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Biotin deficiency stimulates survival pathways in human lymphoma cells exposed to antineoplastic drugs

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Abstract

Cells may respond to nutrient deficiency or death signals with nuclear translocation of the transcription factor nuclear factor κB (NF-κB), which activates transcription of anti-apoptotic genes. Here we tested the hypothesis that biotin deficiency stimulates NF-κB-dependent survival pathways in human lymphoma cells, enhancing resistance to antineoplastic agents. Lymphoma (Jurkat) cells were cultured in biotin-deficient (0.025 nmol/L) and biotin-supplemented (10 nmol/L) media. If cells were treated with antineoplastic agents (taxol, doxorubicin or vinblastine), nuclear translocation of two NF-κB proteins (p50 and p65) was >25% greater in biotin-deficient compared with biotin-supplemented cells. The transcriptional activities of the following NF-κB-dependent reporter genes were 16–59% greater in biotin-deficient compared with biotin-supplemented cells treated with various antineoplastic agents: (1) reporter expression driven by a TATA box and five NF-κB repeats and (2) reporter expression driven by the regulatory region of the anti-apoptotic *Bfl-1* gene. Collectively, these findings are consistent with activation of survival pathways in biotin-deficient lymphoma cells. Finally, cells were treated with antineoplastic agents for 48 h and cell survival was monitored at timed intervals. Biotin deficiency was associated with enhanced survival of cells treated with doxorubicin and vinblastine, but did not affect survival of cells treated with taxol. Collectively, these observations suggest that biotin deficiency may enhance resistance of cancer cells to antineoplastic agents.

Keywords: Antineoplastic agents; Biotin; Chemotherapy; Human; Jurkat cells; NF-κB; Survival

1. Introduction

In mammals, biotin serves as a coenzyme for acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase (PCC), and 3-methylcrotonyl-CoA carboxylase [1]. These enzymes catalyze essential steps in the metabolism of glucose, amino acids and fatty acids [1]. Histones (DNA-binding proteins) also contain covalently bound biotin [2–4].

Evidence has been provided that biotin affects the expression of a large number of genes in mammals [5–7]. Members of the nuclear factor κB (NF-κB) family of transcription factors mediate some of the effects of biotin on gene expression [8]. In mammals, the NF-κB family has the following five members: c-Rel, p50, p52, p65 and RelB [9]. In nonstimulated cells, NF-κB dimers are trapped in the cytoplasm by binding to inhibitor of NF-κB: IκBα and IκBβ. IκB masks the nuclear localization sequences in NF-κB [9]. Cell stress triggers phosphorylation and proteasome-dependent degradation of IκB [10,11]. The liberated NF-κB dimers translocate to the cell nucleus; binding of NF-κB to regulatory regions of genes causes transcriptional activation [9]. The nuclear translocation of dimers containing p50 and p65 is greater in biotin-deficient than in biotin-supplemented human lymphoma (Jurkat) cells [8].

Many NF-κB-dependent genes promote survival of stressed cells [10,11]. For example, evidence has been provided that the increased nuclear translocation of NF-κB in biotin-deficient cells compared with biotin-supplemented controls is associated with (1) transcriptional activation of the NF-κB-dependent anti-apoptotic *Bfl-1/A1* gene, (2) increased

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survival under serum-starved conditions and (3) with increased survival in response to tumor necrosis factor α, a known activator of cell death [8]. Likewise, many cancer cells activate NF-κB in response to treatment with antineoplastic drugs [12–14], mediating expression of anti-apoptotic genes such as *Bfl-1/A1* [15,16]. The products of these genes enhance survival of cancer cells [12,17,18], causing resistance to chemotherapy. Theoretically, activation of NF-κB in response to biotin deficiency might further enhance the resistance of cancer cells to chemotherapy. This is of practical relevance for health care professionals, given the high prevalence of nutrient deficiencies in cancer patients and during chemotherapy [19–21].

In the present study, we tested the hypothesis that biotin deficiency stimulates NF-κB-dependent survival pathways in human lymphoma cells treated with antineoplastic agents. Jurkat cells were used as a model, given that interactions between biotin and NF-κB have been well established in this lymphoma cell line [8].

