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Nutrition and Cancer

Biotinylation of K12 in Histone H4 Decreases in Response to DNA Double-Strand Breaks in Human JAr Choriocarcinoma Cells[1,2]

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ABSTRACT We tested the hypothesis that biotinylation of K12 in histone H4 plays a role in the cellular response to double-strand breaks (DSB) of DNA in human cells. DSB were caused by treating choriocarcinoma JAr cells with etoposide. Biotinylation of K12 in histone H4 decreased by 50% as early as 10–20 min after initiation of treatment with etoposide. Biotinylation returned to initial levels 30–40 min after the addition of etoposide to the medium. Temporal patterns of K12-biotinylation were similar for human lymphoma cells. Phosphorylation of S14 of histone H2B and poly(ADP-ribosylation) of glutamate residues on histone H2A are known markers of DSB in DNA; these modifications increased 10–40 min after alterations in K12-biotinylation occurred. Decreased biotinylation of K12 of histone H4 was specific for DSB but was not detectable in response to single-strand breaks or the formation of thymine dimers. Biotin-deficient choriocarcinoma cells exhibited a 40% decrease in rates of survival in response to etoposide compared with biotin-sufficient controls. These studies suggest that the lack of biotinylation of K12 in histone H4 is an early signaling event in response to DSB. J. Nutr. 135: 2337–2342, 2005.

KEY WORDS: • *biotin* • *DNA damage* • *double-strand breaks* • *histone H4*

In eukaryotic cell nuclei, DNA associates with histones H2A, H2B, H3, and H4 to form nucleosomal core particles. Histones may undergo various post-translational modifications in their N-termini. These modifications include acetylation (1–3), methylation (4), phosphorylation (4), ubiquitination (4), and poly(ADP-ribosylation) (5–7) of ϵ-amino groups, guanidino groups, carboxyl groups, and hydroxyl groups. Distinct patterns of modifications of individual histones, nucleosomal core particles, and chromosomal domains play unique roles in chromatin structure, cell signaling, and genomic stability (8,9). Histone modifications may affect each other in synergistic or antagonistic ways. For example, acetylation of K23 mediates methylation of R17 in histone H3 (10).

There is increasing evidence that histones are also covalently modified by the vitamin biotin (11–13), mediated by holocarboxylase synthetase (14) and biotinidase (11). The following biotinylation sites were identified in human histones: K9 and K13 in histone H2A (15); K4, K9, and K18 in histone H3 (16); and K8 and K12 in histone H4 (13). Biotinylation of histones plays a role in processes such as cell proliferation (12,14), gene silencing (17), and the cellular response to UV-induced DNA damage (17).

Various cellular events and environmental insults may cause DNA damage. For example, UV light may trigger the formation of thymine dimers, whereas the antineoplastic agent etoposide inhibits topoisomerase II, mediating double-strand breaks (DSB)[4] of DNA. Damaged DNA causes chromosomal abnormalities, potentially leading to malignant transformations. The risk for propagation of premalignant cells is reduced by activation of the following pathways in response to DNA damage: *1*) activation of DNA repair mechanisms, which mediate the removal of DNA lesions (18); and *2*) activation of apoptotic cascades, which mediate the elimination of premalignant cells if DNA damage is beyond repair (19). Histone modifications play a crucial role in these processes. For example, DNA damage causes poly(ADP-ribosylation) of histones, recruiting DNA repair proteins to sites of DNA damage (20). These proteins participate in base excision repair (21) and nucleotide repair pathways (22).

Here, we tested the hypothesis that biotinylation of histone H4 plays a role in the cellular response to DSB of DNA. Specifically we determined *1*) whether etoposide-induced DSB are associated with alterations of K12 biotinylation in histone H4 in cells originally derived from various human tissues; *2*) whether putative changes of K12 biotinylation in histone H4 are specific for DSB or whether similar changes are observed in response to the formation of thymine dimers and single-strand breaks; and *3*) whether biotin concentrations in culture media affect cell survival in response to DSB in DNA.

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[2] Supplemental Table 1 is available as Online Supporting Material with the online posting of this paper at www.nutrition.org.

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[4] Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole; DSB, double-strand breaks; TUNEL, TDT-mediated dUTP nick end labeling.

