (from 25 mmol/L stock solution in DMSO). Cells were collected after 48 h, washed extensively with PBS, and incubated with streptavidin-Alexa Fluor 488 for cytofluographic analysis. To test inhibition of protein cross-linking, cultured cells were incubated with increasing concentrations of the competitive TG2 inhibitor MDC. MDC (25 mmol/L stock in DMSO) was diluted in culture medium before it was added to cell cultures at the specified concentrations for the duration specified.

**Confocal laser scanning microscopy.** Cells were stained using immunofluorescence methods described above and then spotted onto glass slides using the cytospin method. The prepared slides were mounted with coverslips using nonfluorescence mounting medium (Dako). Slides were analyzed using a Bio-Rad Olympus confocal microscope with appropriate filters and under constant conditions of laser voltage, iris aperture, and photomultiplier tube amplification.

**Fluorescence microscopy of γH2AX and 3B9 colocalization.** Chamber slides were seeded with PANC-1 cells and incubated overnight in RPMI 1640 containing 10% FCS before replacement with medium containing cytotoxic agents as described in the figure legends. After 3 h, cells were fixed and permeabilized using paraformaldehyde and methanol as described above. Cells were washed and blocked with 5% bovine serum albumin solution in PBS and then stained with 3B9 and anti-γH2AX antibodies as described above. Cells were washed and blocked with 5% bovine serum albumin solution in PBS and then stained with 3B9 and anti-γH2AX antibodies as described above. Coverslips were mounted using nonfluorescence mounting medium and slides were analyzed using the Bio-Rad Olympus fluorescence microscope.

![Figure 1](image-url)
Bioinformatics analysis. Oncomine is a database of microarray data, which holds data from 962 studies, of which 209 were analyzed. The database contains 14,177 microarrays from 35 cancer types (information publicly available at the Web site). Several cancer signatures have been deduced from large-scale analysis of data in the database (40–44). We analyzed the 209 studies using the advanced analysis module limiting results to overexpressed genes only, and gene enrichment was done using the following options: (a) InterPro for analysis of motifs in the overexpressed genes, (b) Gene Ontology molecular function for analysis of functions of overexpressed genes, and (c) Gene Ontology cellular component for cellular compartmentation of the overexpressed genes. Two variables were used to describe the gene sets deduced from the above analysis, (a) odds ratio and (b) P value, which were provided in the database.

Statistical analysis. Statistical comparisons were done using GraphPad Prism v4 (GraphPad Software). Generally, two-way ANOVA was used to deduce significant differences among the results. The Bonferroni post-test comparison was used to report P values. P values are denoted thus: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

La is overexpressed in human malignant cells and is induced by DNA-damaging drugs. The 3B9 target antigen La is overexpressed in malignant cells in comparison to the corresponding primary cell type (Fig. 1). In addition, data mining of Oncomine, which is a cancer gene expression database, indicated that nucleolar proteins and proteins, which contain the RNA recognition motif and which includes La, were overexpressed at mRNA level in many human cancers and in normal cells transfected with oncogenes, such as c-Myc (see Supplementary Data). We also inferred that La expression was cell cycle dependent. Binding of 3B9 to permeabilized Jurkat cells, which were synchronized by double-thymidine block, was maximal during S phase (data not shown).

Loss of cell membrane integrity among etoposide-treated Jurkat cells was measured by propidium iodide binding and increased over time. Unlike the time-dependent accumulation of 3B9 mAb (Fig. 2A, bottom), permeable dead cells did not accumulate Sal5 isotype control mAb specific for a Salmonella

