

Nevertheless, when applied to animal models, zinc deficiency was mostly investigated in juveniles to induce pronounced delay in development and other pathological alterations. In contrast, this study was carried out in a non-growing rat model. Thus, the model reflects the common nutritional situation of adult individuals living at maintenance metabolism.

Over the past years it became clear that in addition to reduced cell proliferation, zinc deficiency is capable to induce apoptosis under various *in vitro* conditions [4–6]. Furthermore, apoptosis was found in rats fed a Zn-deficient diet for 34 weeks [7]. Apoptotic cell death could also be detected in skin lesions of patients with acquired zinc deficiency [8]. In above-cited articles apoptosis was mainly proven by detection of DNA fragmentation. This phenomenon reflects late stages of apoptotic cell death. In the present study, we show that early apoptosis-related factors, caspase-3 and Fas, are elevated on transcriptional level in distinct organs of rats after feeding a Zn-deficient diet.

Material and methods

Animals

Thirty-one adult, non-growing female rats were reared with a purified, phytate enriched diet for 9 weeks up to an average body weight of 212 g, and were then fed restrictively according to the maintenance requirement for energy for another week (8 g per head and per day). The diet was supplemented with sufficient amounts of minerals, trace elements and vitamins to meet recommendations. Feeding conditions were maintained for another 29 days (experimental period) except for dietary Zn content. For animals in the control group, Zn remained at 58 µg/g while for the Zn-deficient animals, Zn was reduced to 2 µg/g by omitting dietary Zn supplementation. Animals receiving the Zn-deficient diet were divided into eight subgroups of three animals each and subsequently sacrificed at day 1, 2, 4, 7, 11, 16, 22 and 29 after onset of Zn reduction feeding ($n = 24$). Controls were sacrificed at day 0 ($n = 3$) and 29 ($n = 4$) for baseline. At necropsy, tissue samples collected (liver, thymus, jejunum and colon) were immediately frozen in liquid nitrogen and stored at -80°C till total RNA extraction was carried out, as described earlier in detail [13].

RNA preparation and reverse transcription

Total RNA was extracted applying single modified liquid separation procedure [9], using 200 mg deep frozen tissue. Thousand nanograms of total RNA was reverse transcribed using random hexamers and 200 U

of MMLV H minus (Promega, Germany) according to the manufacturers protocol.

Primer design

For primer design *Clustal alignment* of sequences of interest available at GenBank database was performed on HUSAR[®] software from DKFZ (Heidelberg, Germany). Primers were synthesised by MWG Biotech (Ebersberg, Germany). Primer sequences were as follows: Caspase-3 FOR 5'-AACCTCCGTGGATT-CAAATC-3', Caspase-3 REV 5'-TTCAAGAG-TAATCCATTTTGTAAAC-3', Fas FOR 5'-AGAAGGGAAGGAGTACAC/TA/CGAC-3', Fas REV 5'-TGCACCTTGGTATTCTGGGTCC-3', glyceraldehyd-3-phosphate-dehydrogenase (GAPDH) FOR 5'-GTCTTCACTACCATGGAGAAGG-3', GAPDH REV 5'-TCATGGATGACCTTGCCAG-3'.

Real-time PCR

In each sample, 25 ng reverse transcribed total RNA (=cDNA) was analysed by real-time PCR in the LightCycler (Roche Diagnostics; Mannheim, Germany) in a total volume of 10 µl containing the indicated end-concentrations of master-mix components: 6.4 µl water, 1.2 µl MgCl₂ (4 mmol/l), 0.2 µl of each forward and reverse primer (0.4 µmol/l) and 1 µl cDNA. Samples were then amplified in a 40-cycle qPCR program with single fluorescence acquisition at the end of each cycle. For determination of specific qRT-PCR products a melting curve was generated at the end of the cycling program. Acquisition of the crossing-point (CP) is essential for further quantification of gene expression. For CP determination the “*second derivative maximum method*”, measuring maximum increase rate of newly synthesised DNA per cycle was used on the basis of LightCycler software 3.5 (Roche Diagnostics). Low CP values indicate high level of gene expression and vice versa.