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MATERIALS AND METHODS

Cell culture. Human choriocarcinoma (JAr) cells and lymphoid (Jurkat) cells (ATCC) were cultured in biotin-supplemented medium (2 μmol/L) as described (23,24). JAr cells were harvested at 60–80% confluence for subsequent analyses. Culture media were supplemented with sodium butyrate (5 mmol/L) for 24 h to inhibit histone deacetylases if samples were analyzed for acetylated histones. For survival studies, biotin-deficient JAr cells were obtained by culturing cells in biotin-deficient medium (0.025 nmol/L) for 3 wk as described (23); cells cultured in medium containing a physiologic concentration of biotin (0.25 nmol/L) were used as control.

DNA damage. Cells were treated with 60 nmol/L etoposide for up to 2 h to cause DSB in DNA (25). Specificity controls were treated as follows: 20 nmol/L doxorubicin for up to 2 h to cause DSB in DNA (26); 0.15 mmol/L zeocin to cause single-stranded DNA breaks (27); and UV light (500 mJ/m^2) for 30 s (28) to cause the formation of thymine dimers. At timed intervals, cells were collected for analyses as described below.

Etoposide is an inhibitor of topoisomerase II, causing DSB at the replication fork and G2 phase arrest (25). Theoretically, etoposide will not cause DSB until the replication fork is formed in the S phase of the cell cycle. Hence, etoposide-resistant controls were generated by treating cells with hydroxyurea (2 mmol/L) for 30 h to cause G1 arrest (29) before treatment with 60 nmol/L etoposide. G1 arrest was confirmed by staining with propidium iodide and flow cytometry (Cell Analysis Facility, University of Nebraska Medical Center) as described (30).

In some experiments, we determined whether biotin deficiency decreased the survival of etoposide-treated JAr cells. In these experiments, biotin-deficient cells and biotin-sufficient controls were treated with 6 nmol/L etoposide, and cell viability was quantified at timed intervals using trypan blue exclusion (31).

Histone analysis. Nuclear histones were extracted from JAr and Jurkat cells using 1 mol/L HCl (12). For analysis of phosphorylated histones, 1 mmol/L sodium vanadate was added to the HCl used for extraction. Equal amounts of histones (as judged by gel densitometry after staining with Coomassie blue) were resolved by using 18% Tris glycine gels (Invitrogen). Proteins were electroblotted onto polyvinylidene fluoride membranes; histone modifications were probed using the following primary antibodies: rabbit anti-human biotinyl-K12 histone H4 (13), rabbit anti-human acetyl-K12 histone H4, rabbit anti-human dimethyl-K4 histone H3, rabbit anti-human phospho-S14 histone H2B, and mouse anti-human ubiquitinyl-histone H2A (Upstate). Antibody binding was visualized using appropriate horseradish peroxidase-labeled secondary antibodies and chemiluminescence; gel densitometry was used for quantification (32). If bands were too faint for detection by gel densitometry, we used half the detection limit for data analysis.

Poly(ADP-ribosylation) of histones is mediated by poly(ADP-ribose) polymerase using NAD as a substrate (20). For analysis of poly(ADP-ribosylation) of histones, JAr cell nuclei were isolated as described (33). Nuclei were incubated with [^{32}P]NAD for 5 min, followed by incubation with 60 nmol/L etoposide for up to 2 h (33) The reaction was quenched using 10 mmol/L nicotinamide (33). Histones were isolated using acid extraction (12), and poly(ADP)ribosylated histones were quantified by autoradiography and gel densitometry.

Analysis of DNA breaks. Cells were grown on microscope coverslips for treatment with etoposide as described above. Positive controls were treated with 1 μmol/L DNase for 10 min at room temperature. DNA breaks were visualized by TDT-mediated dUTP nick end labeling (TUNEL) using a commercial kit (ApoAlert-DNA Fragmentation Assay; BD Biosciences). DNA strand breaks appear in green color due to the incorporation of fluorescein-labeled nucleotides at nicked ends. Images were obtained using an Olympus FV500 confocal microscope (Microscopy Core Facility, University of Nebraska-Lincoln).

Immunocytochemistry. Intracellular histone H4 was visualized using standard procedures of immunocytochemistry (34). Anti-human biotinyl-K12 histone H4 antibody (13) and Cy™2-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) were used for staining. The cytoplasmic compartment was stained using rhodamine phalloidin (Molecular Probes), and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). S14-phosphorylated histone H2B is a known marker for DNA damage (34) and was visualized using rabbit anti-human phospho-S14 H2B and Cy2-conjugated donkey anti-rabbit IgG (positive control). Images were obtained by using an Olympus FV500 confocal microscope (Microscopy Core Facility, University of Nebraska-Lincoln).