http://www.oncomine.org
Antigen (Fig. 2A, top), indicating that binding was antigen specific. Similar results were obtained for the human La-specific SW3 mAb, goat anti-human La polyclonal antibody (Santa Cruz Biotechnology, Inc.), and affinity-purified La-specific polyclonal human autoimmune serum (data not shown). Similar accumulation was also observed using other mAb specific for intracellular antigens, such as poly(ADP-ribose) polymerase, proliferating cell nuclear antigen, actin, tubulin, and fodrin (data not shown), or after using other DNA-damaging agents, such as cyclophosphamide, cisplatin, and staurosporine (data not shown). The 3B9 mAb bound La in the 7-AAD–stained nucleoplasm of control permeabilized cells, whereas 3B9 bound La diffusely in the cytoplasm of cisplatin-treated Jurkat cells in which 7-AAD+ nuclear fragmentation was apparent (Fig. 2B), which indicated that cisplatin induced an apoptotic death among Jurkat cells. We confirmed this finding by showing loss of mitochondrial membrane potential after cisplatin treatment of Jurkat cells (data not shown). These results indicated that dead cells contained a reservoir of intracellular antigens potentially amenable to targeting by mAb for diagnostic and therapeutic purposes.

Notwithstanding these results, significant differences in binding levels of the mAb specific for the different intracellular antigens were observed after cisplatin-induced apoptosis among Jurkat cells. We compared the antigen-specific MFI of cisplatin-treated Jurkat cells with the MFI of control untreated Jurkat cells, which were fixed and permeabilized (Fig. 2C). In comparison with control cells, antigens such as H2A, nucleophosmin, and La that is estimated as 2 × 10^7 copies per cell (45, 46) were at least retained in Jurkat cells after cisplatin-induced cell death, whereas other highly abundant antigens, such as poly(ADP-ribose) polymerase, which is estimated as 2 × 10^6 copies per cell (47), were lost or not available for recognition by specific antibody. In addition, it is apparent that, using the measure of the antigen retention index and in comparison with nucleophosmin and other intracellular antigens, La antigen and H2A were not only preferentially retained but also “induced” or “created” after DNA-damaging drugs.

Fig. 3. 3B9 binding is augmented by DNA-damaging drugs. A, Jurkat cells were incubated with increasing concentrations of cisplatin in the absence (●) or presence (●) of 100 ng/mL TSA. At 48 h, cells were analyzed for 3B9 binding. Inset, after 3 h of incubation with cisplatin in the absence (●) or presence (●) of 100 ng/mL TSA, cells were fixed, permeabilized, and stained for γH2AX. B, Jurkat cells were left untreated (control, white columns), deprived of serum (starved, striped columns), or treated with 20 μg/mL cisplatin (black columns). After 6 h, cells were fixed, permeabilized, and stained for γH2AX, and after 24 h, cells were stained with Annexin V (AnnV) or 3B9. Columns, mean percentage of positively stained cells for each condition (n = 2); bars, SE. Inset, MFI for 3B9 binding to 7-AAD+ events was plotted as mean ± SE for each condition. ***, P < 0.001. C, Immunoblots of soluble and chromatin fractions from a chromatin-binding assay after Jurkat cells were treated with 20 μg/mL cisplatin for 3 h (+) or not (○). D, Adherent cultures of PANC-1 cells were incubated in slide chambers for 3 h with 20 μg/mL cisplatin. Cells were fixed, permeabilized, and stained with 3B9 and anti-γH2AX. Images were acquired for (i) γH2AX staining (red) and (ii) 3B9 binding (green) and (iii) overlaid to investigate colocalization (orange yellow). iv, 3B9 and γH2AX overlays were superimposed on transmission images of cells to show nuclear localization of detected antigens. Images representative of four independent experiments, which produced similar results.
cisplatin treatment (Fig. 2C). Moreover, the type of death stimulus influenced the level of 3B9 binding. Significantly greater 3B9-specific binding to the dead malignant cells was observed after cisplatin treatment than after serum deprivation (Fig. 2D). Similarly, treatment of Jurkat cells in vitro with other primary necrotic stimuli, such as heat (56°C for 1 h), 0.1% H2O2, 5% ethanol, 0.1% HgCl2, and 1% sodium azide, followed by 48 h in culture produced lower 3B9-specific binding than the binding observed after fixation and permeabilization of the Jurkat cells using paraformaldehyde and methanol. Importantly, all 3B9 binding to primary necrotic cells at 48 h after induction was significantly lower than 3B9 binding 48 h after treatment of Jurkat cells with the DNA-damaging drug cisplatin (data not shown). Finally, overexpression of La in permeabilized malignant cells with respect to the corresponding primary cells was still observed after both malignant and primary cells were treated with cisplatin (Fig. 2E).

