Differentiation of Human U937 Promonocytic Cells Is Impaired by Moderate Copper Deficiency

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Copper (Cu) deficiency suppresses macrophage activities in animals and humans. Our previous studies indicated that the induction of Cu deficiency in differentiated U937 monocytic cells impairs respiratory burst and bactericidal activities and lipopolysaccharide-mediated secretion of inflammatory mediators. The current investigation examined the roles of Cu in the monocytic differentiation process. Human U937 promonocytic cells were exposed to a high affinity Cu chelator (5 *μM* 2,3,2-tetraamine [tet]) for 24 hr before inducing differentiation by treatment with 1,25-dihydroxyvitamin D_3 plus interferon-γ (DI). This procedure decreased cell Cu by 55% without compromising cellular Zn, Fe, or general metabolic activities. Lower Cu status significantly attenuated the expression of maturation markers Mac-1 (CD11b), ICAM-1 (CD54), and LPS-R (CD14). This change was associated with a marked suppression in respiratory burst activity and killing of *Salmonella*. To examine if the adverse effect of inadequate Cu on the DI-induced differentiation represented a more general defect, U937 cells were treated with phorbol 12-myristate 13-acetate (PMA). Lower Cu status also suppressed PMA-mediated differentiation of U937 cells. Supplemental Cu, but not Zn or Fe, blocked the tet-induced declines in cell Cu, expression of maturation markers, and respiratory burst and bactericidal activities. These results demonstrate that Cu is essential for the monocytic differentiation process that contributes to the competency of the host's defense system. [Exp Biol Med Vol. 226(3):222–228, 2001]

Key words: copper; cell differentiation; monocytes; U937 cells

In the monocytic lineage, progenitor cells differentiate into monocytes and macrophages. Conditions that impair differentiation are likely to decrease the contribution of monocytes and macrophages to host defense. It is well established that copper (Cu) deficiency adversely affects the functions of neutrophils, another phagocytic cell type, in both humans and animals (1–4). Limited studies also show that dietary Cu deficiency suppresses macrophage activities in laboratory rodents (5). However, it is unclear if the observed suppression is caused by the lower Cu status of the macrophages, the immaturity of cells resulting from abnormal differentiation, or a combination of impaired differentiation and functional activities. We recently reported that the induction of Cu deficiency in differentiated U937 monocytic cells suppresses respiratory burst, bactericidal activity and lipopolysaccharide-mediated secretion of inflammatory mediators without altering metabolic integrity (6). This led us to question if inadequate Cu status also impairs the differentiation of promonocytes into mature monocytic cells. The importance of Cu in cellular differentiation has been reported previously for selected subsets of leukocytes. Cu insufficiency suppresses the maturation of splenic T helper cells in rats (7) and neutrophils in mice (8) and humans (9). Chelator-induced Cu depletion has also been shown to inhibit the differentiation of human hemopoietic progenitor cells (10, 11).

The goal of the present study was to examine the essentiality of Cu in the monocytic differentiation process. The human U937 promonocytic cell line was selected as the cell model since it is widely used to study the differentiation of promonocytes into monocyte-like cells (6, 12–14). The investigation was conducted in two phases. First, conditions establishing moderate Cu deficiency (i.e., no evident impact of decreased Cu status on general metabolic integrity) before inducing cellular differentiation were defined by exposing the non-differentiated cells to a low concentration of 2,3,2-tetraamine (tet). This high affinity Cu chelator has been used successfully to decrease cellular Cu content of several cell lines including U937 (6, 15, 16). Then, the influence of lower Cu status on the differentiation of U937 cells was examined by supplementing cultures with 1,25-dihydroxyvitamin D_3 (D_3) and interferon-γ (IFN-γ), which are physiological mediators that act synergistically in inducing differentiation (17). A parallel series of studies were also performed by exposing U937 cells to phorbol 12-myristate 13-acetate (PMA), a potent differentiation agent that directly activates protein kinase C (18), to determine if

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the phenomenum observed in D_3 and IFN-γ induced differentiation represents a more general influence of inadequate cell Cu on responsiveness. The results of the present study show that Cu deficiency attenuates the expression of Mac-1 (CD11b), ICAM-1 (CD54), and lipopolysaccharide/lipopolysaccharide binding protein receptor (LPS-R, CD14), the arrest of cell cycling, and the respiratory burst and bactericidal activities of differentiating U937 cells.