Biotin-dependent carboxylase. Biotin-dependent carboxylases are good markers for cellular biotin (35). Biotinylated carboxylases in cell extracts were resolved by PAGE and probed using streptavidin peroxidase (24)

Statistics. Homogeneity of variances among groups was tested using Bartlett's test (36). If variances were heterogeneous, the data were log-transformed before further statistical analysis. Significance of differences among groups was tested by one-way ANOVA. Fisher's Protected Least Significant Difference procedure was used for post-hoc testing (36). Effects of treatment over time (survival experiment) were analyzed by repeated-measures ANOVA, followed by pairwise comparison of time points (e.g., biotin-deficient vs. biotin-sufficient cells at time 6 h) by paired *t* test (36). StatView 5.0.1 (SAS Institute) was used to perform all calculations. Differences were considered significant if $P < 0.05$.

RESULTS

Biotinylation of K12 in histone H4 decreased rapidly but transiently in response to treatment with etoposide in JAr cells. Specifically, biotinylation of K12 in histone H4 decreased by ~50% as early as 10–20 min after initiation of treatment with etoposide (**Fig. 1** and online Supplemental Table 1). Biotinylation of K12 returned to pretreatment levels within 30–40 min after addition of etoposide to culture media.

Biotinylation of K12 in histone H4 appears to be an early signaling event in response to DNA damage. This notion is based on a comparison of the time course of K12-biotinylation with the time courses of other known markers of DNA damage (7,34). First, phosphorylation of S14 in histone H2B increased from barely detectable levels at zero time to detectable levels ~0 min after the addition of etoposide to culture media (Fig. 1 and online Supplemental Table 1). Second, poly(ADP-ribosylation) of histone H2A increased from barely detectable levels at zero time to maximal levels 60 min after the addition of etoposide to culture media. Hence, these 2 markers of DNA damage were detectable 10–40 min after alterations in K12 biotinylation occurred.

DSB of DNA caused specific time-dependent modifications of histones as opposed to global changes in histone modifications. For example, treatment of JAr cells with etoposide did not affect the abundance of K4-dimethylation of histone H3 and poly(ubiquitination) of histone H2A (Fig. 1 and online Supplemental Table 1), strongly suggesting that changes in biotinylation of K12 on histone H4 were an early indicator of DSB in DNA in response to etoposide treatment. The occurrence of DSB of DNA was confirmed by TUNEL assay. DNA breaks were detectable ~60 min after the addition of etoposide to culture media, but some damage probably occurred earlier (**Fig. 2**).

Biotinylation of K12 in histone H4 did not change in response to etoposide treatment in G1 phase-arrested JAr cells (**Fig. 3**). We compared temporal patterns of K12-biotinylation in G1 phase-arrested JAr cells and a mixed population of asynchronous JAr cells. G1 arrest by hydroxyurea treatment was confirmed using flow cytometry (Fig. 3, *top row*). Note that etoposide causes DSB only during the S phase of the cell cycle (25). Consistent with this notion, treatment with etoposide did not affect biotinylation of K12 in histone H4 in G1-arrested cells, but caused a substantial decrease in K12-biotinylated histone H4 in asynchronous cells (Fig. 3, *bottom row*).