Relative quantification and statistics

Data analysis and computing was performed on SigmaPlot 2000 (SPSS Science, Chicago, USA). Prior to processing, data were normalised by CPs of a non-regulated housekeeping gene, i.e. GAPDH. Deviation of CPs (ΔCP) in the control group and treatment group was determined. Finally, the relative expression ratio (R) of mRNAs was calculated according to the following equation, assuming optimal PCR efficiency of 2:

$$R = 2^{\Delta\text{CP}(\text{target gene})} / 2^{\Delta\text{CP}(\text{GAPDH})}$$

Relative gene expressions of caspase-3 and Fas of each animal were plotted as a function of days of zinc

depletion. A coefficient of correlation analysis, according to a polynomial equation ($y = a + bx + cx^2$), was carried out for overall treatment period. A trend of significance was defined at $p < 0.05$. Additionally, the relative expression software tool REST [10] was applied to determine the relative up- or down-regulation of target gene expression in the extreme treatment group ($n = 6$; day 22 and 29) compared to the control group ($n = 7$). Data collected from control animals, regardless of whether obtained at day 0 or day 29, were statistically processed in one group ($n = 7$). In this group, up- or down-regulation was calculated by a pair wise fixed reallocation randomisation test, implemented in the REST Software [10].

Results

Specificity of real-time PCR products was confirmed by high-resolution 2% gel electrophoresis and additionally by inspection of product melting temperature carried out at the end of each LightCycler run. Both genes of interest proved to have transcript-specific bands after gel electrophoresis and one specific single melting point. No primer-dimer formation could be observed. As no significant differences in gene expression could be found in control animals after day 0 and day 29, data were statistically processed together in one control group ($n = 7$).

GAPDH mRNA expression was used as a reference to compare the mRNA expression of the target genes in control and treated animals according to the used relative quantification model [10]. The GAPDH expression itself showed no significant regulation upon zinc deficiency. In addition, its variation was as low as those of the target genes ranging from 2.7% to 6.8% in all treatment groups. GAPDH served as optimal house-keeping gene for normalisation in this zinc-deficiency study.

Expression levels of apoptosis-related genes tend to differ with regard to different organs investigated. Regarding mean CP values (data not shown) in control animals, a slightly higher absolute expression level of caspase-3 was found in liver, whereas the lowest expression was found in colon. Same patterns were observed after overall inspection of the treatment group. As to Fas expression, the highest level was detected in thymus and the lowest was measured in jejunum. In contrast to caspase-3, that was expressed at similar levels in all organs under study, the level of Fas expression was by far higher in thymus than in the other organs. This organ showed 8-fold higher expression calculated from the absolute CP value (mean: 26.73) in comparison to liver, jejunum and colon (mean CP: 29.23, 31.53, 31.39, respectively).

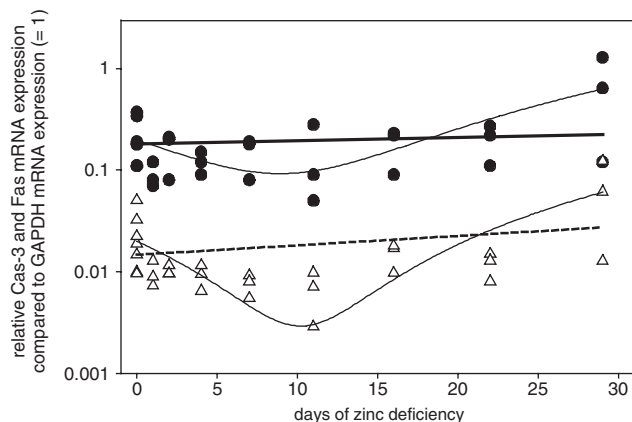


Fig. 1. Polynomial regression ($y = a + bx + cx^2$) plot for relative caspase-3 (black dots) and Fas (white triangles) expression levels versus the constant GAPDH expression in jejunum over 29 days of Zn-deficient diet ($n = 31$). Caspase-3 expression ($p = 0.006$) as well as Fas expression ($p = 0.022$) show highly significant decrease until day 11 and afterwards increase until day 29 of Zn deficiency. Solid and dashed lines indicate non-significantly different baseline levels in control animals.

Basic analysis of the CP values obtained from different organs revealed a coefficient of variance (CV) for Fas mRNA expression ranging from 1.6% to 5.0%. CV for caspase-3 ranges from 2.0% to 6.8%.

