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LABORATORY INVESTIGATION

Aluminum inhibits hemoglobin synthesis but enhances iron uptake in Friend erythroleukemia cells

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Aluminum inhibits hemoglobin synthesis but enhances iron uptake in Friend erythroleukemia cells. Aluminum (Al) overload in dialysis patients and experimental animals is associated with the development of anemia. However, the precise mechanisms of erythrocyte Al uptake and toxicity are poorly understood. Al accumulation, hemoglobin (Hb) synthesis and cell growth were evaluated in dimethysulfoxide (DMSO)-induced Friend erythroleukemia cells (FEC), a model system for erythroid differentiation. FEC were grown in media containing either Al citrate, transferrin-aluminum (Tf-Al), Tf or no additions. Al accumulation occurring only in cells grown in Tf-Al containing media was detected at 24 hours and increased linearly up to 96 hours after induction. By 96 hours, 200 ± 36 μg Al/liter lysed cells were detected in Tf-Al grown cells versus 5 ± 1 μg Al/liter lysed cells in cells grown in Al citrate ($P < 0.001$). Tf-Al inhibited Hb synthesis at 72 hours after induction. At 96 hours 50 ± 15% cells were benzidine positive when grown in Tf-Al compared to 76 ± 15% in Al citrate ($P < 0.001$). FEC grown in increasing concentrations of Tf-Al (100 to 500 μg/ml) showed inhibition of Hb synthesis at lower concentrations of Tf-Al at 100 μg/ml than for cell growth at 300 μg/ml. Higher concentrations of Tf-Al (>300 μg/ml) did not further inhibit Hb synthesis or cell growth. Iron (Fe) and Tf uptake were increased in Al loaded FEC compared to control cells. The increased Tf uptake was probably the result of increased Tf receptor expression on FEC since Tf cell cycling time was unchanged. These data indicate that Al utilizes the Tf uptake pathway for entry into erythrocyte precursors. Al is toxic at sites distal to Fe uptake, possibly at the heme and/or globin synthetic pathways, resulting in decreased Hb synthesis and cell growth.

Al overload in dialysis patients is associated with the development of encephalopathy [1], and osteomalacia [2] and a microcytic anemia [3–13]. Following the initial report by Elliot and Macdougall [3], a number of studies have reported the occurrence of Al induced anemia in dialysis patients [4–13]. Removal of Al from dialysate and/or treatment with the chelator deferoxamine has resulted in the reversal of this anemia [6, 9–13], pointing to Al as the etiologic agent in this process. We have also shown that an increase in erythrocyte Al levels occurred with this anemia and chelation with deferoxamine and/or removal of diaysate Al resulted in decreased erythrocyte Al content and amelioration of anemia in hemodialysis patients [12, 13].

More direct evidence for Al as an etiologic agent in anemia comes from animal studies in which anemia has been shown to develop in Al loaded normal and uremic animals [9, 14–16]. Although it is clear that Al causes anemia in man and animals, the precise mechanisms of Al uptake and toxicity in erythrocytes are poorly understood. Except for the report by Mladenovic [17] in which Al and Tf inhibited human erythroid colony (CFU-E) growth, to our knowledge no other investigations into Al uptake, transport and toxicity in erythrocytes have been reported. Since Al like Fe binds to Tf [18–20], it could utilize the Tf-binding Fe-uptake route for entry into erythrocytes. Al in erythroid precursor cells could interfere with cellular Fe uptake and transport and/or disrupt heme and/or globin synthesis. The well established Friend erythroleukemia cell (FEC) line [21, 22], which can be induced by dimethylsulfoxide (DMSO) to undergo a coordinated program of Fe uptake and Hb synthesis resembling the final stages of erythroid differentiation, serves as a model system to test the proposed mechanisms of erythrocyte Al uptake and toxicity. Present studies describe Al uptake and its effect on cell growth, Hb synthesis and Fe uptake in DMSO induced FEC in culture.

