Injury-Associated Differential Regulation of Histone Expression and Modification in the Thymus of Mice

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One of the key events in the regulation of gene expression is chromatin remodeling involving histone regulation. We investigated the effects of burns on the expression of histone that might be associated with altered molecular and pathological profiles in the thymus. A markedly decreased expression of histone variant H2A.1 mRNA was identified in the thymus after burn during a differential display experiment. Subsequently, we examined the histone expression (mRNA and protein) and posttranslational modification in the thymus after burn. Also, changes in proliferating cell nuclear antigen (PCNA), a central molecule in chromatin assembly, was examined. Reverse-transcription polymerase chain reaction analysis revealed a transient decrease in the expression of several histone variants (H2A.1, H1(r1), H3-B, H3-1, and H4-D) mRNAs in the thymus at 1 day after burn. A decrease in histone subtypes H2A, H2B, H3, and H4, but not H1, was demonstrated 1 and 3 days after burn according to the results of Western blot. Furthermore, there were different levels of decreases in acetylated and dimethylated forms of histone H3 1 and 3 days after burn. In addition, decreased levels of PCNA were evident in the thymus 1 day after burn. Changes in the expression of histones and PCNA may reflect mere decrease in proliferating cells and/or a reorganization of the chromatin structure associated with altered transcriptional activities, eventually contributing to the phenotypic changes in the thymus after burn. Exp Biol Med 229:327–334, 2004

Key words: differential display; histone isotypes; proliferating cell nuclear antigen (PCNA); thymus; injury

Immune suppression associated with systemic inflammation is a common complication of burn. Alterations in the cellular and molecular profiles in the immune system may contribute to the local and systemic responses to burn (1). A reduction in the number of lymphocytes in the thymus, spleen, and blood has been detected in patients and experimental animals with burns (2, 3). Phenotypic alterations in the immune system after burn also include the differential regulation of cytokines, transcription factors, and cell cycle progression (4–6). In particular, increases in apoptosis and the expression of tumor necrosis factor-α (TNF-α) and decreased numbers of S-phase cells have been observed in the thymus 1 day after burn (7). Recent studies suggested that increased activities in transforming growth factor-β and glucocorticoids are involved in the regulation of a variety of genes in the thymus after burn (8, 9).

An ample amount of data suggest that histones play crucial roles in cellular and molecular events such as chromatin assembly, transcription, DNA replication, chromosome segregation, and DNA damage repair (10, 11). Histones, which are divided into five subtypes (H1, H2A, H2B, H3, and H4), make up the most abundant moiety of the chromosome (12). In addition, histones create and maintain the structure of chromatin, whose spatial configuration influences the accessibility of transcriptional machinery to regulatory elements. The modification (e.g., acetylation, phosphorylation, and methylation) of histones by various stress signals changes the structure and function of the chromatin, leading to the transcriptional regulation of a wide range of genes (13). One of the defining features of the histone complex is the timely increase in gene expression of replication-dependent histone subtypes during cell cycle progression. Recent studies have provided evidence of how the cell cycle machinery is directly linked to the periodic histone synthesis (14, 15).

During the progression of the cell cycle, the newly synthesized DNA strands are assembled into chromatin (16). The deposition of newly synthesized histones onto the daughter DNA strands and ordered packaging into the nucleus are mediated by several factors, such as chromatin assembly factor-1 (CAF-1), proliferating cell nuclear antigen (PCNA), and ATP-dependent chromatin assembly and spacing factor (17). PCNA is a replication fork-associated protein that forms a ringlike, noncovalent structure around

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Table 1. Description of Strain and Number of Mice in Each Burn Experiment

Analysis	Strain	Number of mice in each group
Differential display and RT-PCR	C57BLKS/J	No burn, 3; burn: 3 hrs, 3; 6 hrs, 3; 1 day, 3; 3 days, 2; 7 days. 3; 14 days, 2; 29 days, 3
RT-PCR	C3H/HeJ	No burn: 3 hrs, 1; 6 hrs, 2; 1 day, 3; 7 days, 2; burn: 3 hrs, 3; 6 hrs, 3; 1 day, 3; 7 days, 3
RT-PCR	C57BLKS/J	No burn: 3 hrs, 4; 1 day, 4; 7 days, 4; burn: 3 hrs, 4; 1 day, 4; 7 days, 4
Western blot	C57BLKS/J	No burn: 1 day, 3; 3 days, 3; burn: 1 day, 3; 3 days, 3
ELISA (PCNA)	C57BLKS/J	No burn: 3 hrs, 5; 1 day, 5; burn: 3 hrs, 5; 1 day, 4

DNA strands and serves as a sliding clamp (18). PCNA is an essential factor for DNA replication and chromatin assembly in regard to the processivity of the DNA polymerase complex and the chaperoning newly synthesized histones to replicating DNA (19, 20).