Coefficient of correlation of relative mRNA expression levels of caspase-3 and Fas (after normalisation by GAPDH expression) was performed on data including the overall period of zinc deficiency. The most striking results were found in jejunum. Caspase-3 as well as Fas expression was significantly down-regulated until day 11 and then up-regulated ($p = 0.006$ and 0.022 , respectively), compared to the control group. The complete correlation is shown in Fig. 1. As to the other organs investigated, the results obtained were as follows: caspase-3 and Fas expression in thymus (Fig. 2) tended to be up-regulated, although the up-regulation was not significant ($p = 0.09$ for caspase-3 and $p = 0.1$ for Fas). In contrast, the caspase-3 and Fas expression in liver (Fig. 3) was slightly down-regulated ($p = 0.10$ for caspase-3 and $p = 0.07$ for Fas). Regarding the relative mRNA expression in colon samples (Fig. 4), a similar trend as in the liver was found, with slight down-regulation of both factors ($p = 0.18$ for caspase-3 and $p = 0.53$ for Fas).

In addition, the relative up-/down-regulation was computed using REST in the extreme treatment group receiving a Zn-deficient diet for 22 and 29 days in comparison to the control group. Applying this quantification tool, alterations in mRNA expression are presented as an “ x -fold” up- or down-regulation. Data are shown in Table 1. With exception of the Fas expression in liver, these data corroborate the results obtained by correlation analysis. Considering the results

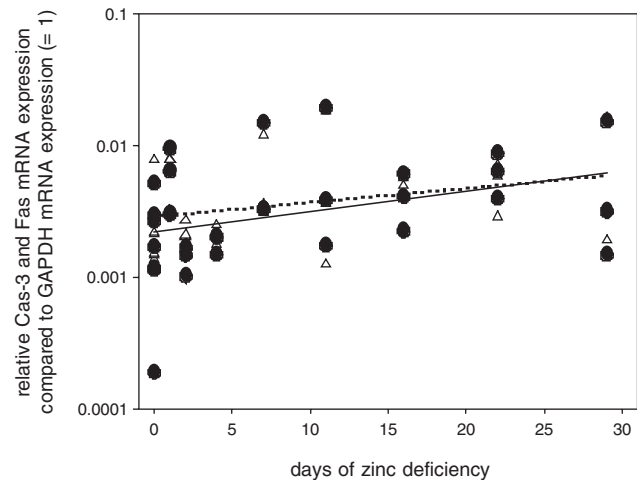


Fig. 2. Regression plot for caspase-3 (black dots) and Fas (white triangles) expression in thymus. Both factors show slight but non-significant up-regulation ($p = 0.09$ for caspase-3 and $p = 0.10$ for Fas) during overall treatment period.

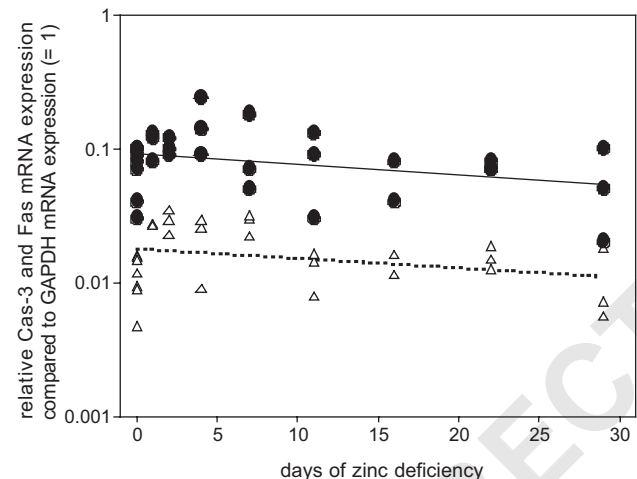


Fig. 3. Regression plot for caspase-3 (black dots) and Fas (white triangles) expression in liver. Both factors show slight but non-significant down-regulation ($p = 0.10$ for caspase-3 and $p = 0.07$ for Fas) during overall treatment period.

of the polynomial regression and the relative expression after 29 days in liver and colon, the mRNA levels in the respective organs seemed to be mostly unaffected.