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Methods

FEC cultures

FEC were grown in RPMI 1640 media containing 10% fetal calf serum and 160 μg/ml penicillin (regular media) in a humidified incubator containing 5% CO_2 in air. FEC were subcultured to yield a clone in which 80 to 90% cells demonstrated Hb production after four days of exposure to 1.5% DMSO (Sigma Chemical Co., St. Louis, Missouri, USA).

Preparation of Tf-Al and Tf-Fe

Purified human Tf (98% pure, Sigma Chemical Co.) was saturated with Al as described by Trapp [18]. Al citrate (Pfaltz and Bauer, Waterbury, Connecticut, USA) was added to Tf in a ratio of 2 mol of elemental Al per mol Tf, in the presence of 0.04 M $NaHCO_3$. Tf saturation with Al was determined by absorbance at 242 and 290 nm [18]. Purified human Tf was saturated with ^{59}Fe, and labeled with ^{125}I as described by Glass et al [23]. Tf saturation with Fe was measured by absorbance at 470 nm.

Hemoglobin synthesis and cell growth in Al-loaded FEC

FEC were plated at 1×10^5 cells/ml in regular media to which 1.5% DMSO alone was added or to which 1.5% DMSO and one of the following were added: Tf (500 μg/ml), Al citrate (340

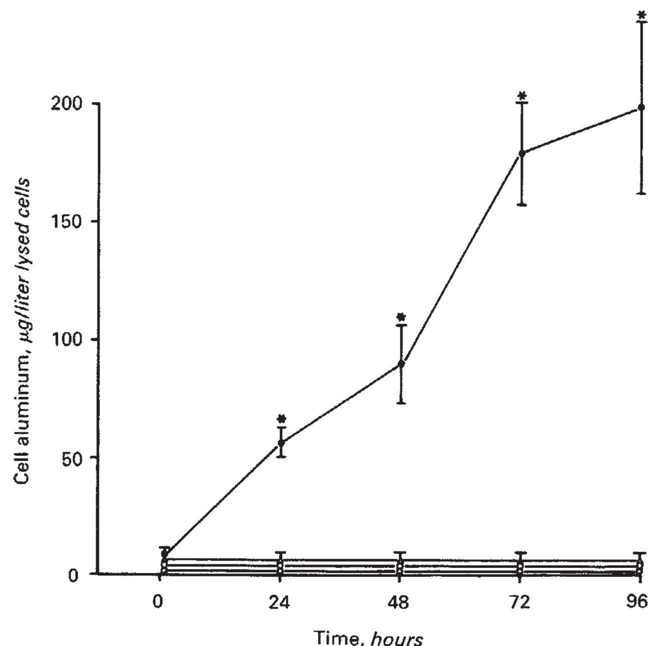

Fig. 1. *Al uptake by DMSO-induced FEC grown in regular media C, (□), media containing Al citrate (○), Tf (△) and Tf-Al (●) at 24, 48, 72 and 96 hours after induction.* Results represent means ± SE of 6 experiments, $^*P < 0.001$, Tf-Al versus C, Tf, Al.

µg/liter elemental Al) or Tf-Al (500 µg Tf/ml containing 340 µg/liter elemental Al). Aliquots of cells grown in the different media were harvested at 24, 48, 72 and 96 hours following initial plating and assessed for Al content as described by us previously [12]; Hb production was measured by percent benzidine stained cells as described by Orkin, Harosi and Leder [24] and cell growth.

In other experiments, FEC were plated at 1×10^5 cells/ml in regular media to which 1.5% DMSO and varying concentrations of Tf-Al (100 to 500 µg/ml) were added. At 96 hours after induction, Hb production was assessed by percent benzidine stained cells, Hb concentration (µg/10^6 cells) was determined as described by Tsiftsoglou et al [25] and cell growth was determined.