Materials and Methods

Materials. Chemicals and tissue culture supplies were purchased from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) unless otherwise indicated. 1α,25-Dihydroxyvitamin D_3 was obtained from Biomol (Plymouth Meeting, PA), and Alamar Blue from TREK Diagnostic Systems (Westlake, OH). Antibodies to cell surface antigens were purchased from Pharmingen (San Diego, CA). Among these antibodies, anti-CD11b, anti-CD54, and anti-CD71 were conjugated with phycoerythin (PE) while anti-CD14 and anti-CD64 were conjugated with fluorescein isothiocyanate (FITC). The negative controls for CD11b, CD54, and CD64 and for CD14 and CD71 were antibodies of mouse isotypes IgG_1,κ and IgG_{2a},κ, respectively.

Cell Culture and Experimental Design. Human U937 promonocytic cells were purchased from American Type Culture Collection (Rockville, MD) and maintained at (1–10) × 10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 25 m*M* glucose, 10 m*M* HEPES, 20 m*M* sodium bicarbonate, 4 m*M* glutamine, 1 m*M* sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml amphotericin B. Cells between 5 and 50 passages after receipt were used for studies.

Non-differentiated U937 cells were cultured in T75 flasks in the medium containing 5 μ*M* tet for 24–72 hr to define the conditions for selectively inducing Cu deficiency with minimal influence on metabolic integrity. The cells were collected by centrifugation and were re-seeded in fresh medium at 2.5 × 10^5 cells/ml every 24 hr. The numbers and sizes of cells in the control and tet-treated cultures were determined every 24 hr with a Coulter counter (Multisizer II, Coulter Electronics, Miami, FL). Samples were also collected for the analyses of cellular trace metals and other characteristics (see below).

To examine the roles of Cu in monocytic differentiation, U937 cells (2.5 × 10^5/ml in T75 flasks) were grown in complete medium alone or with 5 μ*M* tet for 24 hr and collected by centrifugation. The cells generally were reseeded either at 1.25 × 10^5/ml in T25 flasks in medium containing 10 n*M* D_3 plus 5 ng IFN-γ/ml (DI) either without or with 5 μ*M* tet or at 5 × 10^5/ml in 12- and 96-well plates in medium containing 10 n*M* PMA without or with 5 μ*M* tet. Test cells also were cultured in T75 flasks for the determination of cellular content of Cu and Zn. The stock solutions of D_3 (20 μ*M*) and PMA (100 μ*M*) were solubilized in ethanol. IFN-γ (10 μg/ml) was prepared in PBS containing 1% FBS. After 48 hr, control and tet-treated cultures were assessed for cellular Cu and other properties related to cell integrity. The degree of differentiation was determined by the expression of maturation markers, DNA cell cycle profile, and respiratory burst and bactericidal activities (see below).

When indicated, medium was supplemented with a trace metal to examine the specificity of chelator-induced changes. Equimolar concentrations of tet and either Cu, Zn, or Fe added to the media. Filter sterilized solutions (1 mg/ml in 2% nitric acid) of Cu, Zn, and Fe were prepared from reference standard solutions. Appropriate aliquots of 2% nitric acid were added to control medium.

Cellular Cu Status. For the analysis of cellular Cu and Zn contents, cells were prepared similarly to that described by Huang and Failla (6). Then 1.0 ml of each cell suspension was extracted with 1.0 ml of 3.0 *N* HCl (made from 10 *N* sub-boiling distilled HCl) and 1.5 ml of 10% trichloroacetic acid (high purity; Fluka Chemicals, Milwaukee, WI). The samples were allowed to stand at room temperature for 2 hr and were then centrifuged to remove the precipitate. Cu and Zn were determined by inductively coupled argon plasma spectrometry (ICAP) by using an Optima 3100XL instrument (Perkin-Elmer, Norwalk, CT) programmed to detect minute concentrations of the elements. The activity of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was determined spectrophotometrically by measuring the ability of aliquots of cell extracts to inhibit pyrogallol autoxidation as described by Babu and Failla (5).

Mitochondrial Activity. Mitochondrial activity was determined by monitoring the reduction of Alamar Blue dye as described by Garrett *et al.* (19) with modifications. Briefly, DI-treated U937 cells were collected, washed with HBSS, resuspended in 10% Alamar Blue in RPMI at 5 × 10^6/ml, and placed in a 12-well plate at 2 ml/well. PMA-treated cells in a 12-well plate were washed with HBSS before adding 2 ml of 10% Alamar Blue in RPMI was added to each well. The experimental plate was incubated at 37°C. Aliquots (200 μl) of medium were transferred to a 96-well plate every 15 min for analysis and returned to the corresponding wells of the 12-well plate in the incubator after each reading. Fluorescence was measured at excitation and emission wavelengths of 530 and 590 nm, respectively, by using a fluorescence microplate reader (Model LS50B luminescence spectrometer, Perkin-Elmer, Norwalk, CT). Regression analysis of fluorescence versus time gave the rate of the reduction of Alamar Blue. This rate is reported as fluorescence units·min^{-1}·mg $protein^{-1}$.