It is possible that the chromatin remodeling and altered cell cycle progression, in association with the alterations in histones, plays an important role in the burn-mediated changes in immune organs. A characterization of the changes in molecular profiles of various histone subtypes and PCNA will help us to better understand the pathogenic mechanisms involved in the thymic phenotype after burn.

Materials and Methods

Animal Experiment. Female mice (C57BLKS/J and C3H/HeJ strains, 12–13 weeks old) from Jackson Laboratories (Bar Harbor, ME) were maintained in the Animal Resources Service at the University of California, Davis, under guidelines of the National Institutes of Health for the treatment of experimental animals. The Animal Use and Care Administrative Advisory Committee of the University of California, Davis, approved the protocol. The burn protocol has been described elsewhere (21). In brief, under the methoxyflurane anesthesia, a Teflon template measuring 2.1 × 4.0 cm was placed over the shaved back of a mouse. Within the cut portion of the template, 0.5 ml of 70% ethanol was ignited and allowed to burn until exhausted, resulting in an ~18% total body surface area (TBSA) full-thickness burn. Buprenorphine (3 μg) and 1 ml of normal saline were given as intraperitoneal injections for pain control and resuscitation. Control mice were shaved, anesthetized, and resuscitated but not burned. The burn experiments were performed in both C57BLKS/J and C3H/HeJ strains of mice. Mice were killed by either cervical dislocation or exsanguination via cardiac puncture, and thymic tissues harvested at several time points after burn were snap frozen in liquid nitrogen and stored at –80°C until further analyses. Table 1 summarizes the strain and number of mice used in each burn experiment.

Differential Display with a Modified 3′ Rapid Amplification of cDNA Ends (RACE) Protocol. Total RNA was isolated using an RNEasy kit (Qiagen Inc., Valencia, CA), and 100 ng total RNA from each sample was subjected to reverse transcription (RT) using Sensiscript reverse transcriptase (Qiagen Inc.). The oligo-dT primer for the RT has an anchor sequence at the 5′ end, and the sequences for oligo-dT and anchor primers were as follows: 5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3′ (oligo-dT primer) and 5′-GGC CAC GCG TCG ACT AGT AC-3′ (anchor primer). Modified 3′ RACE analysis was done to identify differentially regulated genes using combinations of several upstream anonymous primers, instead of commercial arbitrary primers designed specifically for the differential display experiment, and the downstream anchor primer described above. No radioactive labeling of polymerase chain reaction (PCR) products was introduced. The sequence of the upstream primer that rendered the identification of the differentially expressed histone H2A.1 variant was as follows: 5′-CTG ACC GGT CCC GCG CCA-3′. *Taq* polymerase from Qiagen, Inc., was used with for the PCR amplification with the following program: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 40 cycles. PCR products were analyzed on 1.5% agarose gel instead of polyacrylamide gel. The comparability between samples was provided by the β-actin amplification of each sample. A set of primers used for β-actin amplification were as follows: 5′-CCA ACT GGG ACG ACA TGG AG-3′ (upstream) and 5′-GTA GAT GGG CAC AGT GTG GG-3′ (downstream).

Cloning and Sequence Analysis. Differentially amplified PCR products were gel purified using a QIAquick Gel Extraction kit (Qiagen, Inc.) and cloned into the pGEMT-Easy vector (Promega, Madison, WI). Plasmid DNAs for sequencing analysis were prepared using a QIAprep Spin Miniprep kit (Qiagen, Inc.). Sequencing was done at Davis Sequencing, Inc. (Davis, CA), using the ABI Prism 377 DNA sequencer (PE Biosystems, Foster City, CA). All DNA sequences were initially analyzed using the Advanced BLAST program (National Center for Biotechnology Information).