2. Materials and methods

2.1. Cell culture

Jurkat cells (clone E6-1) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in the following biotin-defined media for at least 5 weeks prior to sample collection (5% CO_2 at 37°C in humidified atmosphere): (1) 0.025 nmol/L of biotin (denoted "deficient"), representing the plasma level of biotin in biotin-deficient individuals, or (2) 10 nmol/L of biotin ("supplemented"), representing the plasma level of biotin in individuals who take typical over-the-counter supplements providing 20 times the adequate intake of biotin [22–24]. Culturing human-derived cells in biotin-defined media for 5 weeks provides for sufficient time to achieve new steady-state concentrations of biotin, as judged by activities PCC and biotinylation of carboxylases [25,26]. Culture media were replaced with fresh media every 48 h. Media were prepared by using customized RPMI-1640 and biotin-depleted fetal bovine serum as described [25]; biotin concentrations in media were confirmed by avidin-binding assay [27] with modifications [25].

Cells were treated with the following antineoplastic agents (treatment times and concentrations of agents are provided in the Results section): (1) Taxol (paclitaxel), the effects of which are mediated by its ability to bind to tubulin, to promote microtubule assembly and to stabilize microtubules by bundle formation [13,28–30]; (2) vinblastine, the effects of which are mediated by the inhibition of microtubule formation, causing arrest in the metaphase [13,31]; and (3) doxorubicin, the effects of which are mediated by intercalation into DNA and inhibition of RNA transcription [13,32,33].

2.2. Biotin-dependent carboxylases

Biotin-dependent carboxylases are good markers for cellular biotin [1]. Biotinylated carboxylases in cell extracts were resolved by polyacrylamide gel electrophoresis and were probed using streptavidin peroxidase [25]. Densitometric analysis of gels was conducted as described [34]. Activities of PCC in cell lysates were quantified as described [25].

2.3. Electrophoretic mobility shift assays

Nuclear extracts from Jurkat cells were prepared as described previously [34]. NF-κB in nuclear extracts was probed by electrophoretic mobility shift assay (EMSA), using a ^{32}P-labeled double-stranded oligonucleotide probe with an NF-κB consensus site [8]. The nuclear abundance of the transcription factor Oct-1 does not depend on biotin and was quantified as a control as described [34]. Additional controls were prepared by omitting nuclear extracts from incubation mixtures or by incubating nuclear extracts with radiolabeled probe in the presence of a molar excess of unlabeled probe.

2.4. Enzyme-linked immunosorbent assays of p50 and p65

Nuclear extracts were prepared as decribed [34]. The abundance of p50 and p65 in nuclear extracts was quantified by using the TransAM NF-κB p50 Activation Assay and TransAM NF-κB p65 Activation Assay (Active Motif, Carlsbad, CA), respectively, according to the manufacturer's instructions.

2.5. Reporter gene constructs

The following reporter gene constructs were used to quantify effects of biotin on the transcriptional activity of NF-κB-dependent genes: (1) a construct of the luciferase reporter gene driven by the 5′ -flanking region of the anti-apoptotic *Bfl-1/A1* gene (basepairs −1374 to +83) was provided by Céline Gélinas (University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ); this plasmid has been denoted as "bfl-1:luc" [16]; (2) a construct of the luciferase reporter gene driven by a basic promoter element (TATA box) and five NF-κB enhancer elements was provided by Rainer de Martin (University of Vienna, Vienna, Austria); this plasmid has been denoted as "NF-κB-Luc"; (3) a promoter-free plasmid containing the luciferase gene (denoted as "pGL3-Basic") was purchased from Promega (Madison, WI); and (4) a construct of the β-galactosidase reporter gene driven by the RSV promoter (denoted as "RSV βgal") was provided by Brett R. White (University of Nebraska-Lincoln, Lincoln, NE).

Transfections with reporter gene plasmids were conducted as described [26]. Forty-eight hours after transfection, cells were treated with antineoplastic agents as described in the Results section. Reporter-gene activities

were quantified as described [26]. Luciferase activities were normalized for transfection efficiency by using β-galactosidase activities. Data are expressed as ratios of luciferase activities in cells transfected with bfl-1:luc or NF-κB-Luc to activities in cells transfected with pGL3-Basic.