3B9 binding reflects DNA damage during drug-induced apoptosis. Among dead Jurkat cells after cisplatin-induced apoptosis, both 3B9-specific binding and staining with the DNA damage marker γH2AX increased with cisplatin dose (Fig. 3A). These effects were augmented by combining TSA with cisplatin (Fig. 3A). Annexin V binding represented another indicator of cell death (Fig. 3B), which was greater among cisplatin-treated than serum-deprived Jurkat cells and which correlated with both an increased proportion of dead cells binding 3B9 (Fig. 3B, inset) and an increased intensity of per cell binding of 3B9 (Fig. 3B, inset). Similarly, γH2AX staining increased in serum-deprived cells and further again in cisplatin-treated cells (Fig. 3B). We inferred that La redistributed to DNA double-stranded breaks after observing increased chromatin-associated La and γH2AX (Fig. 3C) and colocalization of γH2AX and 3B9 (Fig. 3D) among cisplatin-treated malignant cells. Next, we studied chemotherapy-resistant PANC-1 cells to evaluate further the relationship between 3B9 binding and the DNA damage response. Neither gemcitabine (Fig. 4A) nor TSA (data not shown) as single agents induced >20% cell death rate among PANC-1 cells. In contrast, gemcitabine and TSA together acted synergistically to increase the levels of PANC-1 cell death (Fig. 4A), γH2AX+ cells (Fig. 4B and C), and 3B9-specific binding (Fig. 4D).

La becomes fixed in dead malignant cells after cisplatin-induced cell death. Jurkat cell cultures contained ≤10% 7-AAD+ cells, which had died spontaneously in culture. Although these cells were 7-AAD+, most did not bind 3B9 (data not shown). In contrast, cisplatin-induced apoptosis produced 7-AAD+ cells that also bound 3B9 (Fig. 5A). Resistance of apoptotic cells to the nonionic detergent Triton X-100 was reported to correlate with activity of TG2, which mediates protein cross-linking (39). During apoptosis, the proportion of 7-AAD+ cells and the level of 3B9 binding to these cells were comparable irrespective of Triton X-100 treatment (Fig. 5A), which suggested that cross-linking was an integral component of apoptosis in this in vitro system. Immunoblot analysis of Jurkat cell lysates showed that 3B9 recognized a 48-kDa band, which is consistent with the known molecular weight of La and which remained unchanged.

![Fig. 4. Combination treatment with gemcitabine and TSA synergizes to produce cytotoxicity, DNA-damaging effects, and augmented 3B9 binding among dead PANC-1 cells. PANC-1 cells were incubated with increasing concentrations of gemcitabine in the absence (white columns) or presence of 100 ng/mL (striped columns) or 200 ng/mL (black columns) of TSA. Analyses were done of (A) viability using 7-AAD at 48 h, (B) DNA damage using γH2AX staining at 3 h, (C) colocalization (yellow) of staining with γH2AX-specific mAb (green) and 7-AAD (red) in PANC-1 cells treated with gemcitabine and TSA (+) or not (-), and (D) 3B9-specific binding at 48 h. Columns, mean of three independent experiments; bars, SE.](https://www.aacrjournals.org/doi/abs/10.1158/1078-0432.CCR-07-0357)
during a 72-h culture period both in amount and integrity (Fig. 5B). After cisplatin treatment, however, 3B9 identified SDS-stable, higher molecular weight, La-containing bands, which accumulated as apoptosis progressed and which may represent La antigen covalently bound in higher-order complexes with itself and/or other proteins. The expected apoptosis-related La cleavage products were also observed (Fig. 5B; ref. 48).