Phenotypic Analysis. Expression of specific surface antigens Mac-1 (CD11b), LPS-R (CD14), ICAM-1 (CD54), FcγRI (CD64), and transferrin receptor (TfR, CD71) was quantified by flow cytometry by using a direct immunofluorescence staining technique (6). Nondifferentiated and differentiated U937 cells were harvested by pipet and transferred to 12 × 75 mm polyethylene test tubes (1 × 10^6 cells per tube). The cells were collected by centrifugation (250*g* for 5 min), washed with PBS containing 10%

FBS and 0.1% sodium azide, and resuspended in 100 μl of the same solution. Aliquots (20 μl) of antibodies at concentrations formulated by the manufacturer were added to tubes to stain the cells. After 30 min of incubation at room temperature in the dark, the cells were collected, washed, and resuspended in 1 ml of PBS containing 2% formaldehyde. One × 10^4 cells per sample were analyzed within 2 days after preparation by FACSCalibur (Becton Dickinson, San Jose, CA). The fluorescence from an isotype control was set as the background. Positive cells were defined as those with fluorescence greater than that of the background. The mean level of expression of the antigen represented the mean fluorescence intensity of the positive cells. Cell size and granularity were also assessed by measuring mean forward scattering and mean side scattering, respectively, from non-stained cells.

Surface TfR expression was used as a marker to assess the degree of differentiation and to determine if the decline in cellular Cu content exceeded the threshold required to induce secondary Fe deficiency (20–22).

Cell Cycle Analysis. DNA content was measured by flow cytometry to characterize the distribution of cells in the various phases of the cell cycle. Fluorescent staining of DNA was performed according to Fraker *et al.* (23) with modifications. Briefly, nondifferentiated and differentiated U937 cells were collected in 12 × 75 mm polyethylene test tubes (1 × 10^6 cells per tube). The cells were pelleted by centrifugation (250*g* for 5 min), washed with PBS, and fixed with 2 ml of cold 70% ethanol at −20°C overnight. Before analysis, the cells were pelleted, washed with PBS, and stained with 500 μl of PBS containing 25 μg of propidium iodide and 100 ng of RNase A. After 30 min of incubation at room temperature in the dark, 1 × 10^4 cells per sample were analyzed by FACSCalibur. The phase distribution of cell cycle was determined by using ModFit LT for Mac V2.0 software (Verity Software House, Topsham, ME).

Respiratory Burst Activity. Respiratory burst activity of the differentiated U937 cells were monitored by measuring the PMA-stimulated production of superoxide anion ($O_2^{\cdot-}$) (6). Briefly, DI-differentiated U937 cells were collected, washed with HBSS, and resuspended at 4 × 10^6/ml in basal RPMI medium without phenol red. Aliquots (250 μl) of the cells were placed in 1.5-ml microfuge tubes. PMA-differentiated cells adherent in 12-well plates were washed with HBSS before adding 250 μl of basal RPMI medium without phenol red. Then, 250 μl PBS with or without 300 U bovine erythrocyte SOD and 500 μl PBS containing 2 μM PMA and 100 μ*M* horse heart ferricytochrome *c* were added to each sample. The samples were incubated at 37°C for 60 min, placed on ice to stop the reaction, and centrifuged at 5,000*g* for 10 min (supernatants in wells were transferred to microfuge tubes before centrifugation). Absorbance (*A*) of the supernatants was measured at 550 and 675 nm to obtain a corrected absorbance ($A_{550} - A_{675}$). After subtracting the corrected absorbance of samples with added SOD from those without the enzyme, $O_2^{\cdot-}$ production was calculated by using the molar extinction coefficient ($E^{1\%}_{1cm}$ = 21 × 10^3 M^{-1}) for ferricytochrome *c* and reported as nmol $O_2^{\cdot-}$ generated per mg cellular protein.