2.6. Caspase-3

Activity of caspase-3 in whole cell extracts [35] was quantified by using the Caspase-3 Colorimetric Assay kit (Clontech, Palo Alto, CA). Caspase-3 activity is expressed in units of nanomoles of *p*-nitroaniline released from aspartate–glutamate–valine–aspartate–*p*-nitroaniline/(microgram of protein × hours).

2.7. Cell survival

Cells were treated with antineoplastic agents for up to 48 h as described in the Results section. At timed intervals, aliquots of cell suspension were collected and live cells were counted by using the trypan blue exclusion test [36].

2.8. Statistical analysis

The paired *t* test was used to test for significance of differences [37]. StatView 5.0.1 (SAS Institute, Cary, NC) was used to perform all calculations. Differences were considered significant if $P<.05$. Data are expressed as mean±S.D.

3. Results

3.1. Biotin-dependent carboxylases

Biotinylation of carboxylases in Jurkat cells paralleled biotin concentrations in culture media. If cells were cultured in biotin-deficient medium, biotinylated pyruvate carboxylase, PCC and 3-methylcrotonyl-CoA carboxylase were barely detectable in cell extracts, using streptavidin peroxidase as a probe (Fig. 1A). In contrast, holocarboxylases were abundant in extracts from cells cultured in biotin-supplemented medium. Note that the biotin-containing α-chains of PCC (molecular mass 80 kDa) and 3-methylcrotonyl-CoA carboxylase (molecular mass 83 kDa) migrate as one single band on the polyacrylamide gels used here. Holocarboxylases were 2.2 to 2.7 times more abundant in biotin-supplemented cells than in biotin-deficient cells, as judged by gel densitometric analysis of Western blots (Fig. 1B). Acetyl-CoA carboxylase was not detectable in cell extracts, consistent with previous studies in Jurkat cells [25].

PCC activities paralleled the abundance of holocarboxylases in Jurkat cells. PCC activities were 2.7±1.3 pmol bicarbonate fixed/(min×10^6 cells) and 39±6.5 bicarbonate fixed/(min×10^6 cells) in biotin-deficient and biotin-supplemented cells, respectively ($P<.01$, $n=5$). Collectively, these findings suggest that biotin concentrations in culture media affect intracellular biotin concentrations.

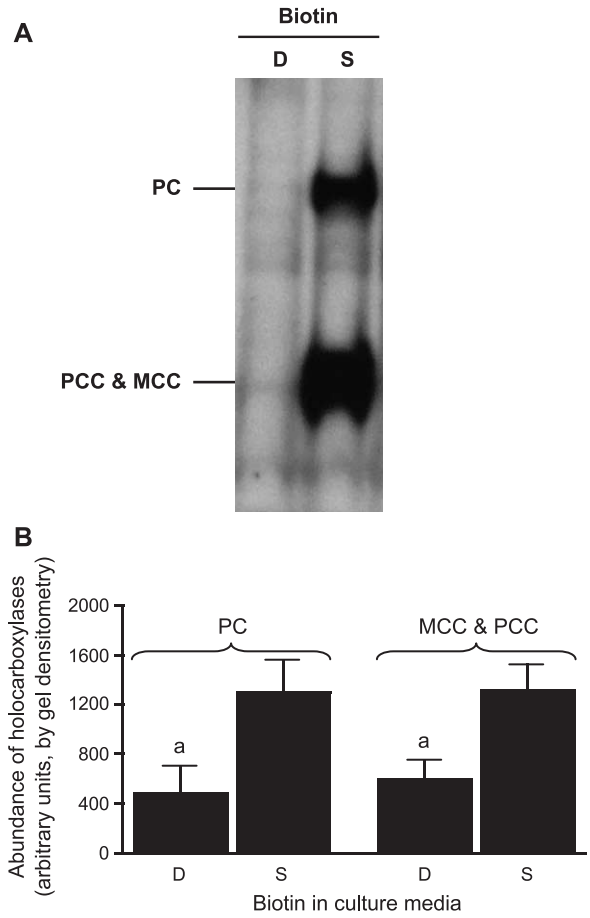

Fig. 1. Biotin supply affects the abundance of holocarboxylases in Jurkat cells. Cells were cultured in biotin-deficient ("D"; 0.025 nmol/L) and biotin-supplemented ("S"; 10 nmol/L) media for 5 weeks. (A) Representative Western blot depicting pyruvate carboxylase (PC), α-chain of PCC and α-chain of 3-methylcrotonyl-CoA carboxylase (MCC). (B) Gel densitometric analysis of Western blots. Values are means±S.D. ($n=5$). [a]Significantly different from the abundance of the same carboxylase in cells cultured in biotin-supplemented medium ($P<.01$).