Next, we hypothesized that inhibition of TG2 activity would reduce 3B9 binding to apoptotic cells because less of the target La antigen would be cross-linked. First, we determined that TG2 activity was maximal at a cisplatin dose of 20 μg/mL after showing a cisplatin dose-dependent incorporation of the TG2 substrate cadaverine-biotin into dying Jurkat cells (Fig. 5C). Then, we used this dose to study the effect of the TG2 inhibitor MDC on the retention of the fluorescent protein-binding dye sulforhodamine in apoptotic compared with viable Jurkat cells (39), which was expressed as a cross-linking ratio. As MDC had a dose-dependent inhibitory effect on protein cross-linking (Fig. 5D), so it inhibited 3B9-specific binding (Fig. 5E). The concentration of MDC (±SE) required to inhibit half of the binding of 3B9 was 260 ± 1, 110 ± 1, and 150 ± 1 μM/L at 24, 48, and 72 h, respectively. Together, these results indicated that the La antigen was retained in apoptotic cells by a generalized protein cross-linking process, which was most likely mediated by TG2.

Protein cross-linking covalently attaches 3B9 to the interior of leaky dead malignant cells during apoptosis. Using etoposide or cisplatin, Jurkat cells were induced to undergo apoptosis in the presence of 3B9 or its Sal5 isotype control mAb. 3B9 binding to apoptotic Jurkat cells was detectable even after stripping of the

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**Fig. 5.** La antigen is cross-linked during apoptosis. A, Jurkat cells incubated in 20 μg/mL cisplatin were removed at specified time points and incubated for 10 min in 1% Triton X-100 solution before 3B9 binding analysis. Data are presented as density plots of staining with 3B9 versus 7-AAD. B, immunoblots of cell lysates of control and cisplatin-treated Jurkat cells were probed with 3B9 for expression of La (top) or actin as a loading control (bottom) at specified times. C, Jurkat cells incubated in the absence or presence of increasing concentrations of cisplatin were labeled with cadaverine-biotin added at 100 μM/L for 48 h. Cells were stained with streptavidin-Alexa Fluor 488 and analyzed by flow cytometry. Points, MFI (n = 3); bars, SE. D, Jurkat cells incubated in the presence or absence of 20 μg/mL cisplatin were incubated with increasing concentrations of MDC. Cells were analyzed at 24 h (white columns), 48 h (striped columns), and 72 h (black columns) for sulforhodamine staining. Inset, dependence of the protein cross-linking ratio on MDC concentration at 48 h (solid line) and 72 h (dotted line) after cisplatin treatment of Jurkat cells. E, Jurkat cells incubated with cisplatin in the presence or absence of increasing concentrations of MDC were stained with 3B9. Columns, net MFI of 3B9-specific binding (n = 3); bars, SE. Inset, dependence of 3B9-specific binding on MDC concentration at 24 h (solid line), 48 h (dashed line), and 72 h (dotted line) after cisplatin treatment of Jurkat cells. Data are representative of three identical and independent assays, which produced similar results.
Jurkat cells with Triton X-100, which suggested that the protein cross-linking process in apoptotic cells included the cross-linking of bound antigen-specific mAb (Fig. 6A). To show that protein cross-linking rather than noncovalent antibody-antigen interactions was responsible for the detergent resistance of 3B9 binding (Fig. 6A), we used 3B9 to probe an immunoblot of recombinant human La antigen and found addition of 1% Triton X-100 to the incubation buffer was sufficient to disrupt noncovalent antibody-antigen interactions (data not shown). Furthermore, and importantly, cross-linking of 3B9 in apoptotic Jurkat cells (Fig. 6A) was more evident than it was in apoptotic CD3⁺-enriched lymphocytes (Fig. 6B). Reflecting the per cell measure of fluorescence intensity given by the MFI, apoptotic Jurkat cells (Fig. 6C) stained much more intensely with sulforhodamine (red) and 3B9 (green) than their counterpart primary CD3⁺-enriched lymphocytes (Fig. 6D). The lack of 3B9 staining of control Jurkat cells indicated that 3B9 binding depended on induction of apoptosis and consequent loss of cell membrane integrity (Fig. 6A and C). Similar results were obtained for the matched pair of U937 monocytic leukemia cells and normal CD14-enriched peripheral blood monocytes (data not shown). Altogether, these data suggested that, along with other proteins in dying malignant cells, both 3B9 and the target antigen La were covalently cross-linked because of a TG2-dependent process.