Bactericidal Activity. The survival of *Salmonella typhimurium* was determined by monitoring the reduction of Alamar Blue dye in a fluorescence microplate assay (6, 24). *S. typhimurium* was grown in LB broth (DIFCO, Detroit, MI) to log phase [(5–10) × 10^8/ml], diluted to 5 × 10^6/ml in RPMI containing 10% human serum, and opsonized at 37°C for 30 min. DI-differentiated U937 cells were collected, washed with HBSS, and resuspended at 1 × 10^6/ml in basal RPMI medium. Aliquots (100 μl) of the cells were transferred to a 96-well plate. PMA-differentiated cells in a 96-well plate were washed with HBSS before adding 100 μl of basal RPMI medium. Then, 50 μl (2.5 × 10^5) of opsonized *S. typhimurium* was added to each well. The plate was centrifuged at 250*g* for 5 min to enhance the contact between *S. typhimurium* and the cells. After 1 hr of incubation at 37°C, 25 μl of 0.7% sodium deoxycholate in PBS was added to each well and the plate was returned to the incubator for 5 min to allow the dissolution of U937 cells. Next, 25 μl of 80% Alamar Blue dye in basal RPMI was added to each well and the plate was returned to the incubator for an additional 2 hr. Fluorescence intensity generated from the reduction of Alamar Blue dye by viable bacteria in each well was measured at excitation and emission wavelengths of 530 and 590 nm, respectively. Wells without bacteria, but with the same quantities of reagents, served as background.

Statistical Analysis. All treatments of an experiment were prepared in triplicate and each experiment was repeated at least twice. Data were analyzed by ANOVA (Excel, Microsoft, Redmond, WA) to determine statistically significant differences ($P \leq 0.05$) and expressed as mean ± SD.

Results

Effects of Cu Deficiency on U937 Promonocytic Cells. The initial task for examining the role of Cu in monocytic differentiation was to selectively reduce the Cu content of U937 cells before exposing cultures to differentiation agents. U937 cells were exposed to 5 μ*M* tet for 24–72 hr and various indicators of cell status were monitored. The presence of 5 μ*M* tet in non-differentiated cultures for 24 hr decreased cell Cu by 49% ($P < 0.05$), without significantly changing Zn content (Table I). Despite the decline in cell Cu content, Cu,Zn-SOD activity was not altered. Similarly, the expression of TfR (CD71) was not increased in tet-treated cultures, indicating that neither the chelator itself nor the reduction in cell Cu induced Fe deficiency. The proliferation rate, cell size and protein content, mitochondrial activity and expression of the surface antigens Mac-1 (CD11b) and FcγRI (CD64) were similar in control and tet-treated cultures of nondifferentiated U937 cells (Tables I and II). ICAM-1 (CD54) was present on all U937 cells, although tet-treatment induced a small, but significant ($P < 0.05$), decrease in its level of expression (MFI, Table II). The tet-induced reduction in cell Cu was blocked

Table I. Cell Cu Content and General Metabolic Profile of U937 Cells Exposed to 5 μM 2,3,2-Tetraamine for 24 hr

	Control	+ Tet
Cell metal, nmol/mg protein		
Cu	0.78 ± 0.04	0.40 ± 0.01*
Zn	3.13 ± 0.07	3.25 ± 0.12
Cu,Zn-SOD, U/mg protein	3.33 ± 0.12	3.13 ± 0.18
TfR positive cells, %	82.1 ± 3.1	83.1 ± 1.8
Doubling time, hr	18.6 ± 0.2	19.5 ± 0.3
Cell size, μm	11.4 ± 0.1	11.1 ± 0.0
Protein, μg/10^6 cells	120 ± 4	121 ± 5
Mitochondrial activity,[a] units·min^{-1}·mg $protein^{-1}$	12.0 ± 0.6	11.8 ± 0.5

Note. Values are mean ± SD for 2–4 separate experiments that were conducted as described in Materials and Methods.
* Mean value for tet-treated cells differed significantly from control ($P < 0.05$).
[a] Arbitrary fluorescence units from the reduction of Alamar Blue by cells.

when equimolar concentrations of tet and Cu, but not tet plus equimolar concentrations of either Zn or Fe, were present in the medium for 24 hr (data not shown). Increasing the time of exposure of cells to 5 μM tet to 48 hr further decreased cell Cu to 38% of control ($P < 0.05$) and was associated with 13%, 19%, and 28% reductions ($P < 0.05$) in Cu,Zn-SOD activity, proliferation rate, and the number of Mac-1 positive cells, respectively. Mitochondrial activity also was reduced ($P < 0.05$) by 13% after 72 hr exposure to tet. These results show that exposure of cells to a low dose of tet for 24 hr decreased cell Cu content without adversely affecting metabolic integrity. Thus, exposure of nondifferentiated U937 cells to 5 μM tet for 24 hr was selected as the standard condition to generate moderately Cu deficient cells for the subsequent investigation of the role of Cu in monocytic differentiation.