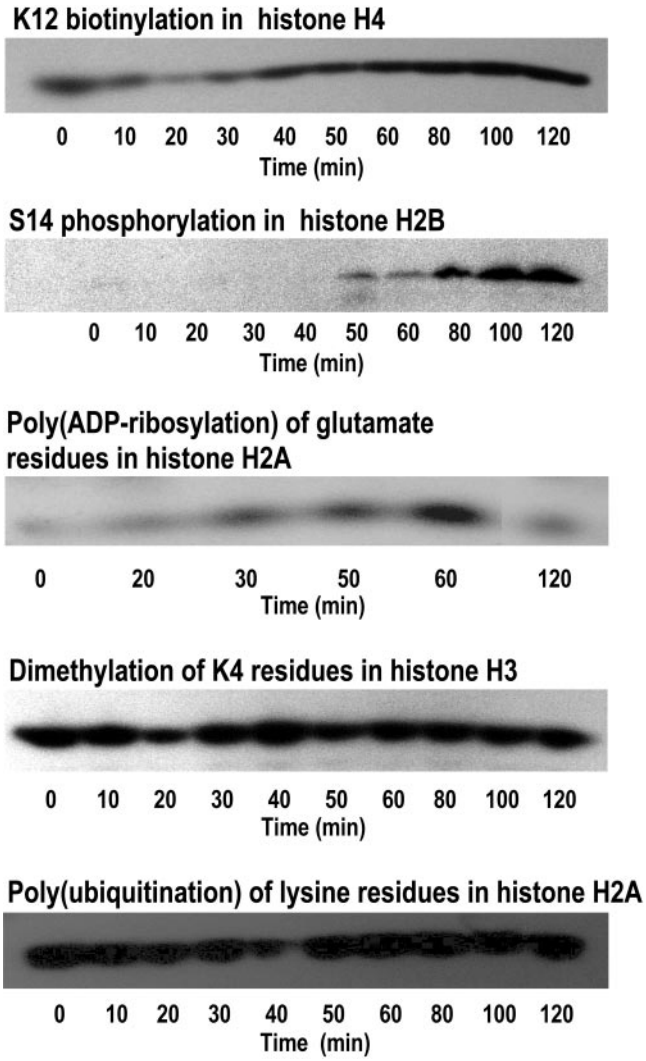

FIGURE 1 Biotinylation of K12 in histone H4 decreases rapidly and transiently in response to treatment with etoposide in JAr cells. Biotinylation of K12 in histone H4, phosphorylation of S14 in histone H2B, dimethylation of K4 in histone H3, and poly-ubiquitination of histone H2A were visualized by Western blot analysis at timed intervals after addition of etoposide to culture medium. Poly(ADP-ribosylation) of histone H2A was visualized using autoradiography after etoposide addition. Representative gels from at least 3 repeats are depicted.

Immunocytochemistry experiments were consistent with the hypothesis that decreased biotinylation of K12 in histone H4 is an early signaling event in response to DSB. First, we confirmed the localization of K12-biotinylated histone H4 in untreated JAr cells (**Fig. 4**A). Preimmune serum did not produce a detectable signal for K12-biotinylated histone H4 (data not shown). Second, we monitored the temporal patterns of K12-biotinylated histone H4 and S14-phosphorylated histone H2B (control) in response to etoposide. Decreased biotinylation of K12 was detectable as early as 20 min after the addition of etoposide to culture media (Fig. 4*B*). The abundance of S14-phosphorylated histone H2B increased ~60 min after the addition of etoposide to culture media (data not shown).

Decreased K12-biotinylation of histone H4 in response to DSB is a ubiquitous phenomenon as opposed to being a tissue-specific event. For example, in lymphoid (Jurkat) cells treated with etoposide, there was a decrease of K12-biotinylation similar to the decrease in JAr cells. K12-biotinylation of histone H4 in Jurkat cells reached a low ~40 min after addition of etoposide to the culture medium (**Fig. 5**A).

Decreased biotinylation of K12 in histone H4 is specific for DSB as opposed to being a global marker for various kinds of DNA damage. First, when formation of thymine dimers in DNA was caused by UV treatment of JAr cells, biotinylation of K12 in histone H4 increased for at least 120 min (Fig. 5*B*). Second, if single-strand breaks of DNA were caused by treatment of JAr cells with zeocin, K12-biotinylation in histone H4 did not change substantially (Fig. 5*B*). In contrast, if DSB of DNA were caused by treatment of JAr cells with doxorubicin, the temporal pattern of K12-biotinylation in histone H4 was similar to the pattern seen after treatment with etoposide (Fig. 5*B*). These observations are consistent with the notion that alterations of K12-biotinylation are specific for the kind of DNA damage, but not for the agent used to cause the damage.

Biotin deficiency was associated with decreased cell survival in response to treatment with etoposide. Here, cells were cultured in biotin-sufficient or biotin-deficient media for 21 d. First, we confirmed that biotin concentrations in culture media affected intracellular biotin concentrations. The abundance of biotinylated pyruvate carboxylase, propionyl-CoA carboxylase (α-chain), and 3-methylcrotonyl-CoA carboxylase (α-chain) was substantially greater in cells from biotin-sufficient medium than in cells from biotin-deficient medium (**Fig. 6**A). Note that propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase were not resolved by the electrophoresis procedure used and migrated as a single band. Acetyl-CoA carboxylase was not detectable in JAr cells, consistent with previous studies in this cell line (23). Next, biotin-defined cells were exposed to 6 nmol/L etoposide, and cell survival was monitored at timed intervals. Cells cultured in biotin-sufficient medium had greater survival rates than cells cultured in biotin-deficient medium (Fig. 6*B*). For example, 6 h after the addition of etoposide to culture media, 64 ± 14% of biotin-sufficient cells were still alive, whereas only 38 ± 11% of biotin-deficient cells were still alive. These effects cannot be explained by the effects of biotin deficiency on proliferation rates, which are moderate (23,24,37).