Discussion

We have discovered that the La autoantigen represents a novel cell death target for the purpose of in vivo targeting particularly in conjunction with antineoplastic treatment that “creates, reveals, or induces” targets for binding in dead cells. Using both cytofluorographic and immunoblot analyses, we found that La protein was overexpressed in various malignant cell lines when its expression was also analyzed in counterpart primary cells. Further evidence for the overexpression of La in malignancy was provided at the transcriptional level by data mining of the Oncomine database (see Supplementary Data). Our inference that La expression increases during the S phase of the cell cycle (data not shown) would be consistent with the requirement for at least a doubling in the amount of the transcriptional and translational apparatus in preparation for cell division.

Comparison with the irrelevant isotype control Sal5 mAb showed that binding of 3B9 to apoptotic cells was antigen specific and did not simply result from passive uptake of IgG. Fluorescence microscopic examination of cisplatin-treated or control permeabilized Jurkat cells showed that 3B9 bound in the cytoplasm rather than in the nucleoplasm of cisplatin-treated cells, which is consistent with the known cleavage of the COOH-terminal nuclear localization signal sequence of La during apoptosis and the subsequent translocation of La.

Fig. 6. The preferential binding of 3B9 to dead malignant cells after cytotoxic drug treatment is detergent resistant and colocalizes with other intracellular proteins. Cultures of Jurkat cells (A) and their counterpart primary CD3⁺-enriched peripheral blood lymphocytes (B) were either left untreated (control) or treated with etoposide or cisplatin for 48 h in the presence of 50 μg/mL of 3B9 or its Sal5 control mAb. After 48 h, cells were treated or not with 1% Triton X-100 before cytofluorographic analysis. Columns, net MFI of 7-AAD⁺ events from four independent assays; bars, SE. Statistical comparisons were made between control and treated cells. Photomicrographs show representative Jurkat cells (C) and CD3⁺-enriched peripheral blood lymphocytes (D), which had been treated with 20 μg/mL cisplatin in the presence of 50 μg/mL 3B9, treated or not with 1% Triton X-100 (Tx-100), and stained with sulforhodamine (red) and Alexa Fluor 488–conjugated anti-mouse IgG (green). Control, nonpermeabilized and untreated Jurkat cells. Bars, 40 μm (PBS) and 20 μm (Triton X-100).
from nucleus to cytoplasm (48). Higher binding of 3B9 to permeabilized malignant cells than permeabilized primary cells also occurred after cisplatin-induced cell death, which suggested that 3B9 would preferentially bind dead malignant cells. We observed that, among mAb specific for various intracellular antigens, 3B9 was striking in its binding preference for cells that were permeable because of cisplatin-induced apoptosis rather than artificial fixation and permeabilization. Similarly, 3B9-specific binding to several types of carcinoma cell, which were induced to die with cisplatin, was significantly greater than when those cells died because of serum deprivation. Again, in comparison with Jurkat cells killed with cisplatin, the binding of 3B9 to Jurkat cells induced to die as a result of various primary necrotic stimuli was reduced.

Together, these results indicate that the mode of cell death is critical for the induction of heightened 3B9 binding and strongly suggest that apoptotic cell death rather than primary necrosis is required. We hypothesize that increased binding of 3B9 to dead malignant cells after treatment with cytotoxic drugs comprises several cell death–related events, which include DNA damage, antigen redistribution, and activation of TG2-mediated protein cross-linking activity.