RT-PCR Analysis. cDNAs were synthesized as described above, and PCR was done using the same conditions as those used for the differential display except for varying annealing temperatures (48°C or 55°C) and number of cycles (25–35 cycles), depending on target sequences (different histone variants). The comparability between samples was determined by RT-PCR amplification of β-actin from each

Table 2. Primer Sequences of Histone Variants

Histone variant (Ref.)	Upstream primer	Downstream primer
rH1(r1) (22)	5′-AGTCGCTCAGGCTGCTTCTAC-3′	5′-CGCTGCCTTCTTGGGCTTAG-3′
H2A.1 (23)	5′-GTGGCAAGGCTCGCGCCAAG-3′	5′-GGTGAGTGAACCGCTGGTC-3′
H2A.2 (24)	5′-CTTTCGTGATGTCCGGTCGTGG-3′	5′-TGGTGGCTCTCCGTCTTCTTG-3′
H3.3A (25)	5′-GAGGTGTCCTTACCATGGCTCG-3′	5′-TGGCATGGATAGCACACAG-3′
H3-B (24)	5′-CTGCAATGGCTCGTACTAAGC-3′	5′-ATGTCCTTGGGCATGATGG-3′
H3-D (24)	5′-ATGGCTCGTACTAAGCAGACC-3′	5′-GTTGGATGTCCTTGGGCATG-3′
H3-I (24)	5′-AGTGTACTGAGATGGCTCG-3′	5′-ACAGGTAGGGGAATTACGCC-3′
H4-D (24)	5′-AAGGACCGACTCTCAGCTCTG-3′	5′-CTTAGCCGCCGAATCCGTA-3′

sample. Sequences of primer sets used for the amplification of different histone variants are listed in Table 2 (22–25). PCR products were analyzed on 1.5% agarose gel.

Total Histone Preparation. Thymic tissues were processed for the total histone preparation using the low-concentration acid extraction procedure. Before homogenization, the wet weight of each tissue was measured for the normalization of histone preparations. Tissues were homogenized in 1× phosphate-buffered saline (PBS) on ice. The homogenates were centrifuged at 12,000 *g* for 10 mins. The resuspension of pellets in hypotonic buffer (10 m*M* HEPES, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.2 m*M* phenylmethylsulfonyl fluoride, and 0.5 m*M* dithiothreitol) on ice for 10 mins was followed by sonication. The cytoplasmic fraction was removed after centrifugation at 3300 *g* for 15 mins and the nuclear fraction (pellet) was resuspended in 0.2 *M* H_2SO_4 overnight at 4°C, followed by trichloroacetic acid (TCA) precipitation and centrifugation for 10 mins at 12,000 *g*. Dried pellets were resuspended in acetic acid/urea sample buffer (5.8 *M* urea, 0.9 *M* glacial acetic acid, 16% glycerol/0.2% methyl green, and 4.8% 2-mercaptoethanol). The total histone preparations were normalized on the basis of wet weights of thymic tissues measured before the homogenization step. The quality of the histone preparations, including the accuracy of normalization, was examined by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

Western Blot Analysis. SDS-PAGE was done on the normalized total histone preparations described above using precast 15% resolving/4% stacking Tris-HCl gels (Bio-Rad, Hercules, CA). Subsequently, gels were transferred onto a PVDF membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Each blocked membrane was incubated with respective primary antibodies against histone isotypes (H1, H2A, H2B, H3, and H4) and modifications (acetyl-histone H3 [lysine 14] and dimethyl-histone H3 [lysine 36]). Primary antibodies against histone isotypes H1, H2A, H2B, and H3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the rest were from Upstate Biotechnology (Lake Placid, NY). Subsequently, the membranes were incubated in the relevant horseradish peroxidase–conjugated secondary antibody. The histone signal was amplified and visualized via chemiluminescence using the ECL Plus Western Blotting detection System (Amersham Pharmacia Biotech, Inc.). The absolute integrated optical density of each band was determined using the Gel-Pro Analyzer (Media Cybernatics, Silver Spring, MD).