Fe and Tf uptake in Al-loaded FEC

FEC were grown in regular media with 1.5% DMSO (control) or with 1.5% DMSO and 500 µg/ml Tf-Al (experimental). Aliquots of cells were harvested from control and experimental media at 24, 48, 72 and 96 hours. Cells were incubated at 1×10^7 cells/ml in PBG containing 1 µM ^{59}Fe, ^{125}I-Tf for 30 minutes in a water bath at 37°C. At five minute intervals 100 µl aliquots of cells were removed and the reaction stopped by diluting with 0.5 ml ice-cold PBG. Cells were washed two times with PBG and cellular radioactivity counted in a gamma counter. The uptake of ^{59}Fe and ^{125}I-Tf was expressed in molecules/cell/minute and Tf cell-cycle time calculated as described [26].

Statistical methods

The results of Al uptake by FEC and Al induced inhibition of Hb synthesis and cell growth were compared using the Duncan's multiple range post hoc test. The results of Fe and Tf uptake in Al loaded FEC were compared using a two-way analysis of variance with paired comparison with time and Al as independent variables, followed by post hoc tests.

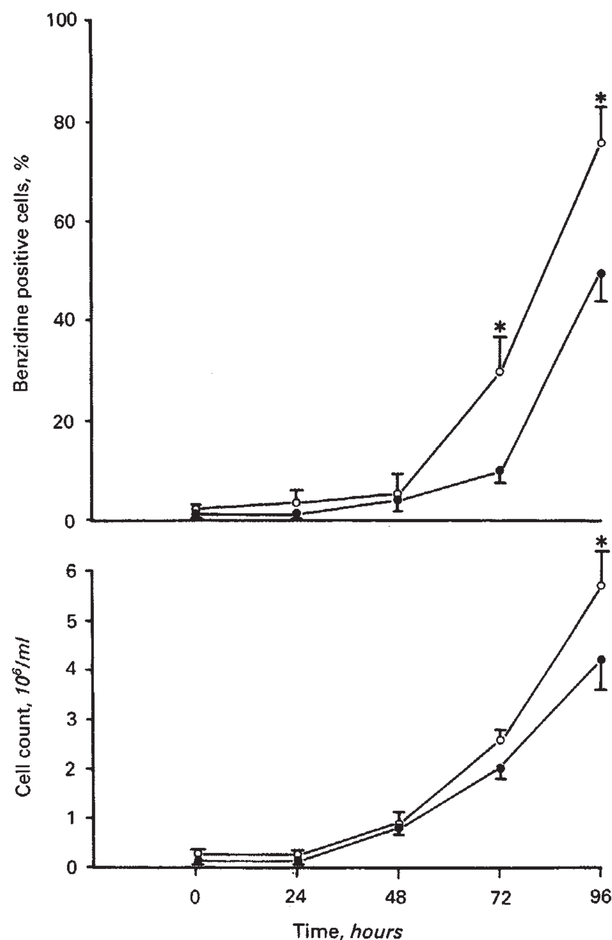

Fig. 2. *DMSO-induced FEC Hb synthesis as assessed by % benzidine positive cells and cell growth in regular media C (○) and media containing Tf-Al (●) at 24, 48, 72 and 96 hours after induction.* Results represent means ± SE of 6 experiments, $^*P < 0.001$, Tf-Al versus C.

Results

DMSO-induced FEC take up Al from Tf-Al

As shown in Figure 1 DMSO-induced FEC grown in media containing Tf-Al showed an increased uptake of Al at 24 hours. Al uptake increased in a linear fashion at 48, 72 and 96 hours after induction with concentrations of 200 ± 36 µg/liter lysed cells at 96 hours. Cells grown in regular media or media containing Al citrate or with Tf without Al showed no Al uptake. To determine if Al accumulation was unique to FEC, Al uptake was measured in rabbit reticulocytes incubated for 30 minutes either with phosphate buffered glucose (PBG), PBG and Al citrate or PBG and Al-Tf. Al content of reticulocytes was 43.8 ± 13 µg/liter ($N = 19$) in the absence of Al, 62.5 ± 13 µg/liter ($N = 9$) in Al citrate and 313 ± 81 µg/liter ($N = 6$) in Al-Tf.