3.2. Nuclear translocation of NF-κB

The following two key observations were made in experiments involving EMSA: (1) the nuclear abundance of NF-κB was greater in biotin-deficient cells compared with biotin-supplemented cells and (2) treatment of cells with antineoplastic agents caused nuclear translocation of NF-κB, consistent with previous studies [13]. In a first series of experiments, effects of biotin and taxol on nuclear translocation of NF-κB were investigated. The nuclear translocation of NF-κB increased in response to treatment with taxol; in dose–response experiments, the abundance of NF-κB in nuclear extracts reached a plateau if cells were treated with about 80–120 μmol/L taxol for 3 h (Fig. 2A). The nuclear abundance of NF-κB was greater in biotin-deficient cells compared with biotin-supplemented cells (Fig. 2A). Subsequent studies were conducted using taxol at a concentration of 80 μmol/L.

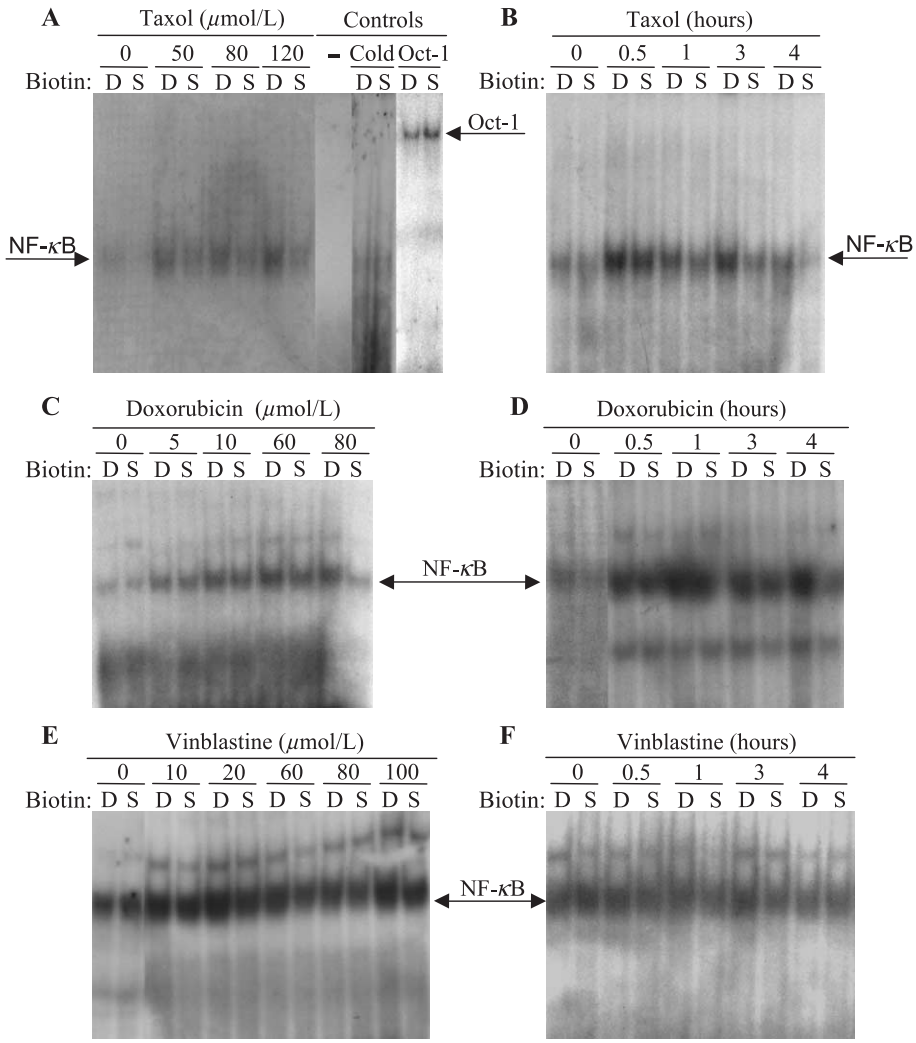

Fig. 2. Biotin deficiency is associated with increased nuclear translocation of NF-κB in Jurkat cells treated with antineoplastic agents. Cells were cultured in biotin-deficient ("D"; 0.025 nmol/L) and biotin-supplemented ("S"; 10 nmol/L) media for 5 weeks. Transcription factors in nuclear extracts were probed by EMSA, using radiolabeled oligonucleotides that contained consensus sites for NF-κB and Oct-1 (control); negative controls were prepared by omitting nuclear extract (denoted "–"). In some cases, nuclear extracts (80 μmol/L of taxol for 3 h) were incubated with radiolabeled oligonucleotides in the presence of a molar excess of unlabeled probe (denoted "cold excess"). Treatments: (A) 0–120 μmol/L of taxol for 3 h; (B) 80 μmol/L of taxol for 0–4 h; (C) 0–80 μmol/L of doxorubicin for 3 h; (D) 60 μmol/L of doxorubicin for 0–4 h; (E) 0–100 μmol/L of vinblastine for 3 h; (F) 80 μmol/L vinblastine for 0–4 h.

The following controls provided evidence for the specificity of the effects described above (Fig. 2A): (1) no band was visible in EMSA if oligonucleotide probes were incubated in the absence of nuclear extract; (2) bands were barely detectable if samples were incubated in the presence of a molar excess of unlabeled oligonucleotide probe; and (3) the abundance of the transcription factor Oct-1 did not depend on biotin concentrations in culture media, consistent with previous studies [34].