PCNA Enzyme-Linked Immunosorbent Assay (ELISA). Thymic tissues were homogenized using mild lysis buffer that contained 1% nonidet 40, 0.15 *M* NaCl, 0.01 *M* sodium phosphate (pH 7.2), 2 m*M* EDTA, 50 m*M* sodium fluoride, 0.2 m*M* sodium vanadate, and 1 μg/ml aprotinin. The tissue homogenates were then centrifuged at ~20,000 *g* for 15 mins, and supernatants were collected. Subsequently, these extract preparations were normalized using the Bio-Rad protein assay kit (Bio-Rad). PCNA levels were measured in thymic extracts using an ELISA kit from Oncogene Research Products (San Diego, CA), according to the manufacturer's instructions. In brief, thymic extracts and detection antibody were pipetted into wells coated with capture antibody, and the reaction mix was incubated for 2 hrs. Streptavidin peroxidase was added to the bound antigen, and the signal was detected using the chromogenic substrate tetramethyl-benzidine. The absorbance was read at 450 nm, and the PCNA levels from thymus extracts were determined from the standard curve. The data was statistically analyzed by Student's *t* test.

Results

Identification of Decreased Gene Expression for the Histone H2A.1 Variant in the Thymus After Burn by Differential Display. To identify genes that contribute to the phenotypic alterations in the thymus (e.g., apoptosis and atrophy) after burn, total RNAs isolated from the thymus of mice at several time points (no burn controls and 3 hrs–29 days) were subjected to the RT-PCR differential display (7). One of these differential display experiments revealed a distinct pattern of increase or decrease of several genes after burn (Fig. 1). Cloning and sequencing analyses of these bands led to the identification of each band, including a mouse histone H2A variant, H2A.1. There was a marked decrease of histone H2A.1 expression in the thymus 1 day after burn compared with control mice. The other regulated bands were not related to histone genes.

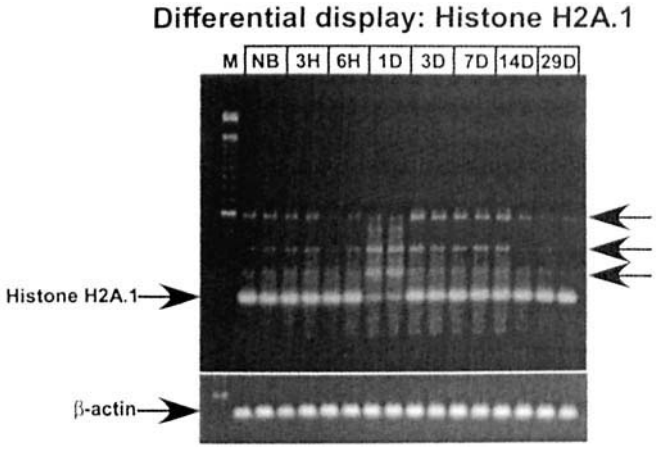

Figure 1. Identification of differentially expressed histone H2A.1 variant in the thymus after burn. Thymus tissues harvested from C57BLKS/J mice at several time points (no burn control [NB], 3 hrs–29 days) after an ~18% TBSA burn were subjected to RT-PCR differential display analysis. Several bands (arrows) were differentially amplified, and subsequent cloning and sequence analyses revealed the identity of each band, including histone H2A.1 variant. The comparability between samples was determined by the β-actin amplification of each sample. M, molecular weight marker (100 bp ladder).

Differential Alterations in the Expression of Histone Variant mRNAs in the Thymus After Burn. The identification of the profound decrease of histone H2A.1 initiated the screening of several other histone variants in the thymus of C57BLKS/J mice after burn. Total RNAs isolated from the thymus were subjected to RT-PCR analysis using primer sets specific for each histone variant (Table 2; Refs. 22–25). The marked decrease in the histone H2A.1 variant found by the differential display was confirmed in the thymus at day 1 (Fig. 2). The decrease in the histone H2A.1 variant was also reproduced in another strain of mice, C3H/HeJ (Fig. 2). In contrast to histone H2A.1 variant, a slight increase in the histone H2A.2 variant was measured in the thymus 1 day after burn (Fig. 2). Furthermore, there was a decrease in histone variants of H1(r1), H3-B, H3-I, and H4-D in the thymus 1 day after burn. However, no significant changes were observed in the expression levels of histone variants H3-3A and H3-D in the thymus after burn.