Effects of Cu Deficiency on the Expression of Differentiation Antigens, DNA Profile, and Functions of U937 Monocytic Cells. The differentiation of U937 cells was induced by exposing the cells to 10 nM D_3 plus 5 ng IFN-γ/ml for 48 hr. The presence of tet in the medium for 24 hr before and during the 48-hr differentiation period decreased ($P < 0.05$) cellular Cu by 55% (1.07 ± 0.06 and 0.48 ± 0.04 nmol/mg protein for control and tet-treated cells, respectively) and Cu,Zn-SOD activity by 18% (9.25 ± 0.59 and 7.61 ± 0.18 U/mg protein for control and tet-treated cells, respectively). The marked increase of Cu,Zn-SOD activity in DI-treated cells compared to nondifferentiated U937 cells (Table I) is consistent with that we recently reported for PMA-differentiated U937 cells (6). In contrast, tet-treatment did not alter ($P > 0.05$) cell Zn content, size, granularity, protein content, and mitochondrial activity (data not shown). Approximately 3% of differentiated U937 cells were TfR (CD71) positive 48 hr after exposure to DI regardless of Cu status. As observed with nondifferentiated cells, these data indicated that tet selectively decreased cell Cu without compromising the general integrity of the differentiated U937 cells.

The results in Table II show that exposure of U937 cells to DI increased ($P < 0.05$) the expression of Mac-1 (CD11b), ICAM-1 (CD54), and FcγRI (CD64) and induced ($P < 0.05$) the expression of LPS-R (CD14). Cu deficiency decreased ($P < 0.05$) both the percentage of Mac-1 positive cells and the mean level of Mac-1 expression on the cell surface. Cu deficiency also decreased ($P < 0.05$) the percentage of LPS-R positive cells and the mean level of expression of ICAM-1. In contrast, Cu deficiency did not affect FcγRI expression ($P > 0.05$). Thus, Cu deficiency selectively altered the expression of differentiation antigens by U937 cells.

The signaling pathways for D_3 and IFN-γ are receptor mediated. To examine whether or not the impact of Cu

Table II. Effects of Cu Deficiency on the Expression of Specific Surface Antigens by Nondifferentiated and Differentiated U937 Cells

Surface antigens	Percent positive cells (%)		Level of expression (MFI)	
	Control	5 μM tet	Control	5 μM tet
Nondifferentiated cells[a]				
Mac-1 (CD11b)	46.6 ± 2.5	45.3 ± 1.6	26.4 ± 2.5	26.6 ± 2.5
LPS-R (CD14)	1.5 ± 1.4	1.1 ± 1.0	14.0 ± 1.4	13.9 ± 0.9
ICAM-1 (CD54)	100 ± 0	100 ± 0	322 ± 3	276 ± 8*
FcγRI (CD64)	65.3 ± 2.7	65.4 ± 2.4	15.5 ± 0.4	15.7 ± 0.4
Differentiated cells[b]				
Mac-1 (CD11b)	93.2 ± 0.1	82.5 ± 3.3*	78.5 ± 2.1	47.8 ± 3.7*
LPS-R (CD14)	17.7 ± 2.1	12.9 ± 3.2*	15.4 ± 1.3	14.5 ± 0.4
ICAM-1 (CD54)	100 ± 0	100 ± 0	1179 ± 176	824 ± 40*
FCγRI (CD64)	96.6 ± 3.4	98.3 ± 1.2	43.2 ± 5.3	36.9 ± 3.6

Note. Data are mean ± SD for 2–4 separate experiments for each surface antigen.
* Values in a row for control and tet-treated cells are significantly different ($P < 0.05$), compared within either percent positive cells (%) or mean level of expression (MFI, mean fluorescence intensity).
[a] Promonocytic (nondifferentiated) U937 cells were incubated in medium without or with 5 μM tet for 24 hr.
[b] Moderate Cu deficiency was induced by exposing U937 cells to 5 μM tet for 24 hr followed by treatment of 10 nM 1,25-dihydroxyvitamin D_3 plus 5 ng IFN-γ/ml for an additional 48 hr, with 5 μM tet present throughout the 48-hr period of differentiation.

deficiency on differentiation is pathway specific, a parallel series of experiments were performed in which U937 cells were exposed to PMA for 48 hr. This compound directly activates protein kinase C, thereby bypassing membrane receptor-mediated events (25). Exposure to 5 μM tet decreased Cu content of PMA-differentiated cells by 57% (P < 0.05) without compromising mitochondrial activity (data not shown). As compared with the non-differentiated U937 cells, the PMA-induced differentiation increased (P < 0.05) the percentage of Mac-1 positive cells (81.8% ± 2.9%) and the mean expression of Mac-1 and ICAM-1 (73.0 ± 6.4 and 543 ± 32 MFI, respectively). Cu deficiency decreased (P < 0.05) Mac-1 positive cells by 19% and the mean expression of Mac-1 and ICAM-1 by 33% and 31%, respectively. In contrast, FcγRI expression was not altered (P > 0.05) by either the PMA-induced differentiation or Cu deficiency. LPS-R was not expressed in response to treatment with PMA in agreement with previous reports (6, 14). Together, these data suggest that Cu deficiency impairs the normal expression of some differentiation antigens of U937 cells regardless of the signaling pathway.