As would be anticipated from treatment with cisplatin, which is a drug known to induce DNA double-stranded breaks, we found a dose-dependent increase in the proportion of Jurkat cells expressing γH2AX, which binds at DNA double-stranded breaks. The proportion of γH2AX+ Jurkat cells increased further when the histone deacetylase inhibitor TSA was added to cisplatin treatment. 3B9 binding to Jurkat leukemia and PANC-1 pancreatic cancer cells was commensurate with the extent of DNA damage. This link was reinforced by increased chromatin binding of both γH2AX and La together with their nuclear colocalization after DNA-damaging treatment. Previously, the combination of TSA and gemcitabine synergized to inhibit growth of the PANC-1 cells, which are resistant to treatment with single agents (49). We found that the DNA-damaging drug gemcitabine (50) synergized with TSA to increase cell death, γH2AX+ DNA damage foci, and 3B9 binding among PANC-1 cells. Together, these data suggest that, like other ribonucleoprotein (38), La may be involved in the DNA damage response (51, 52).

The extent of DNA damage was also related to protein cross-linking activity, which was manifested as cisplatin dose-dependent incorporation of the TG2 substrate cadaverine into apoptotic Jurkat cells. Interestingly, our data support a functional role for TG2 activity in the retention of 3B9 binding in apoptotic Jurkat cells. La binding of 3B9 resisted treatment with nonionic detergent, and high molecular weight and SDS-insoluble La-containing material was detected after treatment of Jurkat cells with cisplatin. Furthermore, 3B9 binding decreased as the concentration of the TG2 inhibitor MDC increased. Altogether, these data suggested that TG2-mediated cross-linking of the 3B9 target La antigen was part of generalized protein cross-linking process occurring in dying cells (39).

Our studies of selective in vitro binding of dead malignant cells by La-specific mAb indicated that tumor-selective binding was likely to be multifactorial in nature. The important factors, which we have defined for La and its specific mAb in vitro and which we hypothesize will enable effective in vivo targeting of tumors, include the following: (a) overexpression of an antigen in malignant cells, (b) cleavage and redistribution of the antigen to cytoplasm after DNA-damaging treatment, (c) “induction” of the antigen by the DNA damage response, (d) accessibility of the targeting moiety to the antigen located inside permeable apoptotic malignant cells, and (e) “fixation” of the target antigen and the targeting moiety itself by TG2-mediated protein cross-linking, which is induced by apoptosis and increased in malignancy.

If such phenomena, which apply to La and 3B9 at least as described herein, were to be reproduced in vivo, then tumor accumulation of 3B9 would be expected to be consequently higher than in other organs. Data presented in the accompanying article (Al-Ejeh et al., 2007) indicate that tumor accumulation in vivo of 111In-labeled 3B9 mAb 72 h after injection was much higher than in normal tissues and was greater still if tumor-bearing mice were treated with cytotoxic drugs rather than if they were not treated. These data suggest that the induction of apoptosis in this tumor model produced sufficient abnormally permeable cells, which were not sequestered by phagocytes and were thus accessible to 3B9 mAb, to result in tumor accumulation of 111In-labeled 3B9 mAb at 72 h after injection. Furthermore, tumor accumulation is directly related to the number of activated caspase-3–positive apoptotic cells in the tumor. Hence, labeled 3B9 may be used as a radioimmunoscintigraphic agent to ascertain if tumors will respond to a given antineoplastic treatment if the tumor cells, which have died because of the treatment, can be detected in situ. Moreover, targeting of abundant and overexpressed antigens, such as La, in malignant cells dying as a result of antineoplastic treatment may facilitate delivery of another modality of treatment, such as therapeutic radiation, attached to a cell death ligand, such as 3B9. We propose a two-step model called the target creation strategy where initial DNA-damaging antineoplastic therapy is combined with subsequent radioimmunotherapy that is directed by a cell death radioligand, such as 3B9. This approach is designed to enhance the therapeutic ratio of the radioimmunotherapy, as new targets for binding of the radioimmunoconjugates are continuously and selectively created within tumors as the targeted radiation further damages nearby viable tumor cells. The therapeutic potential of this strategy may be further improved by the incorporation of new agents that are potent radiosensitizers, such as the histone deacetylase inhibitors (53, 54).

Acknowledgments

We thank Dr. Ghafar Sarvestani (Institute of Medical and Veterinary Science) for technical assistance with confocal microscopy and Dr. Tom P. Gordon for helpful advice and antibody reagents.

4 Al-Ejeh et al., in preparation.

References


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