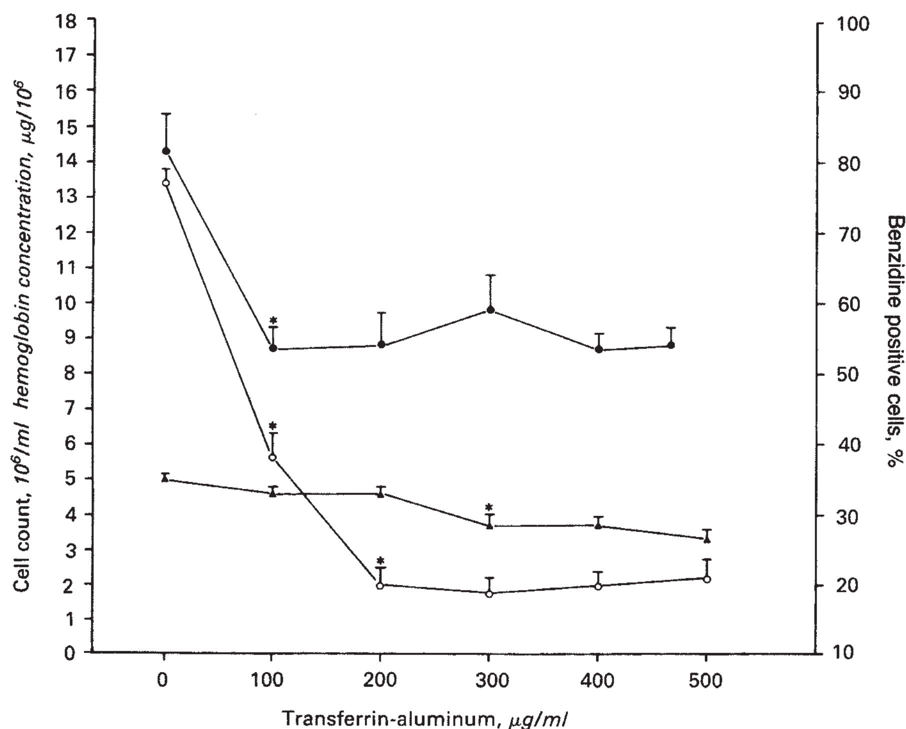

Fig. 3. *Hemoglobin synthesis assessed by Hb concentration (●) and % benzidine positive cells (○), and cell growth (▲) in DMSO-induced FEC grown in varying concentrations (100 to 500 µg/ml) of Tf-Al at 96 hours after induction.* Results represent means ± SE of 6 experiments, *$P < 0.001$. The first significant decrease is denoted by *$P < 0.001$. In the measurement of benzidine positive cells, a further significant decrease was observed and is also noted by an *.

Tf-Al inhibits DMSO-induced FEC Hb synthesis

As shown in Figure 2, Hb production as assessed by the percentage of benzidine positive cells did not increase until 72 hours after induction in cells grown in regular media, at which time Hb production was significantly decreased in cells grown in Tf-Al containing media ($P < 0.001$). At 96 hours following induction, the percentage of benzidine positive cells in the Tf-Al group still lagged behind the regular media. There was no difference in the percentage of benzidine positive cells between cells grown in regular media and those grown in Al citrate or Tf containing media.

Tf-Al inhibits DMSO-induced FEC growth

As shown in Figure 2 DMSO-induced FEC exhibited a small but significant decrease of cell growth in media containing Tf-Al compared to cells grown in regular media at 96 hours after induction. The slight effect on cell proliferation appears to lag behind the effect on Hb production. No differences in cell number were seen when cells grown in regular media were compared with those grown in media containing Al or Tf at similar time intervals.

Hb production and cell growth in FEC grown in varying concentrations of Tf-Al

As shown in Figure 3 Hb synthesis as assessed by the percentage of benzidine positive cells and measured Hb concentration was inhibited at a lower concentration of 100 µg/ml of Tf-Al. Cell growth inhibition was not seen until a threefold higher concentration of Tf-Al or 300 µg/ml of Tf-Al was present. Higher concentrations of Tf-Al did not cause further reductions in Hb concentrations or cell growth.