Next, time–response experiments were conducted. NF-κB in nuclear extracts reached a plateau 0.5–3 h after addition of taxol (80 μmol/L final concentration) to culture media (Fig. 2B); again, nuclear abundance of NF-κB was greater in biotin-deficient cells compared with biotin-supplemented cells. In subsequent studies, cells were treated with taxol for 3 h.

Effects of biotin on the nuclear translocation of NF-κB were not specific for taxol treatment, but similar effects were seen if cells were treated with other antineoplastic agents. Both doxorubicin and vinblastine caused a dose- and time-dependent nuclear translocation of NF-κB (Fig. 2C–F). Nuclear translocation of NF-κB reached a plateau if cells were treated with 10 μmol/L doxorubicin (Fig. 2C) for 4 h (Fig. 2D); likewise, nuclear translocation of NF-κB reached a plateau if cells were treated with 10 μmol/L vinblastine (Fig. 2E) for 4 h (Fig. 2F). Nuclear translocation of NF-κB was greater in biotin-deficient compared with biotin-supplemented cells treated with doxorubicin and vinblastine (Fig. 2C–F).

In previous studies, we demonstrated that p50 and p65 represent the biotin-dependent fraction of NF-κB proteins in Jurkat cells; p52 and RelB are not detectable in nuclear

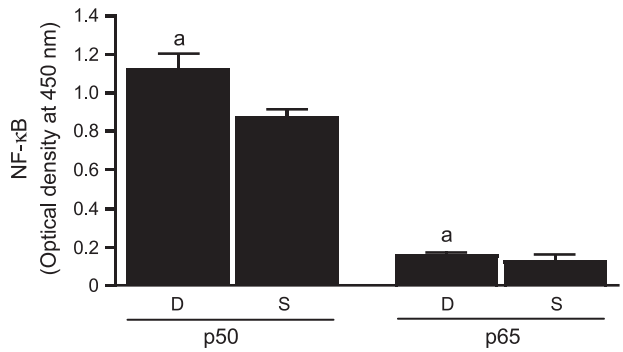

Fig. 3. Biotin affects the nuclear abundance of p50 and p65 in Jurkat cells treated with 80 μmol/L of taxol for 3 h. p50 and p65 in nuclear extracts were quantified by using enzyme-linked immunosorbent assays. D=biotin-deficient medium (0.025 nmol/L); S=biotin-supplemented medium (10 nmol/L). [a]Significantly different from the abundance of the same protein in cells cultured in biotin-supplemented medium ($P<.05$).

extracts from Jurkat cells, whereas the abundance of c-Rel does not depend on biotin [8]. In the present study, the nuclear abundance of p50 and p65 was quantified by enzyme-linked immunosorbent assay, using nuclear extracts from cells treated with 80 μmol/L taxol for 3 h. Nuclear abundance of p50 and p65 was 29% and 25% greater, respectively, in biotin-deficient cells compared with biotin-supplemented cells (Fig. 3).