DISCUSSION

Cells are constantly exposed to environmental insults that may cause DSB of DNA. Cells respond to DSB by activating repair pathways that depend on alterations of chromatin structure (38). Histone modifications play a key role in mediating these chromatin remodeling events. Previous studies identified some of the histone modifications that occur in response to DSB in mammalian cells, e.g., phosphorylation of S14 in histone H2B, phosphorylation of S139 in histone H2AX, and poly(ADP-ribosylation) of glutamate residues in histone H2A (20,34,39). The present study expands the list of damage-dependent histone modifications by unveiling a temporal pattern of K12-biotinylation in histone H4 in response to DSB in human cells.

This is the first time that a correlation was shown between the biotinylation of a distinct lysine residue in a human histone and a physiologic event that requires chromatin remodeling. In previous studies, streptavidin peroxidase was used as a probe for biotin to provide circumstantial evidence for biological functions of histone biotinylation in cell proliferation and UV-induced DNA damage (12,17). Streptavidin binds to biotin in general and does not permit pinpointing distinct biotinylation sites in histones, e.g., K8 and K12 in histone H4 (13). In contrast, the site-specific antibody to

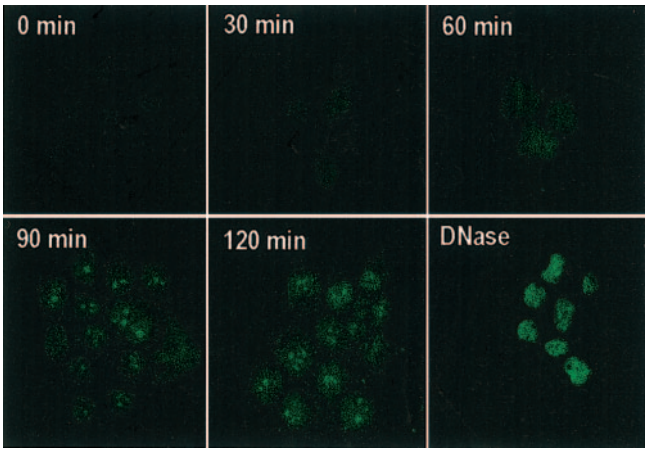

FIGURE 2 Treatment with etoposide causes DNA breaks in JAr cells. Cells were treated with etoposide and DNA breaks were visualized at timed intervals using the TUNEL assay. Positive controls were treated with DNase I for 10 min.

K12-biotinylated histone H4 as a probe used in this study indicates the physiologic relevance of biotinylation of K12 in histone H4 in DNA repair signaling.

Specifically, this study provides evidence *1*) that a transient decrease of K12-biotinylation in histone H4 is an early signaling event in response to DSB; *2*) that signaling by biotinylation of K12 is a universal rather than a tissue-specific mechanism in humans; *3*) that biotinylation of K12 in histone H4 decreases specifically in response to DSB but does not decrease in response to single-strand breaks or formation of thymine dimers; and *4*) that biotin deficiency is associated with decreased cell survival in response to DSB.

The decreased biotinylation of K12 in histone H4 in response to DSB occurs before phosphorylation of S14 in histone H2B and potentially ahead of or in concert with poly(ADP-ribosylation) of glutamate residues in histone H2A. Currently, it is unknown whether phosphorylation of S14 and poly(ADP-ribosylation) of glutamate residues depend directly on biotinylation of K12 in histone H4. This uncertainty is being addressed in ongoing investigations in our laboratory. Moreover, it is not known whether biotinylation of K12 itself depends on other damage-induced modifications of histones such as phosphorylation of H2AX (40). Note that histone H2AX also is a potential target for biotinylation (15). Theoretically, biotinylation of K12 in histone H4 might mediate either DNA repair or apoptosis in response to DSB. We favor the former explanation, given that biotin deficiency was associated with decreased survival of etoposide-treated cells compared with biotin-sufficient controls in the present study. In this context, the following question must be addressed: Why