The adverse impact of moderate Cu deficiency on the apparent differentiation of U937 cell into the monocytic phenotype was supported by several additional observations. First, we evaluated the effects of Cu status on the percentages of cells at various stages of the cell cycle in nondifferentiated and differentiated cultures. The percentages of nondifferentiated U937 cells in G_1/G_0, S, and G_2/M phases of the cell cycle were 42.2% ± 0.8%, 46.1% ± 0.3%, and 11.7% ± 0.6%, respectively. Differentiation was associated with an increase in the percentage of cells arrested in G_1/G_0 phase and a decrease in S phase (Table III). Treatment of nondifferentiated U937 cells with PMA, but not DI, also increased (P < 0.05) the percentage of cells in G_2/M phase. Cu deficiency increased (P < 0.05) the percentage of cells in the S phase and decreased (P < 0.05) the percentage of cells in the G_1/G_0 phase after exposure to either DI or PMA for 48 hr. Second, assessment of monocytic function represents another criterion to measure the degree of differentiation. Moderate Cu deficiency significantly suppressed (P < 0.05) $O_2^{\cdot-}$ production and the killing of *S. typhimurium* in the cells exposed to either DI or PMA for 48 hr (Fig. 1A,B).

Finally, the specificity of the chelator was further examined by the addition of equimolar concentrations of tet and Cu, Zn, or Fe to the medium before and during the PMA-induced differentiation. Supplemental Cu, but not Zn or Fe, blocked the tet-induced declines in cellular Cu (data not shown), the expression of Mac-1 and ICAM-1 (Fig. 2A), and the respiratory burst and bactericidal activities (Fig. 2B).

Table III. Effects of Cu Deficiency on Cell Cycle Profile of Cultures of U937 Monocytic Cells

Phase (%)	DI-mediated differentiation		PMA-mediated differentiation	
	Control	5 μM tet	Control	5 μM tet
G_1/G_0	55.9 ± 1.1	52.7 ± 0.9*	61.9 ± 0.7	54.3 ± 1.3*
S	33.4 ± 0.8	36.1 ± 0.7*	13.5 ± 0.6	22.9 ± 0.6*
G_2/M	10.7 ± 0.9	11.2 ± 0.8	24.5 ± 1.3	22.8 ± 0.7

Note. Control and tet-treated cells were incubated in medium containing either 10 nM 1,25-dihydroxyvitamin D_3 plus 5 ng IFN-γ/ml (DI) or 10 nM phorbol 12-myristate 13-acetate (PMA) for 48 hr. Cell cycle analysis was performed by measuring DNA content using flow cytometry. Values are mean ± SD, n = 3.

* Percentage of Cu-deficient cells with DNA content at the indicated stage of the cell cycle differs significantly (P < 0.05) from that of Cu adequate control cells treated with the same differentiation agent(s).

Discussion

The attenuated expression of selective differentiation antigens and the increased percentages of cells in the S phase of the cell cycle support the hypothesis that inadequate Cu status impairs the normal differentiation of U937 into monocyte-like cells. This finding is similar to previous reports about impaired development of rat T helper cells (7),