3.3. Transcriptional activities

The nuclear abundance of NF-κB was paralleled by transcriptional activities of NF-κB-dependent reporter genes. If cells were treated with 80 μmol/L of taxol for 3 h, the transcriptional activity of plasmid NF-κB-Luc was 59% greater in biotin-deficient cells compared with biotin-supplemented cells (Fig. 4). The expression of luciferase in plasmid NF-κB-Luc is driven by five NF-κB repeats; thus, NF-κB-Luc is a universal model for the transcriptional activity of NF-κB-dependent genes. Next, we used plasmid bfl-1:luc to determine specifically whether expression of the anti-apoptotic *Bfl-1* gene depends on biotin. If cells were treated with 80 μmol/L of taxol for 3 h, the transcriptional activity of plasmid bfl-1:luc was 16% greater in biotin-deficient cells compared with biotin-supplemented cells (Fig. 4). This effect was not specific for taxol treatment: if cells were treated with 80 μmol/L of doxorubicin for 4 h, the transcriptional activity of plasmid bfl-1:luc was 59% greater in biotin-deficient cells compared with biotin-supplemented cells (Fig. 4).

3.4. Cell death

First we determined whether biotin plays a role in the survival of lymphoma cells treated with antineoplastic agents. Biotin concentrations in culture media did not affect the survival of cells treated with 80 μmol/L of taxol (Fig. 5A). A substantial fraction of cells survived treatment with taxol, regardless of biotin status: approximately 40% of cells were still alive 48 h after taxol had been added to culture media. In contrast, biotin affected survival of lymphoma cells treated with doxorubicin and vinblastine. If cells were treated with 60 μmol/L of doxorubicin for up to 48 h, biotin deficiency was associated with a decreased rate of cell death (Fig. 5B). Survival of biotin-deficient cells was significantly greater compared with biotin-supplemented cells for the period spanning 1–5 h after addition of doxorubicin to media. Notwithstanding these effects of biotin on cell survival, 100% of biotin-deficient and biotin-supplemented cells had died 48 h after addition of doxorubicin to media. If cells were treated with 80 μmol/L of vinblastine, biotin deficiency was also associated with a decreased rate of cell death (Fig. 5C). Survival of biotin-deficient cells was significantly greater compared with biotin-supplemented cells 4 and 5 h after addition of vinblastine to media. Notwithstanding these effects of biotin on cell survival, 100% of biotin-deficient and biotin-supplemented cells had died 48 h after addition of vinblastine to media. Control cells were not treated with antineoplastic agents. The number of control cells increased by about 200% during the observation period of 48 h (Fig. 5D); biotin did not affect proliferation rates in untreated cells.

Finally, we determined whether effects of biotin on cell death were mediated by the proapoptotic enzyme caspase-3. Activities of caspase-3 were approximately six times greater in cells treated with taxol, doxorubicin or vinblastine compared with nontreated controls (data not shown). Caspase-3 activities were not significantly different in biotin-deficient cells compared with biotin-supplemented cells, regardless of the antineoplastic agent used (data not