Changes in the Expression of Histone Protein Subtypes and Histone H3 Posttranslational Modification in the Thymus After Burn. The integrity of normalized thymic histone preparations was determined by SDS-PAGE followed by Coomassie blue staining (Fig. 3). The staining pattern identified the typical histone profile from all samples examined. There were three distinct groups of histone bands, H1 (~31 kD), H2A (~14 kD)/H2B (~15 kD)/H3 (~17 kD), and H4 (~10 kD), respectively. There was a marked decrease in histone subtypes H2A, H2B, H3, and H4 1 and 3 days after burn, whereas no significant change was observed in the histone H1 subtype 1 and 3 days after injury (Fig. 3).

Because the posttranslational modification status of histones plays a crucial role in the chromatin remodeling that is associated with the transcriptional regulation of a wide range of genes, we examined whether burn alters the posttranslational modification status of histones in the thymus (26–29) and measured changes in the thymic levels of acetyl-histone H3 (lysine 14) and dimethyl-histone H3 (lysine 36) in response to burn. A substantial decrease was observed in both 1 and 3 days after burn (Fig. 3). Densitometric analyses of Western blot data provide values of relative changes in each histone expression and H3 posttranslational modifications compared with control mice.

Decrease in Thymic PCNA Levels 1 Day After Burn. Close links have been established between histones and PCNA in cellular events, such as cell cycle–associated nucleosome assembly. The discovery of the differential regulation of histone expression and posttranslational modification led to the investigation into changes in the levels of PCNA in the thymus after burn. No significant changes in levels of PCNA in thymic extracts were observed 3 hrs after burn (control, 32.2 ± 0.4 U/ml vs. burn, 29.9 ± 0.8 U/ml; Fig. 4). However, there was a significant decrease of PCNA levels 1 day (control, 23.8 ± 1.2 U/ml vs. burn, 14.5 ± 0.4 U/ml; $P < 0.01$). Of interest, the decrease in PCNA paralleled the alteration in the expression and posttranslational modification of histones in the thymus after burn as described above.

Discussion

Burn decreased the expression and posttranslational modification of all histones examined in the thymus, except for some variant mRNAs (H2A.2, H3-3A, and H3-D) and the H1 protein. One potential interpretation might be that certain signaling events triggered by burn alter interactions among the intracellular molecules involved in histone gene expression. In particular, injury-associated pathophysiologic alterations (e.g., cytokine production, increased proteolytic activity, the induction of glucocorticoids, and oxidative stress) in the thymus may influence the expression of histones (3, 8, 30, 31). It is of interest to note that the altered expression of several histone variant mRNAs in the thymus parallels our previous findings of increases in apoptosis and TNF-α induction 1 day after burn (7). The chromatin condensation and DNA fragmentation in the cells undergoing apoptosis might be linked to alterations in histone expression and posttranslational modification (4, 32).

Because the biosynthesis of histones is known to be coordinated with changes in the progression of the cell cycle, one can speculate that the simultaneous decrease of several histone subtypes at day 1 is associated with the status of cell cycle progression (33–35). Our recent report of the decrease in the fraction of cells during the S phase in the thymus 1 day after burn suggests that the decrease of histones solely represents their bystander roles associated with decrease in DNA abundance without any specific biological effects (4). It is also probable that the substantial decrease of these histone subtypes may contribute to alterations in the transcriptional