Fe and Tf uptake in Al-loaded FEC

To determine if decreased Hb production was the result of decreased Tf and/or Fe uptake the rate of ^{125}I-Tf and ^{59}Fe uptake was measured at various times after DMSO induction in cells grown in the presence or absence of Tf-Al. As shown in Figure 4 significantly increased uptake of Fe and Tf occurred when assessed at 24, 48, 72 and 96 hours in DMSO-induced FEC grown in Al-Tf containing media compared to control media. Calculated Tf cell cycling time was similar for Al-loaded FEC and control FEC at 5.0 ± 0.2 minutes. These data suggest that Tf receptors were operating normally and that Tf-Al caused increased expression of Tf receptors.

Discussion

A major fraction of Al in serum is bound to protein, primarily Tf [18–20]. Citrate has been suggested as the most probable small molecular binder for the non-protein bound fraction of serum Al [27]. Therefore, Al uptake was evaluated in FEC grown in control media and in media containing Tf-Al, Tf and Al citrate. Aluminum was taken up by FEC grown in media containing Tf-Al and not Al citrate. A linear increase in cellular Al concentration occurred in FEC grown in media containing Tf-Al. Similar uptake of Al was obtained in rabbit reticulocytes exposed for 30 minutes to Tf-Al with no uptake from Al citrate, providing further evidence that cells acquire Al via Tf mediated endocytosis.

Aluminum accumulation in FEC resulted in inhibition of Hb synthesis and cell growth (Figs. 2 and 3). Comparison of the time-response curves obtained for FEC grown in Tf-Al and for FEC grown in media with no added Al showed decreased Hb accumulation in Al-loaded FEC at 48 and 72 hours (Fig. 2)

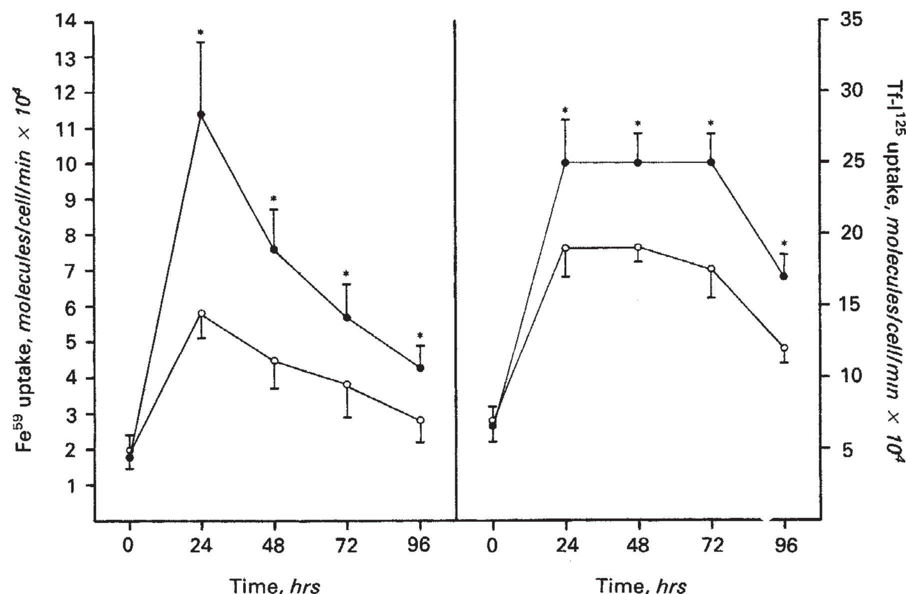

Fig. 4. *Fe^{59} and Tf uptake in DMSO-induced FEC grown in media containing Tf-Al (●) compared to FEC grown in regular media C (○).* Results represent means ± SE of 5 experiments. *In the uptake of Tf-Al vs. C, Fe uptake at 24 hours $P < 0.03$, 48 hours $P < 0.02$, 72 hours $P < 0.007$, 96 hours $P < 0.01$ and *Tf uptake at 24 hours $P < 0.004$, 48 hours $P < 0.02$, 72 hours $P < 0.0001$, 96 hours $P < 0.02$.