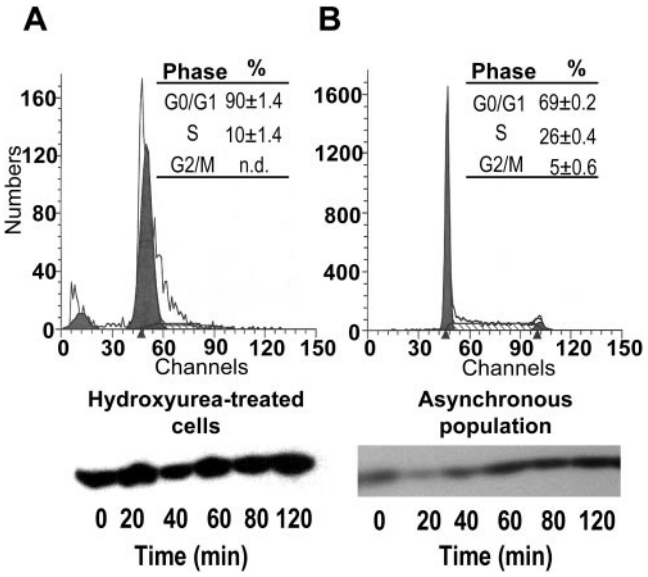

FIGURE 3 Etoposide does not affect biotinylation of K12 in histone H4 from G1 phase-arrested JAr cells. Cells were arrested in G1 phase using hydroxyurea before treatment with etoposide. *Panel A*: G1-arrested cells; *panel B*: asynchronous cells (control). Cell cycle arrest was confirmed using flow cytometry (*top row*), and biotinylation of K12 in histone H4 was visualized by Western analysis at timed intervals after etoposide treatment (*bottom row*); n.d. = not detectable.

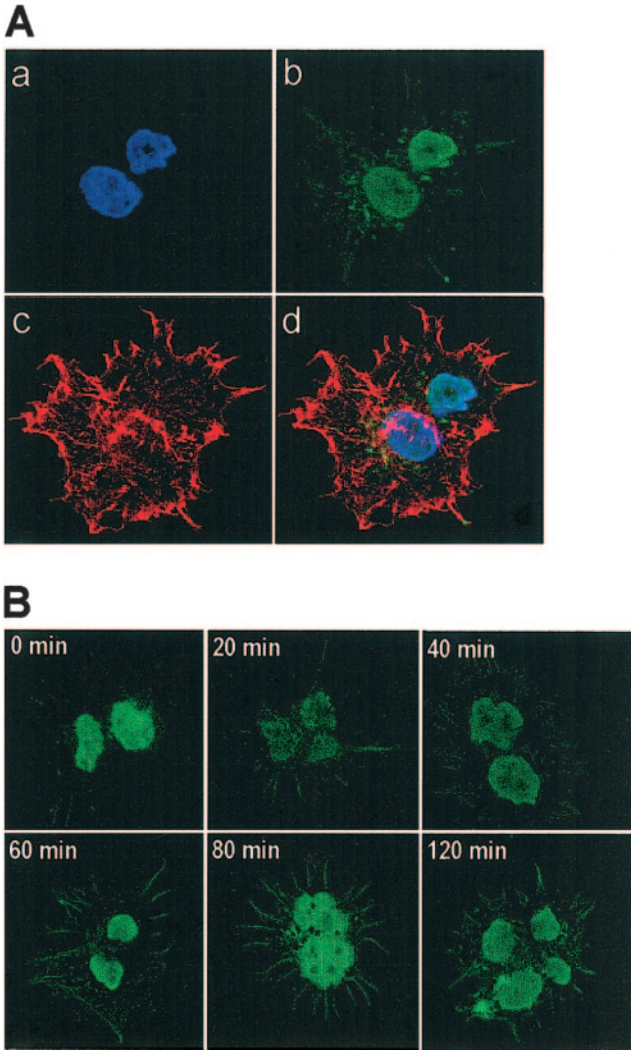

FIGURE 4 Immunocytochemistry experiments are consistent with a rapid and transient decrease in K12-biotinylated histone H4 in JAr cell nuclei in response to treatment with etoposide. *Panel A*: Staining of biotinylation of K12 in histone H4 and cellular compartments in etoposide-free JAr cells: a = staining of nuclei using DAPI; b = staining of K12-biotinylation of histone H4, using a site-specific antibody; c = staining of cytoplasmic compartment using rhodamine phalloidin; d = merged image. *Panel B*: Time course of K12-biotinylation in histone H4 after addition of etoposide to culture media.