Discussion

The physiological status of the animals included in this experiment was characterised by the absence of growth retardation and constant feed intake adapted to maintenance requirement of energy. As reported elsewhere [11], metabolic markers as well as plasma levels of growth-related hormones, including expression levels of their receptor proteins, remained unaffected during the

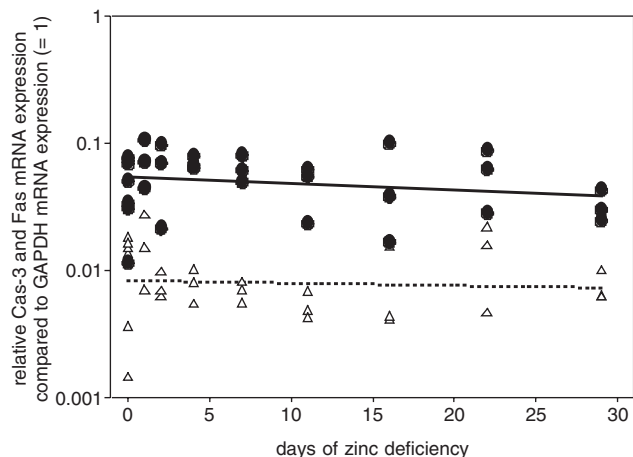


Fig. 4. Regression plot for caspase-3 (black dots) and Fas (white triangles) expression in colon. Both factors show slight but non-significant down-regulation ($p = 0.18$ for caspase-3 and $p = 0.53$ for Fas) during overall treatment period.

Table 1. Results of relative quantification by REST in four different organs after 22 and 29 days of Zn-deficient diet ($n = 6$) in comparison to untreated control rats ($n = 7$)

	Caspase-3	Fas
Liver	1.5-fold down-regulation	2.2-fold down-regulation
Thymus	2.7-fold up-regulation	2.0-fold up-regulation
Jejunum	2.4-fold up-regulation	2.4-fold up-regulation
Colon	1.2-fold down-regulation	1.2-fold down-regulation

Jejunum and thymus showed the strongest up-regulation in mRNA expression of both transcripts—caspase-3 and Fas.

entire experiment. Nevertheless, Zn deficiency was evident from Zn mobilisation from storage tissues (especially skeleton), reduced Zn plasma levels and decreased alkaline phosphatase activity [12,13]. The present animal model also gives insights to alterations of mRNA expression of apoptosis-related factors in adults living at subclinical Zn deficiency without catabolic metabolism. This is a common but often unrecognised scenario in industrial countries [2]. Comparing the results after REST quantification and regression analysis in Sigma Plot, one has to keep in mind that each program makes use of another calculation algorithm, which may lead to different results, especially when differences in expression are only mild. Application of REST only allows comparison of two time points (beginning and end of the experiment). Thus slight inter-group differences during the overall treatment period are not reflected in this calculation.

The observation that absolute expression levels of Fas were highest in thymus reflects the fact that apoptosis of T-lymphocytes is mainly regulated via the Fas and Fas-

ligand interaction pathway [14]. In contrast to other groups [7,15] which determined apoptosis at very late stages by detection of DNA fragmentation using the TUNEL method (in situ cell death detection kit, Roche, Germany), this study could show elevated levels of caspase-3 and Fas mRNA expression. Both factors are involved in early stages of apoptosis. Interestingly, the mRNA expression was altered in jejunum and thymus although the caspase-3 and Fas-related induction of apoptosis is normally regulated on protein level. In particular, members of the caspase family are stored as pro-enzymes and subsequently activated by cleavage of the pro-domain when cells receive “death-signals” [16]. On the other hand, transcriptional regulation of caspases in an experimental pneumococcal meningitis model was found by von Mering et al. [17]. Ciementi et al. [15] were able to show that caspase-3 maintained inactive when Zn levels were normal. The fact that caspase-3 and Fas expressions are predominately up-regulated in jejunum from day 11 onward can be explained by a high rate of mitosis and therefore a high Zn requirement in this organ. Furthermore, the small intestine plays a key role in Zn uptake [18], and might therefore react as first site of action after onset of Zn depletion. The down-regulation of mRNA expression from day 1 to day 11 may be explained by a pronounced release of Zn from storage tissues, which is an early reaction of Zn metabolism to Zn deficiency [13]. In contrast, the cell-turnover and therefore the rate of mitosis in colon epithelial cells is much slower compared to jejunum. This might explain unaffected mRNA expression levels in this organ. In tissues storing Zn at high levels, such as the liver, an increase in caspase-3 and Fas expression could not be detected. This is in good accordance with the results published by Nodera et al. [7]. They were additionally able to show by means of TUNEL that the number of apoptotic cells in thymus were elevated prior to the onset of morphological alterations in this organ.

The present study provides first evidence that even in mild zinc deficiency, apoptosis-related factors are elevated on transcriptional level. Effects are most prominent in organs with high cell-turnover and low capacity of zinc storage.

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