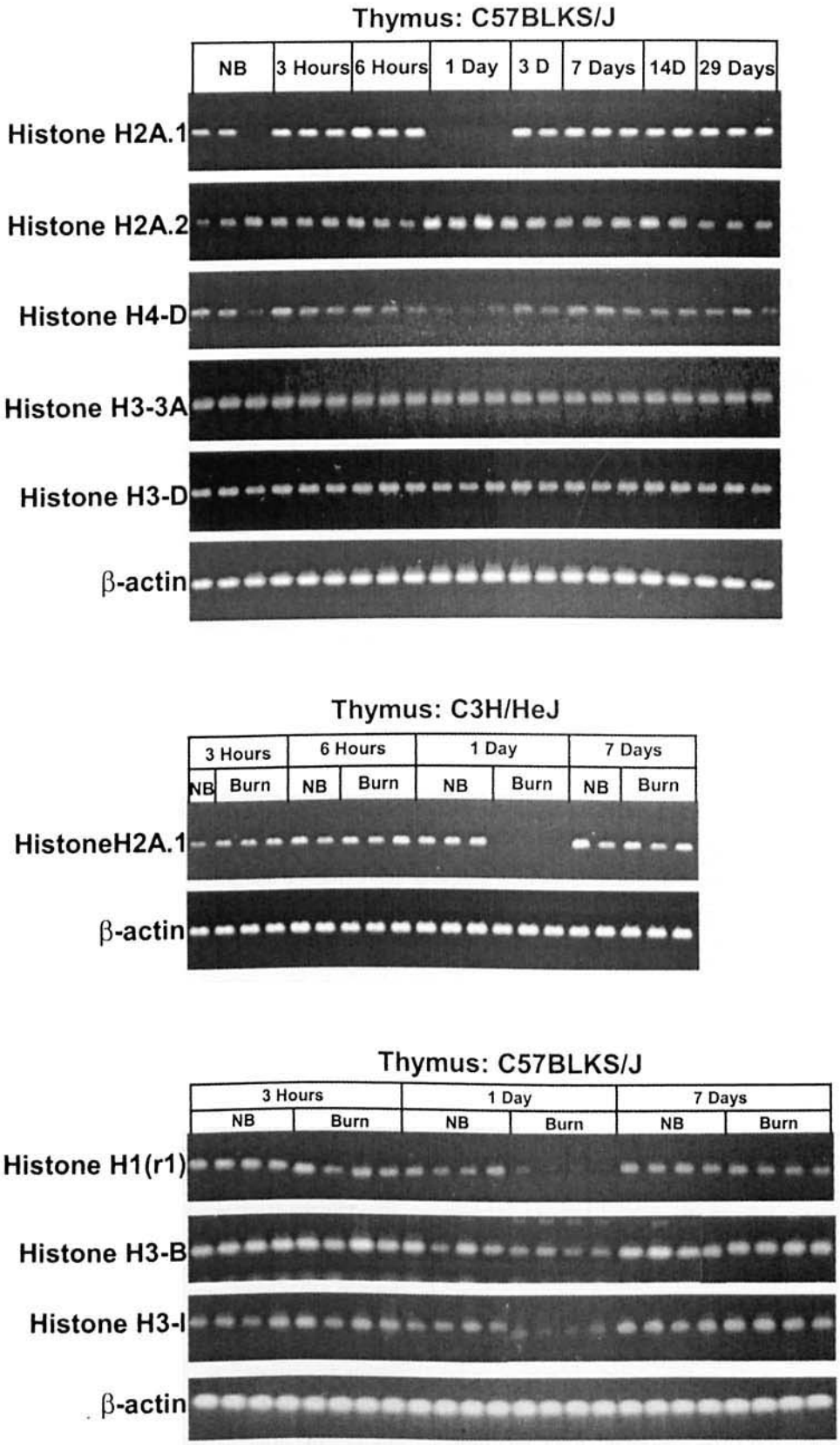

Figure 2. RT-PCR analysis of the expression profile of histone variants in the thymus after burn. Thymus tissues collected from C57BLKS/J and C3H/HeJ strains of mice at several time points (3 hrs–29 days, 3 hrs–7 days, or 3 hrs–3 days) after an ~18% TBSA burn were subjected to the RT-PCR screening of histone variants. Several variants (H2A.1, H1(r1), H3-B, H3-I, and H4-D) were markedly decreased at day 1, when histone H2A.2 levels were slightly increased. The comparability between samples was provided by the β-actin amplification of each sample. NB, no burn control.