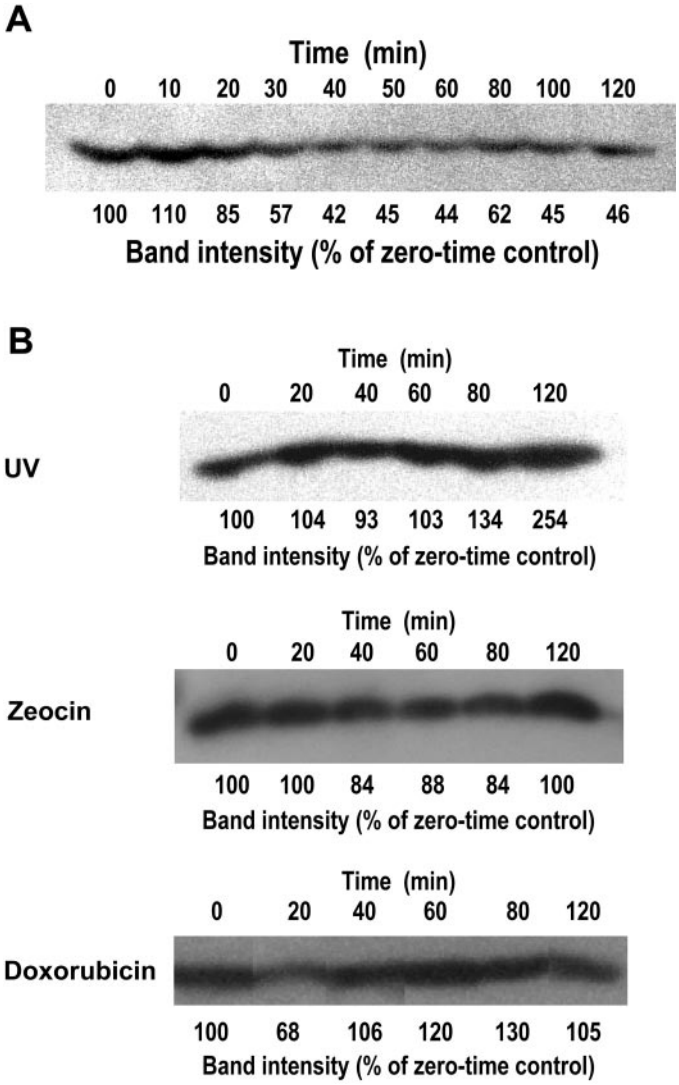

FIGURE 5 Alterations of K12-biotinylation in histone H4 are specific for DSB, but are not specific for JAr cells. (*A*) Jurkat cells were treated with etoposide, and K12-biotinylation in histone H4 was monitored at timed intervals using Western blot analysis; band intensities were quantified by gel densitometry. (*B*) JAr cells were treated with UV, zeocin, and doxorubicin to cause the formation of thymine dimers, single-strand breaks, and DSB, respectively. Biotinylation of K12 in histone H4 was monitored at timed intervals using Western blot analysis; band intensities were quantified by gel densitometry.

would biotin deficiency decrease cell survival in response to DSB, although the damage-related signaling event actually causes a decrease in K12-biotinylation? We speculate that a sufficient supply of biotin is essential to allow for an efficient rebiotinylation of K12 after its rapid transient debiotinylation. We further speculate that biotinylation of K12 plays a role in the reassembly of nucleosomes after DNA repair (41).

Current models of chromatin remodeling in response to DSB are consistent with the findings reported here. Specifically, it was proposed that DSB are associated with a rapid, yet transient, relaxation of chromatin structures mediated by histone modifications (42). Relaxation of chromatin gives DNA repair factors access to sites of DNA breaks. Subsequently, chromatin recondensation prevents dissociation of the 2 broken ends of DNA before repair is mediated by ligases. Note that K12-biotinylated histone H4 is associated with chromatin condensation such as in pericentromeric heterochromatin (A. M. Oommen and J. Zempleni, unpublished data). Hence, a rapid decrease in K12-biotinylation in response to DSB is likely associated with an opening of chromatin structures, giving repair proteins access to sites of damage. Subsequent rebiotinylation of histone H4 is likely associated with recondensation of chromatin. Note, however, that this is an untested hypothesis.

Is the response to DSB breaks comparable in transformed human cell lines and in primary human cells? Previous studies suggested that the frequency of DNA breaks in response to X-rays is similar in transformed and primary cells (43). Also, the kinetics of joining DNA ends is similar in transformed and primary cells. These observations are consistent with the notion that transformed human cells maintain a normal response to DSB breaks. Nevertheless, it remains to be formally demonstrated that pathways of DSB repair are identical in the