followed by decreased cell growth at 96 hours (Fig. 2). The dose-response curves (Fig. 3) obtained by growing FEC in increasing concentrations of Tf-Al showed a sharp decrease in Hb accumulation at concentrations of Tf-Al (100 μg/ml) in media which were threefold lower than Tf-Al concentrations (300 μg/ml) that inhibited cell growth. Both the time-response (Fig. 2) and dose-response (Fig. 3) curves indicate that Hb synthesis was more sensitive than cell growth to the toxic effect of Al. The elemental Al concentrations in Tf-Al added to media (68 to 340 μg/liter) were similar to Al concentrations in the plasma of dialysis patients with Al induced microcytic anemia previously reported by us [12, 13].

Since both Al and Fe are taken up by FEC bound to Tf, Al could potentially interfere with Fe uptake by FEC. To test this hypothesis Fe and Tf uptake by Al loaded FEC was compared with Fe and Tf uptake in control FEC at 24, 48, 72 and 96 hours after DMSO induction. Fe and Tf uptake in Al loaded FEC were always significantly higher compared to control FEC. Tf cell cycling time was similar in Al loaded and control FEC, suggesting that endocytosis of Tf receptor was unaltered. Therefore the increased Fe uptake in Al loaded cells resulted from increased Tf receptor expression. Tf uptake at 37°C measures both surface bound and internalized Tf and therefore indicates that total cellular Tf receptor levels are increased and not merely redistributed. The signal for increased Tf-receptor expression in Al loaded cells could be decreased heme or hemoglobin synthesis, decreased Fe in a critical compartment or Al accumulation itself. Relative rates of uptake of Fe and Al by FEC from Tf-Fe and Tf-Al added simultaneously to media were not done because of the lack of an Al isotope with a sufficiently long half-life.

Mladenovic [17] has previously shown that Al and Tf and not Al alone inhibited human erythroid colony (CFU-E) growth. The model system described by us confirms her observation that Al binding to Tf is necessary for its inhibitory effect on cell growth but also differs in several aspects. FEC clearly accumulated Al from Al-Tf whereas no Al uptake could be demonstrated in CFU-E. The effect of Al on CFU-E was inhibition of colony growth whereas in FEC hemoglobin synthesis was affected greater than cell growth. Lastly, the FEC culture allows us to explore the possible toxic effects of Al on the separate processes of Fe transport, heme and globin synthesis.

The increased Fe and Tf uptake by Al loaded FEC suggests that Al does not affect Fe uptake by FEC. Al could, however, interfere with intracellular Fe transport after it dissociates from Tf. Fe could accumulate in an inaccessable intracellular compartment viz bound to ferritin or within lysosomes as shown in the liver [28]. Alternatively, Al could inhibit heme and/or globin synthesis. Inhibition of erythrocyte ALA dehydrase by Al has been shown in vivo [29] and in vitro [30]. Increased tissue porphyrin deposits have been shown to occur in Al loaded animals [31] and increased erythrocyte protoporphyrin levels have been detected in dialysis patients with Al associated anemia with reduction in protoporphyrin levels following Al chelation [11]. These studies suggest a direct toxic effect of Al on heme synthetic enzymes. There is less evidence implicating Al toxicity on globin synthesis. Since Al destabilizes and cross-links with DNA at low pH [32], Al could potentially intefere with globin transcription.

In summary, this report represents the first direct evidence that Al gains access to the cell bound to Tf. Contrary to speculations that Al possibly inhibits Tf and Fe uptake our studies show that initial uptake of Tf and Fe are not involved. Indeed, these steps are accelerated suggesting that the effect of Al is distal to Fe uptake processes and cycling of Tf. Finally, although in humans accumulation of Al results in a microcytic, hypochromic anemia, no in vitro models exist prior to our studies, including those of Mladenovic [17], in which a hypochromic anemia is induced by Al. Therefore, the model described, the DMSO induced FEC which closely mimic erythroid progenator cells, allows for dissection of the mechanisms whereby Al causes a hypochromic anemia.

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