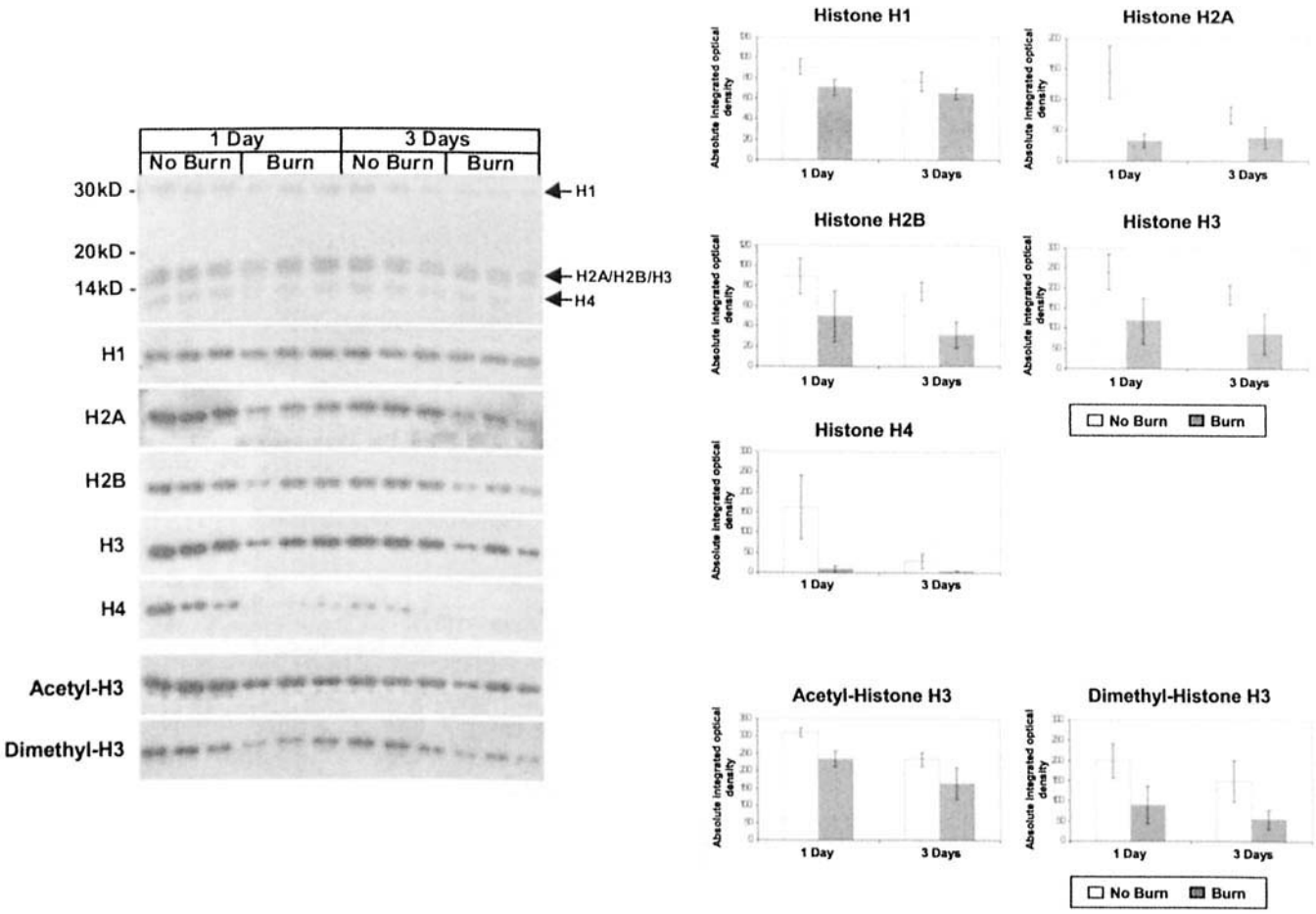

Figure 3. Western blot analysis of the expression profile of histone subtypes and histone H3 posttranslational modification in the thymus after burn. Thymic tissues (C57BLKS/J) harvested 1 and 3 days after an ~18% TBSA burn were processed for total histone preparations. Coomassie blue staining of total histone preparations demonstrated three distinct groups of histone bands that consisted of H1 (~31 kD), H2A (~14 kD)/H2B (~15 kD)/H3 (~17 kD), and H4 (~10 kD). Western blot analyses revealed a marked decrease of histone subtypes H2A, H2B, H3, and H4 1 and 3 days after burn, whereas no apparent regulation of histone H1 subtype was observed. In the posttranslational modification study, a substantial decrease in acetyl-histone H3 (lysine 14) and dimethyl-histones H3 (lysine 36) was observed at 1 and 3 days. Densitometric analyses of Western blot data provide values of relative changes in histone expression and posttranslational modification compared with no burn controls (NB).

activities of a wide range of genes in the thymus by controlling the access of regulatory factors to various transcription elements as a result of altered chromatin configuration (36, 37).

The increase of histone H2A.2 variant at day 1 suggests that histone H2A.2 is more likely to be involved in gene regulation through chromatin remodeling rather than the cell cycle response in the thymus after burn. In fact, it has been reported that changes in chromatin structure represented by a decrease in histone H2A.1 and an increase in histone H2A.2 were observed during subcutaneous passages of Friend murine tumors (38). Furthermore, Friend murine tumors with a higher malignant potential had a lower ratio of H2A.1:H2A.2, which suggests different roles of these histone variants in chromatin reorganization and, possibly, gene regulation. On the other hand, because of the long lifespan and low turnover rate of histones, the proteolytic degradation of damaged histones, which might be responsible for decreased levels of histones after burn, may be essential to maintain the integrity of the genome. It is important to have functional histones for their well-controlled reversible associations with DNA during transcription, replication, and repair of damaged DNA (39, 40).