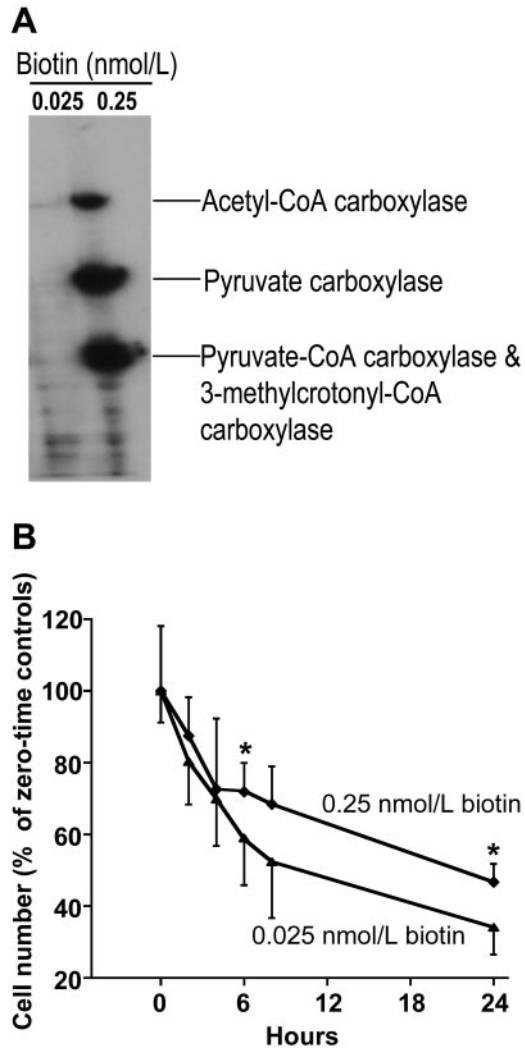

FIGURE 6 Biotin deficiency decreases survival of JAr cells in response to treatment with etoposide. (*A*) Extracts from etoposide-free cells grown in media containing 0.025 and 0.25 nmol/L biotin were resolved by gel electrophoresis; transblots were probed with streptavidin to visualize biotin-dependent carboxylases. (*B*) JAr cell were treated with etoposide and cell survival was monitored at timed intervals using trypan blue exclusion. Values are means ± SD, $n = 10$. *Different from cells cultured in medium containing 0.025 nmol/L biotin, $P < 0.05$.

choriocarcinoma cells used in this study and in primary placental cells.

Taken together, the findings presented here are important for health professionals, based on the following lines of observation. Dietary biotin deficiency is relatively rare in humans, but certain subgroups in the general population are at increased risk for developing biotin deficiency. First, catabolism of biotin is increased during pregnancy (44); consistent with this observation is the fairly high prevalence of marginal biotin deficiency in pregnant women (45,46). Second, some drugs interfere with biotin metabolism. For example, certain anticonvulsants and lipoic acid impair biotin transport and distribution, potentially causing biotin deficiency (35). Third, biotin transporter deficiency may cause intracellular biotin depletion (47). Fourth, mutations of the 2 enzymes (biotinidase and holocarboxylase synthetase) that mediate biotinylation of histones are relatively prevalent in humans. For example, the prevalence of biotinidase deficiency is 1 in 60,000 live births in humans (48). Currently it is unknown whether dietary biotin deficiency or drug-induced biotin deficiency, and mutations of genes coding for biotin transporters, biotinidase, and holocarboxylase synthetase are associated with an impaired ability to repair damaged DNA in humans. Similarly, it is unknown whether supplementation with pharmacologic dose of biotin enhances an individual's DNA repair capabilities.

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ONLINE SUPPLEMENTAL TABLE 1

The abundance of modified histone in response to treatment with etoposide in JAr cells. Histones were quantified by Western blot analysis and gel densitometry at timed intervals after addition of etoposide to culture medium.[1]

	Time after etoposide (min)									
Histone[2]	0	10	20	30	40	50	60	80	100	120
	% of zero-time control									
K12bio H4	100	45 ± 8.6*	53 ± 10*	91 ± 22	96 ± 20	120 ± 20	99 ± 18	139 ± 21	100 ± 8.3	100 ± 17
S14p H2B	100	112 ± 46	73 ± 13	126 ± 59	321 ± 154	511 ± 299	580 ± 389	880 ± 335*	1250 ± 122*	1474 ± 314*
K4Me2 H3	100	101 ± 7.4	98 ± 15	98 ± 18	106 ± 25	103 ± 14	104 ± 15	103 ± 15	87 ± 3.2	97 ± 11
Ub H2A	100	101 ± 7.5	98 ± 15	100 ± 11	93 ± 13	96 ± 10	98 ± 9.3	88 ± 5.9	100 ± 2.5	114 ± 14

[1]Values are means ± SEM; n = 3 - 5. *Different from zero-time control, $P < 0.05$.

[2]Abbreviations used: K12bio H4, K12-biotinylated histone H4; S14p H2B, S14-phosphorylated histone H2B; K4Me2 H3, K4-dimethylated histone H3; Ub H2A, poly(ubiquitinated) histone H2A.