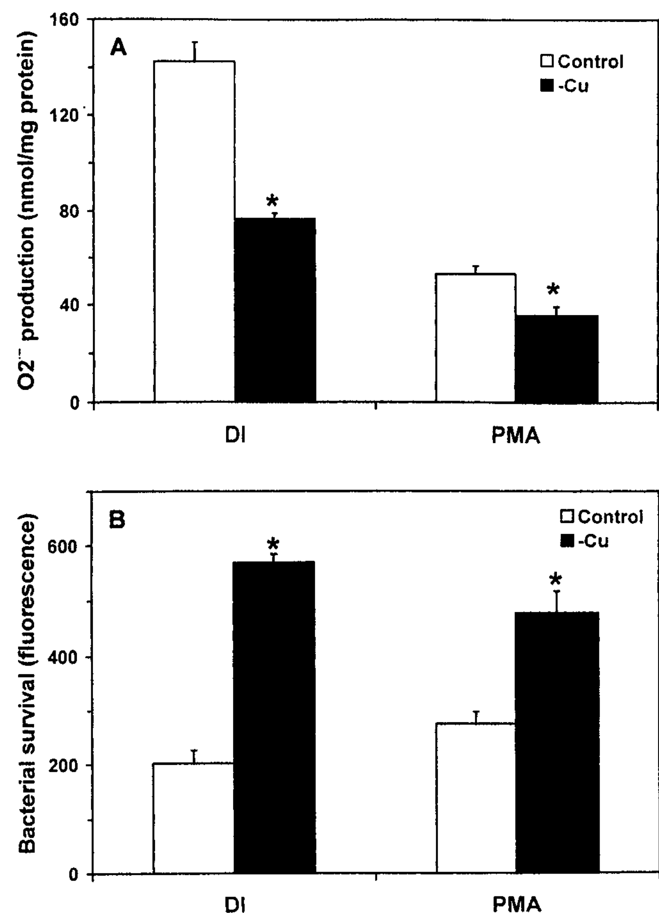

Figure 1. Cu deficiency impairs respiratory burst and bactericidal activities of differentiated U937 cells. Cu deficiency was induced by exposing U937 cells to 5 μM 2,3,2-tetraamine (tet) for 24 hr followed by treatment with 5 μM tet and either 10 nM 1,25-dihydroxyvitamin D_3 plus 5 ng/ml IFN-γ (DI) or 10 nM PMA for an additional 48 hr. Respiratory burst activity (A) and survival of *S. typhimurium* (B) were determined as described in Materials and Methods. Data are mean ± SD for 6 cultures per treatment. Significant differences from the respective controls (P < 0.05).

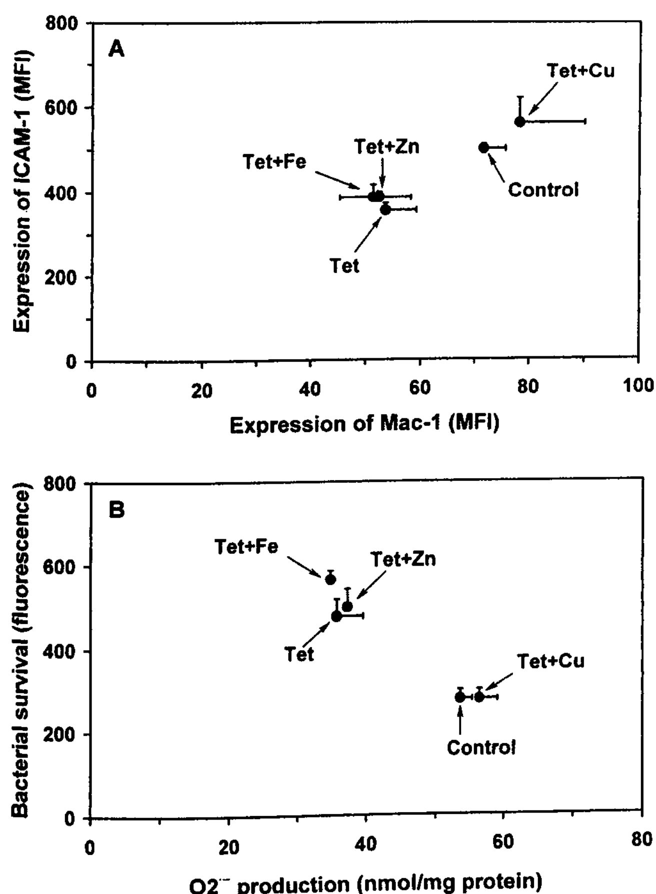

Figure 2. Supplemental Cu, but not Zn or Fe, blocks the tet-induced declines in expression of differentiation antigens and cell activities. Metal supplementation was provided by the addition of either 5 μM Cu, Zn, or Fe to the medium containing 5 μM tet before and during the 48 hr PMA-induced differentiation. The mean expression (MFI, mean fluorescence intensity) of surface antigens Mac-1 (CD11b) and ICAM-1 (CD54) (A), respiratory burst activity (B), and survival of *S. typhimurium* (B) were determined as described in Materials and Methods. Data are mean ± SD for 3–6 cultures per treatment. The indicated properties of the cells exposed to no chelator (control) are similar ($P > 0.05$) to those of tet + Cu, but differ significantly ($P < 0.05$) from those exposed to tet alone, tet + Zn, and tet + Fe.

murine neutrophils (8), and human granulocytes and macrophages (9, 11). Likewise, rat embryos develop abnormally when cultured with Cu-deficient serum (26).