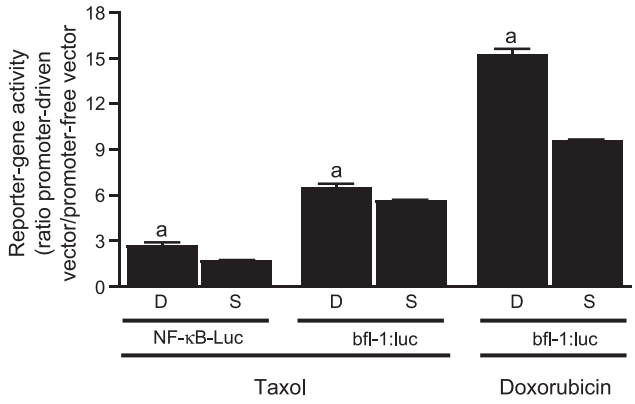

Fig. 4. Biotin supply affects the transcriptional activities of NF-κB-dependent reporter genes. Jurkat cells were cultured in biotin-deficient ("D"; 0.025 nmol/L) and biotin-supplemented ("S"; 10 nmol/L) media for 5 weeks. Cells were transfected with plasmids NF-κB-Luc and bfl-1:luc. Forty-eight hours after transfection, cells were treated with 80 μmol/L of taxol for 3 h or 80 μmol/L of doxorubicin for 4 h. Reporter (luciferase) activities are expressed as fold-increase over an empty vector (normalized for transfection efficiency). Values are means±S.D. ($n=3$). [a]Significantly different from the transcriptional activity of the same plasmid in cells cultured in biotin-supplemented medium ($P<.05$).

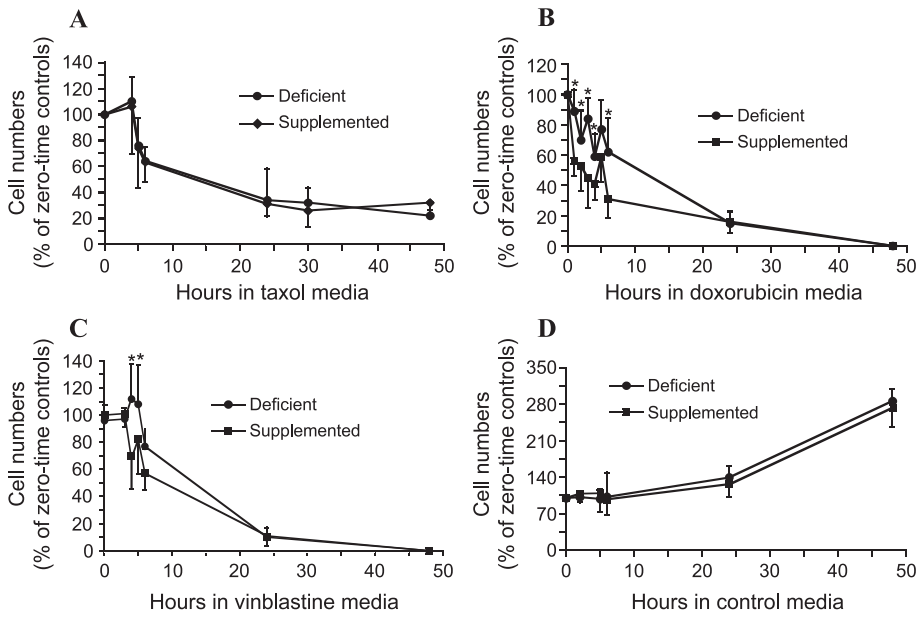

Fig. 5. Biotin supply affects survival of Jurkat cells treated with doxorubicin and vinblastine but not of cells treated with taxol. Jurkat cells were cultured in biotin-deficient (0.025 nmol/L) and biotin-supplemented (10 nmol/L) media for 5 weeks. Treatments: (A) 80 μmol/L of taxol; (B) 60 μmol/L of doxorubicin; (C) 80 μmol/L of vinblastine; (D) no antineoplastic treatment (control). Values are means±S.D. ($n=5$). *Significantly different from cells cultured in biotin-supplemented medium ($P<.05$).

shown). Collectively, these data suggest that the effects of biotin on the resistance to antineoplastic agents are not mediated by caspase-3.

4. Discussion

This study provides evidence (1) that the nuclear translocation of NF-κB proteins p50 and p65 is greater in biotin-deficient compared with biotin-supplemented lymphoma cells treated with antineoplastic agents; (2) that p50 and p65 cause transcriptional activation of anti-apoptotic genes such as *Bfl-1/A1*; and (3) that biotin deficiency is associated with resistance of lymphoma cells to doxorubicin and vinblastine.