The acetylation of histone is involved in the relaxation of the chromatin structure, which allows for an easier accessibility of transcriptional factors to target elements. The methylation of lysine residues in the N terminus of histone subtypes has been implicated in either transcriptional activation or repression (41). The methylation of histone H3 (lysine 36) and acetylation of histone H3 (lysine 14) were substantially decreased in the thymus after burn in the present study. These changes may imply alterations in cytosolic events controlling the posttranslational modification of histones in the thymus after burn. These alterations in histone modifications may reflect a reorganized chromatin structure, which, in turn, establishes an altered environment

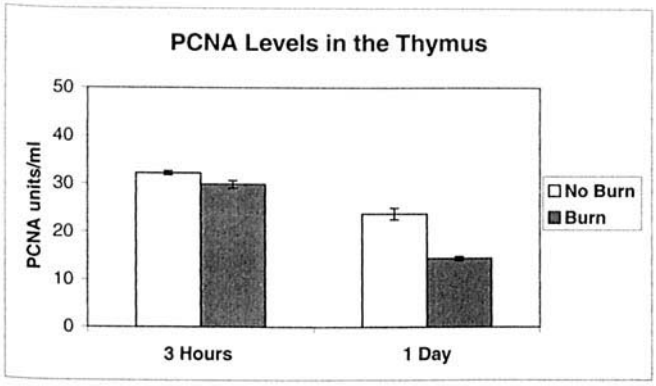

Figure 4. Decrease in PCNA levels in the thymus after burn. Thymic tissues (C57BLKS/J) collected 3 hrs and 1 day after an ~18% TBSA burn were processed as tissue extracts and subjected to PCNA ELISA. The absorbance was measured at 450 nm, and relative PCNA levels (in U/ml) were determined from the standard curve. The PCNA levels in the thymus were significantly decreased 1 day after burn ($P < 0.01$), whereas no apparent changes were noted at 3 hrs.

for transcriptional activities in the thymus in response to burn. It is also probable that oxidative stresses developed in the thymus may change the profile of histone modifications in association with amino acid oxidation, the formation of protein-protein crosslinks, and DNA-protein crosslinks (42, 43). It needs to be noted that the regulation patterns of histone H3 and its posttranslational modifications are similar each other, which suggests that the decrease of histone H3 expression is reflected on changes in histone H3 post-translational modifications.

A marked decrease in PCNA levels 1 day after burn parallels our previous findings, such as an increase in apoptosis and a decrease in the fraction of cells in S phase (4, 7). PCNA is one of the key molecules controlling DNA replication, nucleosome assembly, and cellular differentiation pathways (e.g., differentiation, senescence, and apoptosis; Refs. 6, 44). For instance, the absence or low-level expression of PCNA renders cells prone to apoptosis. On the other hand, the abundant expression of PCNA allows for DNA replication and/or repair. CAF-1, in association with PCNA and other replication fork–associated factors, chaperones histones (mainly H3 and H4 isotypes) to replicating DNA strands (45, 46). Substantial decreases in PCNA levels may reflect a defect in the nucleosome assembly pathway linked to DNA replication and cell cycle progression. The inefficient deposition of a tetramer of histones H3 and H4, which is the first step of nucleosome assembly, caused by decreased levels of PCNA after burn may hinder the second step as well where two histone H2A-H2B heterodimers are added to yield an octamer core (47).

Decreases in the expression and posttranslational modification of histones and PCNA in the thymus may merely reflect the decreased number of cells in the S phase and increased apoptosis after burn. Alternatively, burn may initiate an array of signaling events that regulate the expression of a wide range of genes controlling the thymic phenotype (e.g., cell cycle arrest of immune cells) through changes in histone expression and posttranslational modification contributing to the altered chromatin structure. In addition, the regulation of PCNA, a DNA replication–associated protein, may play key roles in controlling cell fate (e.g., replication, differentiation, senescence, and apoptosis) after burn.

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