Other trace metals, particularly Zn and Fe, also play critical roles in the development of immune cells. For example, Zn deficiency eliminates murine pre-B cells in bone marrow by stimulating apoptosis (27). In addition, Fe deprivation fails to induce G_1 phase cell cycle arrest and expression of differentiation-related genes in PMA or sodium butyrate-treated U937 cells and human HL-60 promyelocytic cells (28). Therefore, it was imperative that we consider the possibility that tet might be affecting the Zn and Fe status of U937 cells. Supplementation studies were performed by adding equimolar concentrations of tet and Cu, Zn, or Fe to the medium. We found that only supplemental Cu blocked the tet-induced declines in the expression of Mac-1 and ICAM-1 and the respiratory burst and bactericidal activities. Moreover, cell Zn content and the expression of TfR on the surface of U937 cells were not altered by tet treatment. These data support the conclusion that tet-induced defect in differentiation was caused specifically by the decrease in cellular Cu content.

Cu deficiency was previously reported to suppress the functions of macrophages (5). It has not been clear if the suppression was the result of a direct effect of Cu deficiency on mature cell functions or an indirect effect of lower Cu status on the differentiation of progenitor cells into monocytes and, ultimately, macrophages. We recently reported that the induction of Cu deficiency in differentiated U937 cells decreased respiratory burst and bactericidal activities and the secretion of inflammatory mediators without impairing their continued maturation, inducing secondary Fe deficiency and compromising metabolic integrity (6). The present studies demonstrate that nondifferentiated Cu-deficient U937 cells also display suboptimal responses to several differentiation signals. Although functional activities are often used to assess the degree of maturation of various cell types, the relative contributions of diminished extent of differentiation verses other effects of Cu deficiency on the lower respiratory burst and killing activities of the differentiating cells are unknown. The present and previous (6) studies with U937 suggest that Cu plays roles in both the monocytic differentiation process and selective defense activities of the cells.

Generally, the capacity of the host defense system depends on both the number of the immune cells and the activities exhibited by these cells when stimulated. Our results show that low Cu status decreases the proliferation rate of U937 promonocytic cells. The cell doubling times increased ($P \leq 0.05$) from 18.6 ± 0.2 hr to 23.5 ± 1.0 hr and 26.1 ± 1.0 hr after 48-h exposure to 5 and 10 μM tet, respectively. Supplemental Cu, but not Zn or Fe, blocked the tet-induced decline in proliferation rate (data not shown). The decrease in cell number caused by Cu deficiency has been reported in subpopulations of leukocytes. The proliferation of mitogen-treated rat T helper cells in laboratory rodents is particularly sensitive to lower Cu status (7, 29). Neutropenia represents a hallmark of Cu deficiency in humans (9). Recently, severe Cu deficiency was reported to inhibit the proliferation of human hemopoietic progenitors (11). Likewise, the proliferation of HL-60 cells is also inhibited by maintenance in Cu-depleted medium for 72 hr (30). Peled and associates (10, 11) have found that moderate Cu deficiency delays the differentiation of human hemopoietic progenitor cells, thereby enabling the expansion of the numbers of progenitor cells *ex vivo*. The number of monocytes is increased in peripheral blood of molybdenum-induced Cu deficient cows, but the degree of differentiation of these cells was not reported (31). The number of resting peripheral blood neutrophils is also increased in Cu deficient mice, but neutropenia results from administration of LPS to these animals (8). Regardless of the different effects of Cu deficiency on the numbers of immune cell

types in various species, the suppressed reactivity of these cells to stimuli is similar (29, 31, 32).

The transformation of U937 promonocytic cells from proliferating to differentiated cells with monocyte-like characteristics requires altered expression of genes. The products encoded by these genes include surface antigens, protein kinases, components of the NADPH oxidase, and enzymes required for killing microorganisms. It is possible that inadequate Cu status selectively attenuates the expression of some of these genes. Indeed, recent studies have shown that Cu deficiency impairs the transcription of the interleukin-2 gene in human Jurkat T cells (15) and increases the transcription of fatty acid synthase gene in rate hepatocytes by increasing the ratio of reduced to oxidized glutathione, i.e., cellular thiol status (33). Elucidation of the role of Cu in the expression of differentiation-associated genes in monocytic cells warrants more detailed studies. It is also important to investigate whether the impaired differentiation of Cu-deficient U937 reflects the impact of decreased Cu status on the differentiation of cells in the monocytic lineage *in vivo.*

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