Previous studies in our laboratory are consistent with the hypotheses (1) that biotin deficiency globally enhances stress resistance as opposed to specifically enhancing resistance to antineoplastic agents and (2) that effects of biotin on stress resistance are mediated by NF-κB. For example, biotin deficiency is associated with increased survival of the fruit fly *Drosophila melanogaster* if exposed to oxidative stress [38]. Likewise, evidence has been provided that biotin deficiency is associated with increased survival of lymphoma cells cultured in serum-free medium or treated with tumor necrosis factor-α; p50 and p65 are likely to play a role in this process [8].

Currently it is unknown whether effects of biotin deficiency on cell survival are mediated by biotin per se or by alterations in metabolite profiles secondary to biotin deficiency. For example, biotin deficiency is associated with accumulation of odd-chain fatty acids (C15:0 and C17:0) caused by low PCC activities [39–41]. It is not unreasonable to propose that some of the effects of biotin deficiency on nuclear translocation of NF-κB are mediated by metabolites such as odd-chain fatty acids rather than by biotin per se. Studies are currently in progress in our laboratory to address this uncertainty.

In the present study, cells were cultured in biotin-defined media for at least 5 weeks. Do the findings presented here also apply to cells that are cultured in biotin-deficient medium for shorter periods? More importantly, do these findings apply to humans consuming a moderately biotin-deficient diet for a brief period? Studies are in progress in our laboratory in which cells are cultured in biotin-defined media for only up to 2 days. In these ongoing studies, effects of biotin deficiency on NF-κB-dependent pathways appear to be even stronger compared with the effects presented here for the 5-week study. Note that consumption of a biotin-deficient diet for 5 weeks causes depletion of cell biotin in healthy adults, whereas consumption of a biotin-deficient diet for only 2 days does not significantly affect cell biotin [42]. Thus, the effects on NF-κB by consumption of a biotin-deficient diet for only 2 days remain uncertain.

Previous studies have suggested that IκBα kinase mediates effects of biotin deficiency on the nuclear translocation of NF-κB [8]. Nevertheless, the exact signaling cascade by which biotin deficiency activates NF-κB is unknown. For example, it is unknown how biotin modulates the activity of IκBα kinase. IκB kinase is composed of at least three subunits: α, β and γ [11]. Various MAP3 kinases (MEKK1, MEKK2, MEKK3 and NIK) are capable of phosphorylating IκB kinase α and β, mediating activation of IκBα kinase [11]; other kinases may also play a role in the phosphorylation of IκBα [11]. It is unknown whether the

availability of biotin affects the activity of any of these kinases or events further upstream in the signaling cascade leading to nuclear translocation of NF-κB [15].

Likewise, biotin-dependent signaling cascades downstream from activation of NF-κB remain unknown. This study provided evidence that caspase-3 might mediate cell death in response to treatment with antineoplastic agents. However, this study also provides evidence that effects of biotin deficiency on cell death are not mediated by decreasing caspase-3 activity. Future studies should examine both the upstream signals mediating activation of NF-κB in biotin-deficient cells and the downstream events mediating survival of biotin-deficient cancer cells during chemotherapy.

The findings presented here are relevant from an oncologist's and nutritionist's point of view, based on the following lines of reasoning. First, this study suggests that biotin status might affect the outcome of cancer chemotherapy. Second, previous studies suggested that nutrient deficiency is fairly common in cancer patients and in patients treated with chemotherapy [19–21]. Notwithstanding the validity of these findings, there are still some uncertainties associated with potential roles for biotin in chemotherapy. First, the present study was conducted using a cell culture model as opposed to working with cancer patients. Future studies will need to determine whether biotin deficiency also causes resistance to antineoplastic agents in animal models or in patients treated with chemotherapy. Second, to our knowledge biotin status has not yet been examined in cancer patients and in patients treated with chemotherapy. Theoretically, it is possible that the prevalence of biotin deficiency is low in cancer patients despite the high prevalence of deficiencies of other nutrients in these patients. Various indicators of biotin nutritional status have been validated in recent studies, including activity of PCC in lymphocytes, urinary excretion of biotin and urinary excretion of organic acids such as 3-hydroxyisovaleric acid [1,43]. These indicators should be used to systematically examine the biotin status in cancer patients.

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